3rd EAVLD Congress
Pisa (Italy)
Palazzo dei Congressi
12-15 October, 2014
European Association of Veterinary Laboratory Diagnosticians
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Dear colleagues,

Personally and on behalf of the Italian Association of Veterinary Laboratory Diagnosticians (SIDiLV), the Organizing Committee and the Scientific Committee, it is a great pleasure to welcome you to Pisa to the 3rd EAVLD Congress.

We are delighted to host this event, which represents an important opportunity to highlight innovations in the field of diagnostics and share experience among members and guests.

As organizers, we are proud to announce that over 200 abstracts have been submitted and more than 300 attendees are expected from Europe and other continents, thus demonstrating the increasing attention to EAVLD and its Congress.

I would like to thank the sponsors, supporting the Congress, for giving us the opportunity of being updated on the most innovative diagnostic devices and on the progress toward their application in field.

We would also sincerely appreciate the EAVLD President and Board, who accepted the proposal to hold the Congress in Italy and for their constant and valuable advice provided during the organization.

Finally, a special acknowledgment to MV Congressi for the timely and rigorous commitment paid to the organization of the meeting.

As you already know, SIDiLV has applied also to host the WAVLD Congress that will be held in 2017 in Sorrento. Somehow, the meeting of Pisa can be considered a test for the next initiative. Therefore any suggestion to improve our reception will be particularly appreciated.

Participants will have the opportunity to enjoy Pisa, that besides being known worldwide for the leaning tower, hosts more than 20 historical churches and is easily connected to Florence and other regional tourist attractions.

Hoping the congress will meet your expectations, I wish you a fruitful work, mutual enrichment and a source of new ideas!

Elena Bozetta
SIDiLV President
Dear colleagues,

A warm welcome to the 3rd EAVLD congress! For all of you a great opportunity to extend your network and make new friends in the field of veterinary laboratory diagnostics. For those of you who have been at previous EAVLD congresses, an equally great opportunity to meet old friends and share new knowledge. After all, this is what EAVLD is all about: a platform to meet and share, in order to increase the overall quality of veterinary diagnostics in Europe.

Also welcome in the beautiful city of Pisa, known worldwide for its leaning tower, but in fact an ancient city with many historical monuments. If you have some time left, I advise you to have a look in and around the old town centre and you will not be disappointed by the beauty of this city.

Many thanks to Gian Luca Autorino and his team for organising this 3rd EAVLD congress. From the start they have taken a very professional approach to organising this congress and I’m sure it will be as successful as the previous congresses. More abstracts were received than for the previous congresses, and the number of attendants is also expected to be higher than before. A sure sign that EAVLD is there for a reason and that there is room for continuous growth!

Also a thank you to the sponsors of this congress. Through their contributions, they allow you to be here for reasonable fees. But through their exhibitions, they also give you a good overview of the current state of the art in the field of veterinary diagnostics. What better place to look into that than at this congress, where all the major players are represented.

At the General Meeting of the EAVLD, held on Tuesday during the congress, many of the original founding board members will step down. They have started EAVLD together in 2009, with the support of their respective institutes. They have made it into the EAVLD of today, with this 3rd congress in a period of 4 years. Their terms have come to an end and a new generation is taking over to further expand on the work of EAVLD. I will hand over the presidency to Miroslaw Pawel Polak, who was elected two years ago as vice-president, and who was the president of the organising committee of the 2nd EAVLD congress. I thank the old board members and wish the new ones a lot of success in their work for the next two years.

Finally, it is you, current and future members of EAVLD, who decide what the future of EAVLD will look like. Please get involved! Come up with ideas for EAVLD and think about how you can participate in its mission to improve the veterinary diagnostics in Europe. Enjoy, learn and if possible, contribute!

Willie Loeffen

EAVLD President
ORGANIZING COMMITTEE

S.I.Di.L.V
Società Italiana
di Diagnostica di Laboratorio Veterinaria
Italian Association of Veterinary Laboratory Diagnosticians

EAVLD
European Association of Veterinary Laboratory Diagnosticians

ORGANIZING SECRETARIAT

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Alfredo Caprioli, Istituto Superiore di Sanità, Rome (Italy)
Nicola Decaro, Department of Veterinary Medicine, University of Bari, Bari (Italy)
Kirsty Line, Animal Health and Veterinary Laboratories Agency (AHVLA), Exeter (UK)
Frederik Widén, The National Veterinary Institute (SVA), Uppsala (Sweden)

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Antonia Ricci, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (Italy)
Fabrizio Vitale, Istituto Zooprofilattico Sperimentale Sicilia, Palermo (Italy)

THE CONGRESS VENUE

PALAZZO DEI CONGRESSI
Via Matteotti, 1 - 56124 Pisa
tel. 050 598212 / 050 598213
www.palazzodeicongressi.pisa.it
SPONSORS

*Special thanks to the following companies who have supported the 3rd EAVLD Congress 2014*

**GOLD SPONSORS**

- BIOMÉRIEUX
- GD
- IDEXX
- Life Technologies
- PRIONICS
- QIAGEN
- Zoetis

**SILVER SPONSORS**

- BioX Diagnostics
Special thanks to the following media partners who have supported the 3rd EAVLD Congress 2014

- rapidmicrobiology
- VETERINARY RESEARCH
SPONSORS INFORMATION

AGROLABO

Since 1986 the Diagnostics Division has grown to become a leading manufacturer of an integrated portfolio of specialist diagnostic tests for companion and livestock animals which includes immunochromatographic, microbiological, haematological, ELISA and IFA tests.

AHVLA SCIENTIFIC

Products and services to scientists around the world
To enhance its scientific activity, the Animal Health and Veterinary Laboratories Agency (AHVLA) in the UK, has developed commercial products and services and made them available to organisations around the world. These services are brought together under AHVLA Scientific.
The products and services available include:
• Specialist laboratory reagents & diagnostic kits
• A world leading range of proficiency testing schemes
• Veterinary research and development projects
• A unique bacterial cell characterisation service
• Testing services for veterinary practitioners and the pharmaceutical industry
• Intellectual property and monoclonal antibody licensing

AHVLA Scientific includes business development managers, a customer service team, scientific research and reagent production groups to ensure that the products and services offered are delivered effectively to our customers. They are also able to bring scientific expertise, high quality service, innovative methods and, in many cases unique products and services to the market.
Our expertise has been developed in the veterinary landscape over the last century. Our scientists have had a significant influence on the development of animal health and food safety strategy in both the UK and Europe and the laboratory has achieved World Reference Laboratory status for many important diseases. These achievements have earned the Agency an international reputation for scientific excellence as it has led the world in developing new techniques for the diagnosis and management of animal disease.

BIOLLOG

Biolog is a pioneer in the development of powerful cellular analysis tools for solving critical problems in veterinary, clinical, pharmaceutical, and biotechnology research and development. In addition to our award winning Microbial Identification Systems, Biolog’s unique Phenotype MicroArray technology can be used to assay cells of all types, from microbial to mammalian.

BIOMÉRIEUX

A world leader in the field of in vitro diagnostics for 50 years, bioMérieux is present in more than 160 countries through 41 subsidiaries and a large network of distributors. 

bioMérieux provides diagnostic solutions for customers in the Veterinary diagnostics field, agri-food, biopharma, cosmetics industries and in the clinical field: from farm to folks.
The aim of the veterinary diagnostics activity is to develop solutions to combat animal diseases and zoonoses while contributing to the prudent use of antibiotics in veterinary medicine.

bioMérieux veterinary diagnostic solution
Time-to-diagnosis and accuracy of treatment are paramount to case management, as well as infection control, the fight against multi-drug resistance and reducing animal healthcare costs. From sample to decision, bioMérieux provides a comprehensive Microbiology range based on manual or automated technology:

- Automated Media Preparation: Masterclave®
- Ready-to-use culture media: ChromID® range
- Automated Inoculation system: Previ® ISOLA
- Identification solutions: VITEK® 2, VITEK® MS (MALDI-TOF technology) & API® range
- Antibiotics susceptibility testing: VITEK® 2 Technology & Etest® range
- Pathogens Detection kits: ADIAVETTM PCR kits

**BIO-RAD**

Bio-Rad produces tests for food safety with a complete line of solutions for food pathogen testing, including a full menu real-time PCR test kits for detection of key pathogens, culture media for nutritive enrichment and RAPID chromogenic media for easy colony identification for detection of pathogens and enumeration of quality indicators. As an instrument manufacturer, Bio-Rad provides instrument options for both low and high volume users, including our iQ-Check Prep automation system.

**BIOVET Inc**

Biovet Inc. develops, manufactures and markets animal diagnostic kits. Additionally, Biovet provides animal health and agro-industry specialists with a unique expertise in the diagnostic field. Biovet operates certified laboratories offering a complete array of innovative diagnostic services for veterinarians. The head office is located in St-Hyacinthe, QC, CANADA and the US branch is in Minneapolis, MN. Biovet employs more than 50 people including 15 scientists. The company is active internationally particularly in North America and Europe.

**BIO - X DIAGNOSTICS**

Bio-X Diagnostics is developing diagnostic reagents for diseases affecting production animals such as cattle, pigs, horses, small ruminants, trout and carp.

Bio-X Diagnostics manufactures and sells a number of original products, including multipathogen antigen diagnostic kits such as a digestive kit, a respiratory kit, an abortion kit and an enterotoxaemia kit. They implement ELISA (Enzyme Linked ImmunoSorbent Assay) technology. These kits are primarily intended for state or private veterinary laboratories, in routine diagnostics but also for research laboratories.

In addition to the ELISA kits, Bio-X Diagnostics prepares and sells reagents for direct or indirect immunofluorescence and indirect immunoperoxidase assays.

To assure a very quick and accurate diagnosis in the field, Bio-X Diagnostics has developed immunochromatography strip test kits. These strips provide a diagnosis in a matter of minutes without using any sophisticated equipment and are meant as a complementary tool to laboratory diagnosis.

Bio-X Diagnostics is ISO 9001:2008 certified and recently moved to its new and very modern facilities in Rochefort, Belgium.

**BOEHRINGER INGELHEIM SVANOVA**

Boehringer Ingelheim Svanova offers ELISA for antibody detection and pen-side tests for antigen detection for a wide range of infectious diseases in livestock animals, poultry and horses. Svanova contributes to a better animal health and food safety with the diagnostic solutions to prevent, monitor and eradicate animal diseases.

Svanova is a small company with 20 employees working passionately on the development, production and distribution of ELISA assays. One of the strengths of Svanova is the close cooperation with the specialists and researchers throughout the world when developing new assays. Svanova’s slogan “when every sample counts” reflects the high targets set for development and production of the assays. All products are manufactured under strict ISO certified methods in Sweden and strict quality testing guarantees the high precision of Svanova’s assays.

Svanova’s roots are deep in the veterinary diagnostic research. Svanova is today part of Boehringer Ingelheim Animal Health.
but arose in 1988 out of the activities of the Swedish National Veterinary Institute. In the beginning the kits were predominantly used in Sweden, but the demand for quality diagnostic solutions grew beyond the borders of Scandinavia and the business gradually expanded globally. Svanovas’ products are being distributed and used worldwide. Our sales partners offer services in local language and provide together with the Svanova team convenient and experienced customer service.

- Please visit us at Svanovas’ booth at the conference,
- visit our website www.svanova.com or
- contact our product experts, customer service our R&D team

Claudia Lindh, Brand Manager, claudia.lindh@boehringer-ingelheim.com
Afsaneh Jalali, Customer Service Manager, afsaneh.jalali@boehringer-ingelheim.com
Malik Merza, Head of R&D, malik.merza@boehringer-ingelheim.com

EUROIMMUN AG

EUROIMMUN produces an extensive range of indirect immunofluorescence, microplate ELISA, immunoblot (EUROASSAY, EUROLINE, Westernblot) and radioimmunoassay test systems for laboratory diagnostics in the areas of autoimmunity, infectious serology and allergology. EUROIMMUN has its headquarters in Luebeck, Germany and supplies its products and services to over 3,000 laboratories worldwide.

EXOPOL

Exopol is a biotechnology company founded in 1993 whose labours are research and development as well as production of qPCR kits and vaccines/autovaccines. Moreover we accomplish with microbiology, parasitology and serology. We offer veterinary diagnosis in addition to an expertise knowledge of animal infectious diseases using it in order to develop our autogenous vaccines and diagnostic kits: Real Time PCR.

Years of research investment and over 20 years of experience in diagnosis have developed Exopol and its activity what has led the launch to the market of our innovative and easy to use qPCR kits which show relevant advantages as the reagents are already stabilized in the wells of an easy to cut plate which is not necessary to keep frozen.

GD ANIMAL HEALTH

GD Animal Health is a leading organisation in animal health and animal production. It supports industrial customers, governments, veterinarians and farmers by providing animal health programmes, consultancy and laboratory diagnostic services. GD Animal Health is unique in combining expertise in the field of animal health management with expertise in laboratory diagnosis. We employ poultry, ruminant and pig experts as well as microbiologists and chemists. In addition, GD Animal Health combines monitoring and eradication of diseases with practical research projects to identify emerging diseases and develop new laboratory tests.

GENEREACH BIOTECHNOLOGY CORPORATION

GeneReach Biotechnology Corporation is dedicated to developing, manufacturing and marketing products for applied nucleic acid detection technology, we offer pathogen detection platforms, including equipments and reagents, to multiple industries. Our products are currently in aquaculture, agriculture, companion animal and livestock industries, manufacturing in compliance with the requirements of Good Manufacturing Practices (GMP) in addition to ISO 9001:3008 and ISO 13485:2003.
HIPRA

HIPRA is a veterinary pharmaceutical company dedicated to the research, production and marketing of products for Animal Health. HIPRA stands out for its strong specialization in Biological products. We research, develop and produce live vaccines and inactivated vaccines with high added value, intended for farm animals -especially swine, ruminants and poultry- to which we apply the latest scientific advances.
HIPRA is the only Animal Health laboratory that has its own line of Diagnostic kits: CIVTEST. These reagents for veterinary diagnostics have been developed by our R&D department in collaboration with various leading laboratories and validated by our own DIAGNOS service.

IDEXX

For more than 25 years, IDEXX Livestock and Poultry Diagnostics has focused on improving the health of the world’s food animals. Personnel around the globe work closely with customers, key opinion leaders, regulatory officials and agency heads to set up diagnostic testing programs. Rigorous quality control includes ISO certification and approvals from the USDA and international regulatory agencies that ensure quality, repeatability and reliability of test results. A global distribution network, an intimate knowledge of local conditions and languages and a seasoned, experienced staff make IDEXX uniquely qualified to provide laboratories, veterinarians and producers with diagnostic tests and technologies they can trust. The IDEXX portfolio addresses more than 50 pathologies affecting bovine, small ruminant, porcine, poultry and equine species.
Livestock and poultry producers, laboratories, veterinarians and dairy processors depend on IDEXX diagnostic technologies to make confident decisions about animal health, disease management and reproductive efficiency, and to ensure consumers have access to safe, healthy food and milk. Reproducibility, reliability and accuracy are three of the reasons why more than 1 billion IDEXX tests—including dairy residue tests and milk-based diagnostics—have been run worldwide since 1985.
For more information, please visit our website at www.idexx.com/production

IDVET

Established in 2004 and located near Montpellier, France, IDvet develops, manufactures and markets immunodiagnostic tests for the detection of infectious diseases in livestock and poultry.
IDvet’s ID Screen® diagnostic kits, based on the ELISA technique, contain all the necessary reagents for the analysis of serum, plasma, meat juice or milk samples.
These high-quality tests are extensively validated, meet international standards and show innovation through simplified protocols, ready-to-use reagents, and improved test performance.

IN3DIAGNOSTICS

In3diagnostic takes its origin from scientific research carried out at academic level, in basic science as well as in knowledge of microbiology and immunology of animal infectious diseases, including zoonoses. Their components come from Department of Veterinary Science of the University of Turin, where they are carrying out their own research activity since more than 20 years.
In3diagnostic offer a platform of diagnostic products called Eradikit®, developed to improve diagnosis of infectious diseases and representing for diagnostic laboratory and sanitary authority new tools for efficient control and eradication of some of the most important animal diseases. All products are based on new generation recombinant technology, which have been validated through peer review publications on international journals and/or patented reagents.
**LIFE TECHNOLOGIES**

Life Technologies™ products harness the power of science to transform lives. As a member of the Thermo Fisher Scientific family of brands, our instruments, everyday tools and services offer high-quality, innovative life science solutions for every lab and application.

Our Life Technologies™ Animal Health portfolio offers products and services for the animal health industry, designed to help understand diseases and the well-being of livestock communities at a more fundamental level.

Our range of diagnostic tools including ELISA, PCR test systems and sample preparation solutions covers most economically important diseases across all major production animal species.

The entire range of Prionics® farm animal diagnostic solutions has recently become part of the trusted Life Technologies™ product portfolio.

We believe that better diagnostics equals better animal health. Visit lifetechnologies.com to learn more.

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**PRIONICS**

Founded in 1997, Prionics headquartered in Zurich, Switzerland, is now part of Thermo Fisher Scientific and the Life Technologies brand. As part of the strongest, broadest global portfolio of diagnostic solutions and services for farm animals, customers now have access to a comprehensive selection of innovative diagnostic tools for many additional diseases more quickly, efficiently and accurately under the Life Technologies brand.

PrioCHECK™, BOVIGAM™ and PARACHEK™ will continue to be manufactured in the same way and to the same high standard to which customers have become accustomed.

We are committed to providing customers with high-quality products and sustainable solutions. Focusing our expertise to discover, develop and market innovative diagnostics solutions for farm animal diseases is at the core of both Prionics and Thermo Fisher Scientific.

To learn more go to lifetechnologies.com/animalhealth.

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**QIAGEN**

QIAGEN is the leading global provider of Sample & Assay Technologies that are used to transform biological materials into valuable molecular information. Sample technologies are used to isolate and process DNA, RNA and proteins from biological samples such as blood or tissue. Assay technologies are then used to make these isolated biomolecules visible and ready for interpretation. QIAGEN markets more than 500 products around the world, selling both consumable kits and automation systems to customers through four customer classes: Molecular Diagnostics (human healthcare), Applied Testing (forensics, veterinary testing and food safety), Pharma (pharmaceutical and biotechnology companies) and Academia (life sciences research).

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**TECAN**

- Provider of instruments and workflow-solutions to the world’s pharma, biotech, diagnostic and forensic laboratories and companies
- Our solutions accelerate, automate and enhance the processes in state-of-the-art life sciences laboratories
- Global leader in laboratory automation for 30 years
- Leading provider of OEM instruments and components
- HQ in Switzerland, 3 manufacturing and R&D sites and a software competence center
  - ~1,200 employees; maintaining a sales and service network in 52 countries
- Listed at the SIX Swiss Exchange (TECN; TECN SW)
VMRD

VMRD was founded in 1981 by D. Scott Adams, DVM, PhD, and currently employs approximately 50 researchers, lab technicians, and support personnel. From its site in Pullman, Washington VMRD develops and manufactures diagnostic test kits and related reagents for distribution in more than 55 countries. As a rapidly growing company VMRD strives to preserve its family focused culture and core values of integrity and quality. Its mission to provide high quality products, services and support for customers and a harmonious and rewarding work environment for employees reflects and enforces the company’s market reputation for delivering best in class products with a uniquely personal touch. As a result of this clear focus VMRD has a global impact on improvements in animal welfare through the diagnostic laboratories, animal producers, government agencies and veterinarians who use its products. Visit www.vmrd.com for more information.

ZOETIS

Zoetis discovers, develops, manufactures and commercializes a diverse portfolio of animal health medicines, vaccines and diagnostics. Zoetis is a 9,800 employees company with 60 years of experience in animal health, selling products in more than 120 countries worldwide. The company has 28 manufacturing sites, all dedicated to delivering a reliable supply of quality products. R&D team is more than 1,100 employees, delivering products for 8 species : cattle (dairy and beef), swine, poultry, sheep, fish, dogs, cats and horses.
With reliable, accurate and fast results, Diagnostics from Zoetis enable our customers to make better, more-informed decisions. We provide advanced, easy-to-use, cost-effective testing tools and resources across multiple species. Backed by a long history of quality, innovation and expert technical support, Zoetis offers a range of diagnostic products to complement the full portfolio of products and services from Zoetis. Our comprehensive animal health solutions support our customers from detection through treatment, prevention and control. Commercial and technical teams from Zoetis will help customers to implement a tailored solution from our complete suite of diagnostics, treatments and vaccines to improve animal health and business performance.
### Sunday October 12th

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<td>18.00-19.30</td>
<td>Welcome reception at the congress venue</td>
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### Monday October 13th - Auditorium

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<td>Registrations</td>
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<td>Opening Ceremony</td>
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<td>Coffee break and poster session</td>
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<td>Oral Presentations - Animal Diseases, Bacterial</td>
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<tr>
<td>14.00-14.45</td>
<td>Oral Presentations - Animal Diseases, Viral</td>
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<tr>
<td>14.45-16.15</td>
<td>Oral Presentations - Animal Diseases, Viral</td>
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#### Session  
**GENERAL SESSION**  
(All aspects of veterinary diagnostics)

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<td>10.15-10.45</td>
<td>Coffee break and poster session</td>
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#### Oral Presentations - Animal Diseases, Bacterial

- **Minimum inhibitory concentration of mycoplasma species: a chance for the practitioner, a challenge for the laboratorist**
  
  Flaminio B., Fincato A., Sturaro A., Santone C., Gobbo F., Catania S.

- **Occurrence of extended-spectrum B-Lactamase (ESBL)-producing Escherichia coli in poultry and turkey farms in Italy**
  
  Lettini A.A., Cibin V., Conficoni D., Keti A., Ricci A.

- **Bactotype® map real-time PCR-combining optimized sample extraction with sensitive detection**
  
  Gaunitz C., Labitzke M., Engemann C., Schroeder C., Sasse O., Hennart S.

- **A multiplex real-time PCR for detection in bronchoalveolar lavage fluid of four bacterial agents associated with bovine respiratory disease**
  

- **Prionics®-check TB strip – a novel lateral flow device (LFD) for the detection of bovine tuberculosis**
  
  Hardegger R., Schroeder B., Kaempfer S., Singh M., Raeber A.

- **IDVET PPD tuberculin antigens and the ID screen® ruminant IFN-G ELISA: a complete solution for the diagnosis of bovine tuberculosis**
  
  Comtet L., Olaagnon L., Pourquier P., Robles-Félicité L., Lecoq A.

- **Bovine tuberculosis: real-time PCR results in Bavaria**
  

#### Lunch and poster session

13.00-14.00  
**LIFE TECHNOLOGIES AND PRIONICS SATELLITE SYMPOSIUM - (Galilei room)**  
Multi-platform testing in the diagnostic laboratory of the future  
Participation reserved only to company’s guests

14.00-14.45  
**Keynote lecture**  
HoBi-like pestivirus: a new threat to cattle industry?  
Nicola Decaro - University of Bari – Italy

14.45-16.15  
**ORAL PRESENTATIONS - ANIMAL DISEASES, VIRAL**

- **Development of a novel ELISA system for detection of antibodies to peste des petits ruminants virus (PPRV)**
  
  Llorente F., Fernandez Pino J., Fernandez Pacheco P., Jimenez Clavero M.A.

- **Broad molecular detection of flaviviruses**
  
  Yacoub A., Metreveli G., Leskova V., Widen F., Falk K., Belak S., Leijon M.

- **A new lateral flow device for specific WNV detection in biological samples**
  
15.30-15.45 Diagnostic tools for rapid detection of ASFV in blood samples: lateral flow assay and real time PCR
Redondo H., Fernández Pinero J., Gallardo C., Sastre P., Ranz A., Rodríguez M.J., Arias M., Rueda P., Sanz A.

15.45-16.00 Rapid insulated isothermal RT-PCR assay for the detection of equine arteritis virus nucleic acid in equin semen

16.00-16.15 New insights into oral fluids as a diagnosis procedure to detect and determine the prevalence of porcine respiratory and reproductive virus syndrome (PRRSV) under field conditions
Robben N., Quijada Carreres A., Vidal A., Moradell L., Fraile Sauce L.

16.15-16.45 Coffee break and poster session

16.45-18.15 ORAL PRESENTATIONS - OTHER ANIMAL DISEASES

16.45-17.00 Evaluation of two rapid scrapie ELISAS for retro-pharyngeal lymph nodes (RLN) in sheep

17.00-17.15 Implementation of a novel serodiagnostic test to measure ascaris suum infections in fattening pigs and to assess the potential impact of this parasite on farm productivity
Vandekerckhove E., Vlaminck J., Geldhof P., Tjäder L., Lindh C., Malik M.

17.15-17.30 Italian diagnostic network on cetaceans strandings: a national monitoring program to investigate the causes of death

17.30-17.45 The prevalence of mycoplasma bovis infection in the cattle from south-Eastern region of Poland
Szacawa E., Dudek K., Bednarek D.

17.45-18.00 Chemiluminescent multiplex elisa for simultaneous detection of MVV/CAE, CLA and Johne’s in goats and sheep

18.00-18.15 Evaluation of PCR methods for the molecular detection of babesia caballi and theileria equi on field samples
Bartolomé Del Pino L.E., Cersini A., Scicluna M.T., Nordini R., Manna G., Antognetti V., Autorino G.L.

18.20-19.00 S.I.Di.L.V General Meeting (S.I.Di.L.V members only)

Tuesday October 14th - Auditorium

Session

DIAGNOSTIC TOOLS IN FOOD SAFETY AND ZOONOTIC DISEASES

9.00-9.45 Keynote lecture
Stefano Morabito- Istituto Superiore di Sanità- Italy
Analytical methods for food testing: an evolving paradigm from culture to genome

9.45-10.45 ORAL PRESENTATIONS - FOOD SAFETY AND ZOONOSES

9.45-10.00 Cleaved lamin A as an efficient marker of anabolic treatment with glucocorticoids in calves
Richelmi G.B., Botta M., Baioni E., Pezzolato M., Pitardi D., Meistro S., Varello K., Maurella C., Bozzetta E.

10.00-10.15 Gone fishing! Magnetic capture and real time PCR (MC-PCR) of echinococcus multilocularis DNA in red fox (vulpes vulpes) faecal samples

10.15-10.30 Brucella suis biovar 2: genetic assessment in Italy

10.30-10.45 Application of the enferplex caprine TB test to a breakdown herd in the UK

10.45-11.15 Coffee break and poster session

Session

QUALITY ASSURANCE AND AUTOMATION
(Standardisation and validation of diagnostic tools, ring trials, robotisation)

11.15-12.00 Keynote lecture
Paul in ‘t Veld - Food and Consumer Product Safety Authority - the Netherlands
Quality Assurance: how to produce reliable test results
### 12.00-13.00  ORAL PRESENTATIONS - QUALITY ASSURANCE AND AUTOMATION

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<td>The network of Italian laboratories for oncology in veterinary medicine: harmonizing diagnosis and data sharing</td>
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### Lunch and poster session  13.00-14.30

#### TECAN Sponsored Symposium (Auditorium)

New trends in veterinary laboratory automation: sample tracking, FDC/SAR automatic analysis and Mass spectrometry sample preparation

Participation reserved only to company’s guests

### 14.30-16.15  ORAL PRESENTATIONS - MOLECULAR DIAGNOSTICS AND EPIDEMIOLOGY

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### NOVEL TECHNOLOGIES IN DIAGNOSTICS (OMICS and biomarkers, whole genome sequencing, molecular typing)

**Wednesday October 15th - Auditorium**

**9.00-9.45**

**Keynote lecture**

*Identification of pathogens by whole genome next generation sequencing*

Marc Eloit – Institut Pasteur, France

**9.45-11.15**

**ORAL PRESENTATIONS - NOVEL TECHNOLOGIES IN DIAGNOSTICS**

**9.45-10.00**

*Virulence of Salmonella Typhimurium influences the shift of porcine intestinal microbiota composition upon experimental infection*


**10.00-10.15**

*A novel parvovirus from horse with polyarthritis*


**10.15-10.30**

*Neurotropic astrovirus in European cattle with non-suppurative encephalitis*


**10.30-10.45**

*Next generation sequencing of the aleutian disease virus genome*

Hagberg E.E., Fahnøe U., Struve T., Gorm Pedersen A.

**10.45-11.00**

*Genome-based phylogenetic analysis of O26 verocytotoxin-producing Escherichia coli*


**11.00-11.15**

*Microarray immunoassays on optical compact disks for allergen-specific IGE detection in veterinary allergy*

Rivas L., Morais S., Venteo A., Maquieira A., Sanz A.

**11.15-11.45**

*Closing ceremony and Farewell coffee-brunch*
KEYNOTE SPEAKERS

**DECARO NICOLA**

Born in Bari (Italy) on November 11, 1973. Graduated in Veterinary Medicine, marks 110/110 (top grades cum laude). Doctorate fellowship in Infectious pathologies of carnivores (2004). Assistant (2002-2004) and Associate (2005-current) Professor of Infectious Diseases of Animals (SSD VET/05), Department of Veterinary Medicine, University of Bari, Italy. Visiting Scientist at the Virology Divisions of the Faculties of Veterinary Medicine of Zurich (2003), Giessen (2004), Utrecht (2005), and at the National Animal Disease Center, USDA, Ames, IA (2013). PhD degree, Utrecht University, The Netherlands (2009). Director of the Specialization School of Infectious Diseases of Animals (2008-current). Member of the editorial boards of Veterinary Microbiology, Veterinary Immunology and Immunopathology, Journal of Veterinary Diagnostic Investigation, Journal of Virological Methods, Journal of Microbiological Methods, Virus Genes, Veterinaria and AIVPA Journal. Author/coauthor of 280 scientific manuscripts (167 on peer-reviewed international journals); h-index = 32.

**ELOIT MARC**

Laboratory for Pathogen Discovery
Institut Pasteur, 75015, PARIS
Marc Eloit has received his DVM from the Veterinary School of Maisons-Alfort and his PhD from University Paris VI. He is Professor of Virology and the former head of the Unit of Virology in Veterinary School of Maisons-Alfort. He joined in 2008 the Institut Pasteur to develop a program of Pathogen Discovery based on High Throughput Sequencing (HTS). He is also the chairman and the CSO of Pathoquest, a spin-off of Institut Pasteur he has founded in 2010, which is dedicated to the identification of pathogens using HTS.

**MCGIVEN JOHN**

NAME: John McGiven
Date of birth: 22/08/73
Position: Senior Research Officer. OIE/FAO/WHO Reference & Collaborating Centre for Brucellosis

Educational and Professional Qualifications
2009-2014: PhD (Biochemistry), Imperial College London.
1999-2001: MSc (Immunology), University of Surrey
1991-1994: BSc (Zoology), University of Liverpool.

Relevant Employment History
2009-to date: Senior Research Officer. Brucellosis Reference Laboratory, Animal Health Veterinary Laboratories Agency (AHVLA).
2003-2009: Higher Scientific Officer. Laboratory Testing Department, Veterinary Laboratories Agency (now AHVLA).
1996-1998: Assistant Scientific Officer. Immunodiagnostic Unit, Veterinary Laboratories Agency

Relevant Publications
- McGiven, J., 2013. New Developments in the immunodiagnosis of brucellosis in livestock and wildlife. Rev Sci Tech (manuscript accepted for publication in brucellosis special edition in April 2013)

Selected Presentations

MORABITO STEFANO
Stefano Morabito is senior scientist at the Unit of Food-borne Zoonoses of the Department of Veterinary Public Health and Food Safety of the Istituto Superiore di Sanità in Rome. The Unit acts as the National Reference Laboratory for E. coli for clinical and veterinary aspects and, since 2006, it has been designated European Union Reference Laboratory (EU RL) for E. coli by the European Commission. He coordinates the activities of methods development and research studies on the pathogenicity of verocytotoxin-producing Escherichia coli (VTEC). He is member of the CEN TC 276 WG dealing with microbial contamination of food and of the ISO SC9. His main research areas include the molecular bases of virulence in VTEC O157 and other VTEC, with particular emphasis on the genomic asset of the strains causing severe disease in humans. His area of expertise include the mobile genetic elements vehiculating the virulence determinants and their evolution and distribution in the different E. coli pathogroups. Other research areas include emergence and phylogensis of the different VTEC clones as well as studies on the characterisation of bacterial toxins and on their capability to disseminate among the different E. coli groups. He is author of about 50 peer-reviewed publications and acts as reviewer for a number of scientific journals and is the Editor of the book “Pathogenic Escherichia coli, molecular and cellular microbiology”, published by Caister Academic press. He is also in the editorial board of the scientific journal Medical Microbiology and Diagnosis.

IN’T VELD PAUL
Paul in’t Veld studied Food Technology at the Agricultural University in Wageningen (The Netherlands) from 1979 to 1987 and specialised in food microbiology and food chemistry. Obtained his PhD in 1998 on the topic: The development and evaluation of microbiological reference material for food microbiology.
Worked, from 1987 to 1999, at the National Institute of Public Health and the Environment (RIVM) mainly on reference materials. Since 1999 he is working at the Netherlands Food and Consumer Product safety Authority (NVWA), the competent authority in The Netherlands. His activities at the NVWA are related to standardisation of methods in general (more specific in validation/verification of (alternative) methods as the convenor of ISO TC 24/SC9/WG3), to coordinate method development activities and support the organisation with microbiological advice on methods. Besides this he is a member of AOAC RI board of directors and a technical assessor for various accreditation bodies.
**Hobi-like pestivirus: a new threat to cattle industry?**

**Decaro, N., Buonavoglia, C.**

Department of Veterinary Medicine, University of Bari, Valenzano (BA), Italy, e-mail: nicola.decaro@uniba.it

Cattle, Hobi-like pestivirus, diagnostic and prophylactic implications

**Introduction**

Bovine viral diarrhoea virus (BVDV) infections in ruminants are associated to a variety of clinical forms, including subclinical infections, immunosuppression, respiratory distress, gastroenteritis, reproductive failures, haemorrhagic disease and mucosal disease (MD) (1). BVDV (family Flaviviridae, genus Pestivirus) is a single-strand, positive-sense RNA virus, whose genome encodes for a polyprotein (NH₂-Npro-C-Eᵐᵒᵗ-E1-E2-p7-NS2-3-NS4A-NS4B-NS5A-NS5B-COOH), which is processed by viral and cellular proteases, thereby generating structural and non-structural products. The single large open reading frame is flanked by the 5’ and 3’ untranslated regions (UTRs).

On the basis of the ability to cause cytopathic effect in the infected cell cultures, two BVDV biotypes are known, cytopathogenic (cp) and non-cytopathogenic (ncp), both involved in the pathogenesis of MD, a fatal outcome of the BVDV infection in persistently infected calves. Typically, an animal affected by MD is infected by both cp and ncp viruses, which are called a virus pair (2). Cp strains have been reported for different pestivirus species, including Hobi-like pestivirus (3).

Based on the current nomenclature of the International Committee on Taxonomy of Viruses (http://www.virustaxonomyonline.com), the genus Pestivirus consists of four recognized species, BVDV-1, BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV) (4). Four additional Pestivirus species have been proposed but remain officially unrecognized (Fig. 1), i.e., Pestivirus of giraffe, associated with abortion in multiparous cows of the Italian prototype strain is able to infect all those species, although only ruminants displayed clinical signs and virus shedding (7). Additional Hobi-like viruses were associated to abortion in multiparous cows of the same herd (8) and to respiratory disease in cattle of a neighbour Italian region (9). In Italy, infection of cattle with Hobi-like virus resulted in the birth of persistently infected (PI) calves (10). More recently, a case of mucosal disease in a PI calf has been reported in that country (11). Evidence of Hobi-like virus in Asia has been reported. Although no clinical sign was noted, seroconversion to Hobi-like viruses were observed in some dairy herds in Thailand (12). In Bangladesh, Hobi-like viral sequences were detected in samples from animals displaying diarrhoea, respiratory distress and/or fever (13).

**Impact on pestivirus testing**

The emergence and spreading of Hobi-like pestivirus have raised several concerns about the ability of commercial and laboratory diagnostic assays to efficiently detect the virus or its antibodies. ELISA kits commercially available for BVDV serology have been reported to be poorly sensitive when used to detect Hobi-like virus antibodies, unless moderate to high antibody titres are present. In addition, due to the antigenic cross-reaction among ruminant pestiviruses, ELISA testing is not able to discriminate between BVDV-1/2 and Hobi-like antibodies. Exposure to this emergent pestivirus can be assessed by virus neutralisation (VN) by evaluating the antibody titres against the different viral species, although this method is cumbersome and not suitable for extensive surveillance programs (5). Antigen capture enzyme-linked immunosorbent assays (ACE) are routinely employed for detection of BVDV PI calves, as well as of acutely infected animals. However, different positivity rates were obtained using these assays according to the viral protein that is detected (Eᵐᵒᵗ or NS3) (14).
Since Hobi-like viruses are easily cultivated in vitro, virus isolation appears as a valid diagnostic tool, although post-testing through immunofluorescence, immunoperoxidase or RT-PCR is required in the case of noncytopathogenic strains and there is no chance to identify the pestiviral species (5).

RT-PCR methods used in BVDV surveillance programs either do not detect at all or have a low sensitivity in detecting the new pestivirus, which could be in addition mistyped as BVDV-2. A real-time RT-PCR (qRT-PCR) assay has been established for identification of Hobi-like strains (15), but this method is not able to detect simultaneously BVDV-1/BVDV-2 which should be required for extensive use in eradication programs. Extensive testing of experimentally-generated Hobi-like PI animals showed that BVDV qRT-PCR, ACE, and immunohistochemistry tests had higher levels of detection compared to Hobi-like specific assays, although the lack of differentiation between BVDV and Hobi-like viruses would make these tests of limited use in a Hobi-like PI control and/or surveillance (14). With the aim to overcome the limitations of diagnostic methods, a nested PCR (nPCR) assay was developed which is able to detect and type all bovine pestiviruses, including the new species (16) (Fig. 2).

**Fig. 2.** Gel electrophoresis of products obtained from RT-PCR (lines 2-5) and nPCR (lines 7-10) assays for detection and typing of bovine pestiviruses (adapted from Ref. 16). Line 1, marker GeneRuler 100bp DNA Ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany); Line 6, marker GeneRuler 1kb DNA Ladder (MBI Fermentas GmbH); lines 2, 7, BVDV-1 strain NADL; lines 3, 8, BVDV-2 strain 232/02; lines 4, 9, Hobi-like strain 1/10-1-Italy; lines 5, 10, negative control.

Impact on pestivirus vaccination

Despite the extensive use of vaccination, pestiviral infections in cattle are still widespread especially in areas where there are no specific control programs. BVDV vaccines contain BVDV-1, BVDV-2 or both antigens, whereas to date, there are no specific vaccines against Hobi-like viruses. Several studies have been carried out to evaluate the cross-reactions existing among bovine pestiviruses. All studies demonstrated that there is only a partial cross-neutralisation between extant BVDVs and the emergent Hobi-like strains. Animals immunised or experimentally infected with BVDV displayed very low VN titres against Hobi-like pestivirus and viceversa. The poor serological cross-reactivity existing between BVDV and the novel pestivirus raised some concerns about the ability of currently available vaccines, mostly containing BVDV-1, to protect effectively against the latter virus (17–19). However, in-vivo cross-protection studies through vaccination of animals with BVDV and challenge using the heterologous strain are needed to address definitively this issue.

Conclusions

Pestivirus infections are responsible for dramatic economic losses for cattle industry due to the negative impact on the animal productions. In Italy, there are scarce data related to the economic losses caused by BVDV infections, but according to what reported for other European countries, these losses should be considerable. A study on the economic impact of BVDV infection carried out in Denmark has showed that these economic losses range between 10 and 40 million US dollars per million of newborn calves (20). The extent of economic losses due to Hobi-like virus infection and the prevalence of exposed animals are as yet unknown and, thus, are goals for future investigations. Further, there is a need for improved diagnostics, as the lack of accurate diagnostic tools for virus or antibody detection and the scarce information regarding the clinical presentation of the disease may lead to misdiagnosis at both the field and laboratory levels.

References


IDENTIFICATION OF PATHOGENS BY WHOLE GENOME NEXT GENERATION SEQUENCING

Marc ELOIT\textsuperscript{1,2}

\textsuperscript{1} Institut Pasteur, Laboratory of Pathogen Discovery, Department of Virology, 28 rue du Docteur Roux, F-75724 Paris, France
\textsuperscript{2} PathoQuest, 28 rue du Docteur Roux, F-75724 Paris, France

As in other medical fields, the availability of next generation sequencing (NGS) techniques is about to revolutionize diagnostics of infectious diseases. The demonstration of the microbial origin of diseases and their diagnosis were initially based on the demonstration of the presence of a given pathogen in a given clinical sample, and was first dominated by culture assay for bacteria and later for viruses. These techniques do not advance prior hypotheses regarding the causative agents except their cultivability. They have been progressively complemented and sometimes replaced by nucleic acid-based tests like PCR. Several strategies have been developed to broaden the range of detection including bacterial typing by sequencing the 16S gene or multiplexed PCR assays. An alternative strategy takes advantage of the increasing availability and speed and decreasing cost per base of NGS offered by deep sequencing machines. It is now possible to use the tools of metagenomics, which is the study of the microbial genetic sequences recovered directly from a given human, animal or environmental sample. In this setting, the sequence of all the nucleic acid (NA) species of the sample are determined and compared with those in databases. This technology has first been used to describe the complexity and the dynamics of microbiomes from different origins. It has also been used to discover new infectious agents. De novo assembly of full length genomes of pathogens can sometimes be achieved directly from the samples, and if not large partial sequences can be subsequently completed by using classical molecular biology tools. Frequently, such metagenomic study uncovered known but unexpected viruses, phages, bacteria, parasites or fungi, which paves the way to application in the field of diagnosis of infectious diseases. Some applications for WG-NGS in microbiology – pathogen discovery, study of variability, surveillance of vectors – have already emerged and some of them will be presented.

In principle, such a whole genome NGS (WG-NGS) would be advantageous also in clinical diagnostics, as there is no need to design specific primers to pre-amplify target sequences. This avoids the very hard work consisting of designing several tens or hundreds of specific primers able to target multiple pathogens. These advantages, however, come with several drawbacks. The main one is that random amplification, currently indispensable for all available sequencing technologies, also amplifies host NA, meaning that searching for microbial NA is like looking for a needle in a haystack. The microbe vs. host NA ratio must therefore be increased using different strategies. Nevertheless, this procedure still requires high depth sequencing, at least if an analytical sensitivity similar to that of diagnostic PCRs is expected. Also, good genome coverage is necessary to predict phenotypes such as resistance to antimicrobials or virulence, as loci of interest are not specifically targeted and success in obtaining the necessary genetic information is unpredictable when partial sequences are acquired.

The analytical sensitivity of WG-NGS is not as easy to evaluate as that of PCR, as it is more critically influenced by matrix properties. In particular, the quantity of host NA, as well as its physical state or association with proteins, may complicate its elimination before sequencing. Also, the analytical sensitivity critically depends on the depth of sequencing. Increasing the depth of sequencing for an optimized sample preparation can decrease the level of detection down to $10^2$ to $10^3$ gc/mL, within the range of most homemade PCRs. Developing a WG-NGS diagnostic pipeline critically relies on two partly interdependent criteria: time to results and database exhaustiveness.

The requirements are not the same for pathogen discovery, when the range of detection should typically include the unknown, and medical diagnosis. In this latter case, it is more important to screen samples against a curated database of known pathogens that could be of interest for the physicians. Typical blast analysis of hundreds of million of reads after de novo assembly into larger contigs against the whole NCBI databases using relaxed criteria, which is classical in pathogen discovery, is too time- and resource-consuming to be used in diagnostics. By contrast, stringent mapping of non-assembled reads on a comprehensive database of pathogens, together with the progressive increase of read length permitted by the evolution of sequencers, speeds up the overall process down to a few hours. Time from sample to results can thus be 2 days or even less. In conclusion, usage of WG-NGS is currently expanding and is progressively shifting from a research tool to a diagnostic tool that may be used in emergency situation like animal or human disease outbreaks or in critical care in human medicine.
NEW DIAGNOSTICS AND VACCINES FOR BRUCELLOSIS - SWEET SOLUTIONS TO STICKY PROBLEMS

John McGiven

Animal Health and Veterinary Laboratories Agency AHVLA, UK

Brucellosis is one of the world’s most significant zoonosis and is caused by infection with members of the genus *Brucella*. These have a cell wall characteristic of Gram negative bacteria including lipopolysaccharides and, in the most significant *Brucella* species, O-polysaccharide (OPS). The major economic and health impacts of the disease arise from livestock, in particular ruminants and swine, where the main clinical feature is reproductive failure. The principle source of infection in the general human population is most often via ingestion of unpasteurised dairy products. Serology is the most cost effective means of disease detection but has significant imperfections including false positive serological reactions (FPSRs) due to antibodies that are raised against other Gram negative bacteria in possession of OPS structures similar to that of *Brucella*. Serology with non-OPS antigens has been largely ineffective and alternative approaches such as bacterial culture, PCR or measurements of cell mediated immunity are impractical, ineffective or unproven.

The OPS from *Brucella* is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyl (D-Rha4NFo) that are variably α-(1®2) and α-(1®3) linked. This structure contains some epitopes that are shared with the OPS from other organisms, notably *Yersinia enterocolitica* O:9, and some that appear to be unique. Previous attempts to harness the unique epitopes using competitive ELISA failed to resolve FPSRs because the unique and common epitopes overlap on the naturally produced antigen causing steric hindrance of specific antibody binding. We hypothesised that the use of smaller, discrete epitope antigens, would improve the specificity of serodiagnosis.

Oligosaccharides were derived from the OPS of *B. abortus*, *B. melitensis* and *Y. enterocolitica* O:9 by partial acid hydrolysis. These were separated and analysed by chromatography with on-line mass spectrometry (MS). OPS specific antibodies were used to select from this pool of oligosaccharides and those captured were evaluated by graphitised carbon chromatography with on-line MS. On the basis of the MS evidence a series of oligosaccharide conjugates comprised of between nine to two D-Rha4NFo units, each containing a single α-(1®3) link with a variable number of α-(1®2) links, were synthesised. These were applied as antigens in ELISA and evaluated for their ability to sensitively and specifically detect anti-*Brucella* antibodies within three different populations of bovine serum: those that were true positive, false positive or true negative according to conventional serology.

The results not only demonstrated that antibodies very effectively bind very small antigens but that these smaller antigens, with diminishing numbers of α-(1®2) links, provide superior capability to differentiate between the true and false positive sera. This discovery also presents an exciting opportunity for the generation of novel vaccines against brucellosis by solving a long standing paradox such that we may now simultaneously exploit the properties of OPS as both the protective antigen and a specific DIVA diagnostic.
Microbiological food testing is based on the use of analytical methods aiming at culturing the pathogens. In spite of the massive developments in the design of methods and technologies for the identification of bacterial pathogens, the field of food testing failed to adapt yet, mainly due to the need of funnelling the methodologies to be included into the food safety regulations towards the channels of standardization.

In recent times, the paradigm of microbiological hazards in food, involving pathogens that are efficaciously identified by their cultural characteristics, has been shaken by the appearance of microorganisms that cannot either be cultivated or distinguished by the non-pathogenic strains belonging to the same species. The appearance of such hazards, and the need to shorten the time required for the pathogens’ identification made the analytical approach for food testing to vault in a new direction. Methods based on the PCR identification of the pathogens’ presence entered the food testing laboratories as screening tools facilitated by the availability of commercial reagents validated against the cultural reference methods.

In the last couple of years international standards based on the PCR appeared in the ISO catalogue and have been included in the regulations concerning the food safety. This marked the conversion of this technique from a technology supporting the cultural approach to a reference technology in its own right.

Although representing a substantial advance in the food testing, in respect to the rapidity and sensitivity, the PCR-based detection of pathogens in a food matrix has drawbacks, represented by the possibility that the indirect evidence of the presence of a pathogen in a food matrix might not indicate a real threat for the consumer’s health. This criticality is well represented by the horizontal method for detection of STEC in food, ISO/TS 13136:2012. The method is based on the PCR detection of a number of genes including genes associated with both the virulence and the serogroups of the STEC mainly isolated from severe cases of human disease. As a whole the method requires the detection of eight genes and necessitates the cultural isolation of single live colonies producing the same PCR signals generated during the screening. In fact, the possibility exists that a composite signal generated by the amplification of virulence and serogroup-associated genes could be made up by single signals coming form different bacterial cells present in the enrichment culture. Additionally, dead cells or free DNA present in the matrix could also generate signals. The ISO/TS 13136:2012 is iconic of the urge to implement new technologies while limiting the production of results based on the indirect evidence of the presence of a microbiological hazard in a food and jeopardizing the sustainability of the food production processes.

Undoubtedly, the molecular approach had an impact on the food testing already. Great efforts are being devoted to make the approach definitely culture-free. One promising strategy is represented by the use of intercalating agents to be added to the enrichment cultures before DNA extraction and amplification. Commercial kits are populating the catalogues of a few companies and the trend seems to be positive.
QUALITY ASSURANCE: HOW TO PRODUCE RELIABLE TEST RESULTS

Paul in ‘t Veld1

1 Netherlands Food and Consumer Product Safety Authority
Quality assurance, accreditation, validation, verification

Introduction
Reliable results are of utmost importance for any laboratory and this is more than just the proper application of an analytical method. A laboratory must be able to demonstrate the quality of their work in order to give their clients or governmental organizations confidence in the results. This can be done by using (in- or externally) validated methods and by applying a quality assurance system such as described in ISO 17025 for accreditation of testing laboratories.

Validation of methods
The first step will be the selection of the method. Does the lab wants to use an (inter)national standardized method, an alternative (rapid) method or an in house developed method? The selected method should be preference be validated externally. Most of the standardized method are however not validated as they are consensus methods. Currently a lot of emphasize is given to validation of reference methods by the EU. Most of the alternative (proprietary and rapid) method have been (independently) validated as it is of the interest of the test kit producer. For this the ISO 16140 was developed. Currently this standard is under revision (expected to be published in 2015). The basis of this type of validation is a comparison of the results of the alternative method against the reference method. The last being a standardized method in most cases. When an in-house method is going to be used a big burden on the laboratory would be to validate this method. Currently protocols for this are developed in order to have a similar (minimum) approach for the labs which is also of importance for accreditation organizations. These protocols are developed by ISO/TC34/SC9/WG3.

Verification of methods
Verification of methods intends to provide evidence that a laboratory is capable of applying a validated method correctly in its laboratory. Protocols for this are developed as well. Many methods in food microbiology are validated for ‘all foods’ or a ‘broad range of foods’, however the number of matrices used in validation is quite limited. Therefore part of the verification procedure will target matrices commonly used in the laboratory but not included in the validation study.

Routine application of the method
The validation and verification of methods give evidence that the method can be applied correctly by the laboratory. This is in most cases a one-time event. Day to day demonstration that the performance is constant over time is also needed. For this quality assurance sample can be used, being spiked samples a low level for presence/absence methods or at countable levels for enumeration methods. For the enumeration method results can be plotted in so called control charts to get a visual impression of the performance over time with the possibility to set limits. This is the so called ‘first line quality control’. Doing so it will not be possible to judge the trueness of the method as there is no comparison with external laboratories. For this participation in proficiency studies (p.t.) is needed. Nowadays more and more commercial organizations are offering a range of matrices and microorganisms that can be tested using p.t. The choice of the provider is crucial as they provide different types and number of samples and their participants in number can vary.
ORAL PRESENTATIONS
ANIMAL DISEASES, BACTERIAL
MINIMUM INHIBITORY CONCENTRATION OF MYCOPLASMA SPECIES: A CHANCE FOR THE PRACTITIONER, A CHALLENGE FOR THE LABORATORIST

Flaminio B. [1], Fincato A. [1], Sturaro A. [1], Santone C. [1], Gobbo F. [2], Catania S. [1]

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Keywords: Mycoplasma, Minimum Inhibitory Concentration, MIC

INTRODUCTION:
Mycoplasmas are small organisms lacking in cell wall that are considered “fastidious” organisms, because their isolation is difficult and time consuming. Mycoplasmas infection can be associated with important diseases in several animal species, such as respiratory and articular disease and also with infertility, and in these cases an antibiotic treatment could represent an useful tool for the reduction of symptoms and economic losses. The choice of the appropriate drug should be done following the indication given by an AST (Antimicrobial Sensitivity Test), in order to have the knowledge of the sensitivity of the field isolate to specific antibiotics; the MIC (Minimum Inhibitory Concentration i.e. the lowest concentration of an antimicrobial able to inhibit the growth and/or metabolism of a microorganism in vitro) with microdilution method results the best technique applicable to these pathogens. Despite time required and the difficulties in its execution it is an important resource for the practitioner.

MATERIALS AND METHODS:
Different pathogenic mycoplasmas strains isolated from animals (avian, ruminants) belonging to different productive categories were firstly identified by a specific 16S-PCR-DGGE (Denaturing Gradient Gel Electrophoresis). Each strain was subjected to three serial cloning in liquid and in solid phase, then 10 ml of inoculum for the MIC test was produced in culture medium for mycoplasmas (Mycoplasma Experience without inhibitors®, Reigate, UK), sub- aliquoted and stored at -80°C for at least 24 hours. The titration was carried out in 96-well plates using a statistical method (MPN, Most Probable Number): the result is expressed as UCC/ml (Unit Changing Color). The MIC inoculum (10³-10⁵ UCC/ml) was dispensed into 96-well MIC commercial plates (Merlin Diagnostika®) and incubated at 37±1°C until detection of growth.

RESULTS:
In the years 2013-14 the MIC tests were performed for 170 Mycoplasma spp. isolates (113 from poultry and 57 from ruminants). In particular we tested 80 M.synoviae (MS) strains, 26 M.gallisepticum (MG) and 54 M.bovis. For MS we noticed a good susceptibility to doxycycline, oxytetracycline, lincomycin, tylosin and tilmicosin and resistance to erythromycin and enrofloxacin. About MG, it was observed the efficacy of tetracyclines and macrolides in the majority of the strains. For M.bovis high MIC values were detected for macrolides and lincomycin whereas good efficacy was observed for enrofloxacin, tetracyclines and florfenicol.

DISCUSSION AND CONCLUSIONS:
The MIC with microdilution method allows to quantify the in vitro sensibility or resistance to antimicrobials of “fastidious” organisms like mycoplasmas. It also has the advantage of testing a strain with several antimicrobials at the same time and defining its sensitivity profile. Moreover the study of MIC in micro and macro areas permits to reduce the selective pressure against certain drugs: our data concerning recent MG infections suggest a return to macrolides efficacy after their replacement with tetracyclines. In conclusion, despite the technical difficulties encountered in mycoplasmas isolation and MIC performing and their time-consuming that represent a challenge for the laboratories, this AST is a chance for the practitioner, because it provides useful information for the best drug choice and the correct therapeutic regimen that have to be applied during a specific outbreak.

This work was supported by the Italian Ministry of Health with the RC IZSVE 15/10 “Le micoplasmosi nel settore avicolo industriale: studio e messa a punto di nuove metodiche e protocolli diagnostici al fine di valutare e studiare il differente ruolo dei ceppi circolanti tra le differenti tipologie di produzioni avicole”.

REFERENCES:
02

OCURRENCE OF EXTENDED-SPECTRUM β-LACTAMASE (ESBL)- PRODUCING ESCHERICHIA COLI IN POULTRY AND TURKEY FARMS IN ITALY

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Keywords: antimicrobial resistance, commensal bacteria, food safety, public health,

INTRODUCTION:
Over the last decade, the increased occurrence of bacterial strains producing extended-spectrum beta (β)-lactamases (ESBLs) and/or acquired AmpC β-lactamases has gained worldwide attention, thus being an important reason of therapy failure and having serious consequences for public health. In particular, commensal bacteria as Escherichia coli, representing a complex reservoir of genes responsible of these resistances, could be involved in the transfer of antimicrobial resistant determinants from food-producing animals to humans via the food chain. Therefore, the Biohaz Panel of EFSA [1], which ranked the most relevant poultry meat-borne hazards, categorized ESBL/AmpC gene-carrying E. coli as of medium to high public health relevance for poultry meat, together with Salmonella and Campylobacter.

Data on the occurrence of this phenomenon in Italy are quite poor, thus, the aim of this study was to conduct a randomized survey for assessing the presence of ESBL-producing E. coli in broilers and fattening turkeys flocks and to further characterize the isolates in terms of antimicrobial resistance profiles.

MATERIALS AND METHODS:
A sampling scheme was developed aiming to assess the occurrence of ESBL-producing E. coli in broiler and fattening turkey farms located in the Veneto region, taking into account the number of farms and available preliminary information on the phenomenon frequency. For each farm, selected according to randomized criteria, one flock was submitted to sampling by using two pairs of boot swabs, as performed in the framework of salmonella control programs (Commission Regulation 200/2012 and 1190/2012). The sampling campaign was carried out from January 2013 to May 2014.

Each sample (two pairs of boot swabs) was incubated at 37° C per 18 ± 2 hours in 250 mL of APTS. 1 µL of pre-enrichment was plated on two MacConkey agar selective plates (added with 1 µg/mL of ceftriaxone and cefotaxime, respectively), thereafter plates were incubated at 37°C for 24 ± 3 hours. One typical well-isolated colony per plate was confirmed biochemically.

The Minimum Inhibitory Concentration (MIC) of the presumptive ESBL-producing E. coli isolates was defined by broth micro-dilution according to ISO standards [1] in combination with the semiautomatic Sentititre System (Sensititre, Trek Diagnostic Systems, UK). The following antibiotics, in accordance with the EFSA recommendations [2] were included in the panel: cefepime, cefoxitin, imipenem, meropenem, cefotaxime, ceftazidime, cefotaxime/clavulanic acid and ceftazidime/clavulanic acid. The strain was considered ESBL-producing in case of “cephalosporin MIC/cephalosporin added with clavulanic acid MIC” resulted greater than 8 [3].

RESULTS:
Samples were collected from 51 and 45 chickens and fattening turkeys farms, respectively.

Out of the 167 E. coli selected from the MacConkey agar plates, 144 were confirmed ESBL-producing, 73 from broilers and 71 from turkeys. Thirty-five (78%) and 44 (86%) of chickens and fattening turkeys farms were classified as positive for the presence of ESBL-producing E. coli, respectively.

All E. coli isolated showed high level of resistances to most of the antibiotics tested (figure 1). In particular high level of resistance to cefepime, cefotaxime and ceftazidime were observed.

DISCUSSION AND CONCLUSIONS:
These results suggest that there is a worrying rate of occurrence of ESBL-producing E. coli in broiler and turkey farms located in the Veneto region, in line with recent evidences [4, 5], suggesting an increasing trend of this phenomenon in poultry farms in European countries. The ESBL-producing E. coli isolates will be further characterized in order to identify the determinant genes responsible of these phenotypes and to better clarify the role that these food-producing animals could have in the transfer of antimicrobial resistant determinants to humans.

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**INTRODUCTION:**
Mycobacterium avium subsp. paratuberculosis (MAP) is the cause of paratuberculosis (Johne's disease), a chronic inflammatory intestinal disease of ruminants, which occurs worldwide. The purpose of this study was to increase the sensitivity of MAP detection by combining a sensitive and specific amplification and detection method with an optimized protocol for the extraction of MAP DNA from fecal samples. Culture from fecal samples is generally regarded as the gold standard for MAP detection in ruminants. However, this is labor intensive and can take up to 16 weeks. Therefore, direct fecal PCR is becoming more widely used which enables test results within hours. The challenges for extracting MAP DNA from fecal samples include MAP clusters in the sample, thick mycobacterial cell walls, and PCR inhibitors.

**MATERIALS AND METHODS:**
A special fecal sample pretreatment was developed by combining three strategies: chemical lysis (Lysis buffer), mechanical disruption (bead-beating), and heat treatment of the sample. After fecal sample pretreatment MAP DNA extraction is performed using either QIAamp® cador® Pathogen Mini Kit or the QIAamp DNA Stool Mini Kit. For amplification and detection of MAP DNA the highly sensitive and easy-to-use bactotype MAP PCR Kit was developed. The kit is a duplex real-time PCR and features a ready-to-use master mix, a heterologous extraction and amplification control and TaqMan® based chemistry that can be used on all real-time PCR cyclers commonly used in veterinary laboratories.

The high analytical sensitivity of the bactotype MAP PCR Kit was demonstrated using a titration series of in vitro DNA [106–100 copies/well] performed in triplicate and analyzed using QIAGEN’s Rotor-Gene Q, Applied Biosystems® 7500 Real-Time PCR System, BioRad CFX96 and Agilent Mx3005P. To evaluate performance of the workflow the Johne’s Disease Fecal Proficiency Panels 2012 and 2013 kindly provided by the U.S. Department of Agriculture (USDA) Veterinary Services were tested. The workflow was compared with a real-time PCR kit from supplier L after extraction using the MagMAX™ Total Nucleic Acid Isolation Kit and PCR on the Applied Biosystems 7500 Real-Time PCR System.

**RESULTS:**
The bactotype MAP PCR Kit is able to detect 5 MAP DNA copies per sample with a correlation coefficient greater than 0.998 and with high efficiency on all instruments tested. The bactotype MAP real-time workflow was evaluated using the 2012 Johne’s Disease Fecal Proficiency Panel. Samples were extracted using the new developed fecal sample pretreatment and sample extraction kits mentioned above. PCR was performed on the Rotor-Gene Q. The bactotype MAP PCR Kit correctly detected all samples of the Panel. The bactotype MAP real-time PCR workflow was further tested using the 2013 Johne’s Disease Fecal Proficiency Panel. Samples were extracted using the fecal sample pretreatment in combination with the QIAamp cador Pathogen Mini Kit. In addition the workflow was tested in comparison with a real-time PCR kit from Supplier L and magnetic-bead sample extraction using the MagMAX Total Nucleic Acid Isolation Kit on BioSprint 96. PCR was performed using the Applied Biosystems 7500 Real-Time PCR System.

The bactotype MAP PCR Kit detected all positive samples in comparison to the alternative method, which failed to detect sample number 1. Furthermore, the CT values revealed better results for 20 from 21 samples using the bactotype MAP PCR Kit.

**DISCUSSION AND CONCLUSIONS:**
QIAGEN’s workflow for detection of MAP in bovine fecal samples combines a sensitive real-time PCR with optimized sample preparation. This method combines a special fecal sample pretreatment, MAP DNA extraction using the QIAamp cador Pathogen Mini Kit or QIAamp DNA Stool Mini Kit, and MAP DNA amplification and detection with the easy-to-use bactotype MAP PCR Kit.

QIAGEN workflow for detection of MAP can detect 5 copies of MAP DNA per sample with high efficiency and weak positive samples from low shedders.

**Keywords:** Paratuberculosis, fecal sample extraction, workflow
A MULTIPLEX REAL-TIME PCR FOR DETECTION IN BRONCHOALVEOLAR LAVAGE FLUID OF FOUR BACTERIAL AGENTS ASSOCIATED WITH BOVINE RESPIRATORY DISEASE


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Keywords: BRD, Calf pneumonia, multiplex real-time PCR

INTRODUCTION:
Bovine respiratory disease (BRD) leads to many health problems in (veal) calves. The cause of the disease is multifactorial and it is believed that stress and management factors combined with infection with bacteria, viruses and mycoplasmas can lead to respiratory problems. Growing concerns about increasing antibiotic resistance in livestock and poultry urged the need for alternative strategies, for example through the application of management measures or vaccination. These approaches require knowledge on the causative agents, however, the availability of rapid and reliable diagnostic tools is limited. Therefore we developed a multiplex real-time PCR for the detection in bronchoalveolar lavage fluid (BALF) of four bacterial agents commonly associated with BRD: Pasteurella multocida, Mannheimia haemolytica, Histophilus somni and Trueperella pyogenes (1).

MATERIALS AND METHODS:
A total of 74 bacterial isolates, representing 39 different species, were available for evaluation of specificity of the multiplex real-time PCR assay. These included: 1) representatives of the target bacteria of the assay, 2) isolates associated with bovine diseases other than BRD and 3) isolates of phylogenetically closely related nontarget species. To determine the sensitivity of the multiplex real-time PCR assay, log cultures were prepared from the four target bacteria and tested in a range from 10^1 to 10^8 CFU/ml in BALF from calves reared under specific pathogen free (SPF) conditions.

BALF samples (n=30) of calves with clinical problems of BRD were examined. BALF samples (10 ml) were spun down in a centrifuge for 10 min at 4600xg and the sediment was used for bacterial culture according to the standard procedures of the laboratory. Identification of the bacteria was done by MALDI-TOF mass spectrometry. Candidate selection for probe/primer design in the V3 16S-region of the four target pathogens was done by Insignia-based querying of a purpose-built database of bacterial 16S rDNA sequences (2), which were extracted from the nucleotide database at NCBI. Specific primers and TaqMan probes were further developed with the AlleleID 7 software. DNA was isolated from the sedimented BALF material using the MagnaPure total Nucleic Acid kit and tested by the multiplex real-time PCR in an ABI 7500 real-time PCR system.

RESULTS:
Results of the analysis of 74 bacterial isolates showed a high specificity of the multiplex real-time PCR; only target bacteria resulted in Ct values below 40. Spiking experiments of the four target bacteria in BALF of SPF calves resulted in positive results from at least 10 CFU/ml, both in single and in multiplex PCRs. Results of the examinations on 30 BALF samples from BRD-infected calves showed all bacteriological positive samples also to be detected by the multiplex real-time PCR (Table 1). The PCR detected a higher amount of P. multocida, M. haemolytica and H. somni in BALF samples compared to the bacteriological examination (Table 1). For T. pyogenes the number of positive and negative BALF samples was found to be similar by both examinations.

DISCUSSION AND CONCLUSIONS:
We successfully developed a multiplex real-time PCR for the detection of BRD associated bacterial pathogens in BALF of calves. The multiplex PCR showed equal or higher sensitivity compared to routine bacteriological examination. The increased detection of the bacterial pathogens by PCR might be explained by the presence of dead target bacteria in BALF, thus impeding bacterial culture. This is currently being investigated using alternative PCRs. This multiplex real-time PCR may be an important diagnostic tool for detection of bacteria commonly associated with BRD problems in calves.

REFERENCES:

Table 1: Results of bacteriological examination and multiplex real-time PCR of BALF samples of calves originating from herds with health problems related to BRD.

<table>
<thead>
<tr>
<th></th>
<th>P. multocida</th>
<th>M. haemolytica</th>
<th>H. somni</th>
<th>T. pyogenes</th>
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<td><strong>BE</strong></td>
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<td>- 5 14 19</td>
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1Bacteriological examination
05
PRIONICS®-CHECK TB STRIP – A NOVEL LATERAL FLOW DEVICE (LFD) FOR THE DETECTION OF BOVINE TUBERCULOSIS

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Keywords: Lateral Flow Device, Serological Tuberculosis Test, Strip

INTRODUCTION:
It is generally accepted that cell-mediated immune (CMI) responses are the principal and earliest immune responses to develop after infection with Mycobacterium bovis, the causative agent of bovine tuberculosis. However, even antibody responses develop considerably later than CMI responses, serological based test assays are useful tools to complete the diagnostic portfolio for bTb testing. Generally, ELISAs are suitable tools for diagnostic testing, but are relative time consuming and need to have a well equipped laboratory. Here we describe the development of a LFD assay, Prionics®-CHECK TB STRIP, to detect specific antibodies directed against MBP70 and MBP83 antigens.

MATERIALS AND METHODS:
The Prionics®-CHECK TB STRIP, in the Prionics LFD comb-format, consisting of 8 combs in a row, is suitable to be used as a Point-of-Care (POC) diagnostic application but also allows high throughput testing in veterinary diagnostic laboratories. In contrast to the design of a classical single test LFD device, the Prionics®-CHECK TB STRIP does not require a sample pad which may act as a sponge holding an excess of sample fluid. As with any LFD device, the technology is based on capillary forces that drive the sample through a porous matrix such as nitrocellulose. The design of the Prionics®-CHECK TB Priostrip encompasses three sprayed lines: the first line (the control – upper line) that captures any particle and thereby shows that reaction conditions and technology worked and the run is valid, the second (MBP70) and third line (MBP83) contain specific antigen molecules to capture antibodies which are directed against one of the two antigens.

RESULTS:
A total of 160 samples have been tested with the prototype Prionics®-CHECK TB STRIP. Fifty (50) samples derived from an Irish and ninety-nine (99) samples from a UK negative herd, defined on historical records of >5 years TB free history based on skin test and BOVIGAM® negative test results. Eleven samples provided by the AHVLA were confirmed as bTb positive reactors (skin test and BOVIGAM® positive test results). The definition of positive test result is: If either the MBP70 or the MBP83 or both sprayed antigen lines show a coloured reaction, then the test result is interpreted as positive. For a negative test result only the control band shows a coloured reaction. Based on 160 measurement points and one distinct threshold determination, the specificity (SP, N=149) of the LFD test assay is up to 99.3% (95% CI = 98.0%-100%), whereas the sensitivity (Se, N=11), tested on confirmed positive bTb reactors, is up to 90.9% (95% CI = 73.9%-100%).

DISCUSSION AND CONCLUSIONS:
The Prionics®-CHECK TB STRIP is a suitable tool to complete the diagnostic portfolio for bovine tuberculosis.

REFERENCES:
Acknowledgement
This work is supported by European Union Commission under Framework Program (FP7-SME-2013 606577) DEMO NOPERSIST.
**INTRODUCTION:**

IGRA (Interferon Gamma Release Assay) is widely used to detect the cellular response to pathogens such as Mycobacterium bovis by measuring the difference between activated and non-activated whole blood interferon gamma (IFNg) signals. IDvet offers a complete solution for the detection of bovine tuberculosis. It includes stimulation antigens and an ELISA kit for detection. The aim of this study is to assess the sensitivity and specificity of this solution.

**MATERIALS AND METHODS:**

PPDa and PPDb tuberculins (IDvet) were used as stimulation antigens. IFNg level was then determined using the ID Screen® Ruminant Interferon Gamma ELISA (IDvet). All ELISA results were standardized and expressed as sample/positive control ratios (S/P). Different freeze-dried IFNg reference materials were created from natural IFNg from different species (bovine, ovine, caprine or buffalo). The stability, repeatability, reproducibility and robustness of the ELISA itself was studied. Specificity for bovine tuberculosis diagnosis was evaluated on cattle from different breeds and countries (n=1077). Sensitivity was evaluated in comparison with confirmatory techniques (PCR, single and comparative intradermal tuberculin test).

**RESULTS:**

The ELISA reagents showed excellent stability. Repeatability and reproducibility were excellent, with CV% between 3 and 8%. Using the reference material, analytical sensitivity was found to be constant and equivalent between runs. Measured specificity was 99.0% (IC95 98.4 – 99.6%) and sensitivity was 88.3% (IC95 81.1 – 95.5%).

**DISCUSSION AND CONCLUSIONS:**

The use of the IDvet PPDs with the IDvet IFNg ELISA gave a measured test sensitivity comparable with published values. Thanks to the use of tuberculins with appropriate potencies, as well as well-defined interpretation criteria, test specificity was high. The performance of the IDvet PPDs and IFNg ELISA meets bovine TB eradication program requirements.

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07

BOVINE TUBERCULOSIS: REAL-TIME PCR RESULTS IN BAVARIA


Keywords: bovine tuberculosis, cattle, real-time PCR

INTRODUCTION:

Bovine tuberculosis caused by Mycobacterium (M.) bovis or M. caprae is a rare and notifiable epizootic disease in Germany. From 01.01.2003 to 31.12.2012 127 outbreaks were observed, thereof 70 in Bavaria [1]. Numerous cases occurred in the administrative district of Oberallgäu in Swabia. According to an emergency regulation for the prevention of bovine tuberculosis starting at 15.03.2013, cattle had to be tested via single intradermal comparative tuberculin test (SICTT). Cattle showing positive or questionable results in the SICTT had to be slaughtered or culled and organ material was tested by an official real-time PCR protocol, prescribed by the federal Friedrich-Loeffler-Institute (FLI). The regulation intends the individual testing of retropharyngeal lymph nodes, lung, gut, liver, spleen, kidney and their corresponding lymph nodes by Duplex-PCRs, leading to the execution of 22 single PCR’s per animal.

MATERIALS AND METHODS:

The FLI protocol stipulates the detection of two target genes (HELI and IS1081) as well as ß-Actin as internal control. Only when both target genes were detected the animal was diagnosed M. tuberculosis-complex-positive. In the case only one target gene was amplified the extraction and PCR were repeated. When the repeated PCR showed the amplification of both target genes, the animal was diagnosed M. tuberculosis-complex-positive, otherwise the result was classified as questionable and culture was started. Culturing of positive or questionable samples was performed in BD BBLTM MGITTM media as well as on Löwenstein-Jensen- and Stonebrink-media with incubation at 37°C for 42 days.

RESULTS:

From 15.3.13 to 31.5.14 6.905 specimens from 740 cattle were tested individually by real-time PCR. All samples derived from slaughtered or culled cattle after SICTT or from euthanized or perished cattle with suspicious lesions or cattle suspicious in meat inspection. 71 animals were tested PCR-positive for M. tuberculosis-complex, 87 questionable and 582 PCR-negative. Most frequently the lung lymph nodes (32x), followed by the mesenteric lymph nodes (31x) and lung (19x) were PCR-positive, followed by various other organs. The 87 questionable PCR-results resulted from animals who showed in one or more samples only one signal (HELI or IS1081): 51 cases; one signal and poor ß-Actin ct-values: 11 cases; and too high ß-Actin ct-values: 25 cases. From all 71 PCR-positive animals M. caprae was isolated. In 12 of the 87 questionable animals M. caprae was found, all 12 cases showed a single signal in the preceding PCR. Also 14 PCR-negative animals were examined by culture, resulting in 4 M. caprae isolates.

DISCUSSION AND CONCLUSIONS:

In the combat against bovine tuberculosis PCR is a valuable tool. Before the emergency regulation for the prevention of bovine tuberculosis from 15.03.2013 the PCR was performed supplementary to culture (except for suspicious animals derived from meat inspection). Hence the diagnostic procedure was very time consuming, but had a higher sensitivity. PCR was performed only with suspicious samples. Since the emergency regulation from 15.03.2013 the diagnosis has been accelerated, however with the disadvantages as lower sensitivity and at expense of money and manpower. For the testing of 740 cattle 6.905 samples were extracted and 13.810 PCRs were performed (calculated without repetitions). M. tuberculosis-Complex DNA was almost exclusively detected in lymphatic tissues, the lung and in organs showing lesions. If the more sensitive culture technique is regarded as secondary in the diagnosis of bovine tuberculosis the less sensitive PCR-protocol should be confined to the affected tissues.

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ORAL PRESENTATIONS
ANIMAL DISEASES, VIRAL
DEVELOPMENT OF A NOVEL ELISA SYSTEM FOR DETECTION OF ANTIBODIES TO PESTE DES PETITS RUMINANTS VIRUS (PPRV)

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INIA-CISA ~ Valdeolmos ~ Spain

Keywords: Peste des petits ruminants, PPRV, Recombinant, Ruminant, ELISA

INTRODUCTION:
Peste des petits ruminants (PPR) is an OIE listed disease affecting sheep and goat caused by PPRV, a Morbillivirus of the Paramyxoviridae. It has caused high mortalities in small ruminant populations in Africa, South Asia and China, where it is endemic (1).

Introduction and spread of PPR in Morocco in 2008 showed the capability of the illness to easily reach distant areas from its endemic distribution. Presence of the illness close to European borders has increased the concern over this disease. Consequently, surveillance programs need to be implemented in Europe using the best available serological detection methods.

With this objective in mind we have developed and standardized an immunoassay test (ELISA) for the detection of antibodies to PPRV in serum, for the application in serological diagnostic and surveillance.

MATERIALS AND METHODS:
Cloning and generation of recombinant baculovirus: Nucleoprotein (NP)-coding region from PPRV strain Nigeria 75/1 was cloned and the protein expressed using a baculovirus system (2). Briefly, viral RNA was extracted from Vero cells infected with the PPRV strain Nigeria 75/1. Complementary DNA was synthesized by reverse transcription. The complete NP-coding region was amplified, cloned and sequenced. The resultant donor plasmid was transformed in E. coli strain (DH10Bac) obtaining a recombinant bacmid. Sf9 insect cell line was transfected with the bacmid to generate the recombinant baculovirus. Recombinant baculovirus expressing PPRV NP were selected by Western blot analysis and protein identity was confirmed by MALDI-TOF.

Preparation of recombinant NP antigen: High-five insect cells were infected with the recombinant baculovirus at a multiplicity of infection of 5 and incubated at 27°C. After two days, cell culture was centrifuged and the cells were collected, resuspended in PBS and sonicated. The lysate was clarified by centrifugation and then the supernatant was used as antigen, immobilized in ELISA plates.

Indirect ELISA: ELISA plates were coated with the recombinant NP. After washing, plates were treated with blocking buffer prior to addition of sera, diluted 1:400 in blocking buffer. After incubation, plates were washed again, and protein G conjugated with peroxidase was added for 1 h. Following another washing step, the plates were revealed by incubation with chromogenic substrate. The cut-off value of the test was determined as the mean percent inhibition values of naive ruminant serum samples plus two standard deviations (SD).

Sera: A panel of 15 reference sera from goats and sheep was used for evaluation of the ELISA.

A total of 140 negative ovine sera were obtained from PPR-free areas in Spain.

Finally, 144 sera from 6 sheep experimentally inoculated with PPR strain Morocco/08 at different times pre- and post-inoculation, were used for the evaluation. These samples were obtained in the course of an experiment carried out at a bio-safety level 3 (BSL3) Containment Research Laboratory in our Institute.

RESULTS:
The results obtained indicated that this NP-based indirect ELISA format is effective for the detection of specific antibodies for PPRV.

All field sera from negative sheep (144) gave negative results, indicating a high specificity of the technique.

Analysis of experimentally infected sheep showed that the method detects antibodies 7 days after PPRV inoculation. These results are similar to the obtained with commercially available diagnostic kits.

DISCUSSION AND CONCLUSIONS:
The use of a recombinant protein obtained using the baculovirus expression system provides an abundant antigen source.

Initial results showed a high specificity and antibody detection level similar to commercial kits.

The method is simple and easy to automate and can be a useful tool for the diagnosis and serological surveillance of PPR.

Acknowledgements:
The authors thank Ana García Casado and María del Carmen Barbero for their technical assistance. This work was supported by Spanish project RTA 2011-00072-00-00 and INIA-MAGRAMA agreement EG13-020.

REFERENCES:
INTRODUCTION:

Flaviviruses are arthropod borne viruses comprising more than 70 species and more than half are serious human and/or animal pathogens. Flaviviruses are distributed worldwide and are increasingly spreading over Europe. For example, in Greece there was a WNV outbreak in 2010 (1), in Italy 2012 (2) and in Madeira a dengue virus outbreak in 2012 (3).

MATERIALS AND METHODS:

The PCR assay presented here is developed using similar techniques as previously described (4-5). Primers were designed from an alignment containing 1159 flavivirus NS5 gene sequences. Two PCRs are involved in a semi-nested format. The first PCR (pre-amplification) include a single degenerate primer pair. The second PCR include 19 tagged primers at low concentration and one labeled detection primer corresponding to the tag-sequence. The reverse primer is the same for both steps. RNA from isolates of West Nile Virus (WNV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV), Dengue Viruses (DENV1-4) and Tick borne Encephalitis Virus (TBEV) as well as the Usutu virus and Bagaza virus were used to evaluate the broad detection capacity of the assay. In addition, 66 clinical samples were used to evaluate the assay. They included human sera and urine, brain samples from birds and horses and mosquito and tick vectors.

RESULTS:

All isolates were successfully detected. The analytical sensitivity of the assay was evaluated using two different panels (WNV and DENV) from the Quality Control in Molecular Diagnostics organization (QCMD; http://www.qcmd.org/) from 2011. The detection limit was 104 copies/ml of WNV and 103 copies/ml of Dengue virus. Several tested pestiviruses, which also belong to the Flaviviridae family, and other RNA viruses such as the Chikungunya virus, which gives similar clinical symptoms as DENV, and the avian viruses APMV 2, 3 & 6, H5N1 & H7N1 and NDV gave no appreciable signal. To benchmark the present broad detection assay, flavivirus detection from the 66 clinical samples were compared with the detection obtained with dedicated TaqMan PCR assays (6-8). This showed 92% were detected with both methods. Only 68% of samples that were detected by the present method were detected by the benchmarking TaqMan systems (6-8) while three samples were negative by the former but detected by latter.

DISCUSSION AND CONCLUSIONS:

A simple broad detection method for flaviviruses was developed. It show excellent sensitivity as evaluated using the QCMD panels for WNV and Dengue virus and also high specificity using a broad range of RNA viruses including pestiviruses. The assay was successful with clinical materials varying from brain tissue to insect vectors. Compared to dedicated standard TaqMan based assays the sensitivity was superior. However, this is largely explained by one of the TaqMan system used (6) failed to detect a set of twelve samples from a recent WNV outbreak in Europe in 2008/2009 (9). Otherwise it is likely that the presently described assay has a comparable sensitivity to TaQMan PCR and the outstanding virtue of this novel method is the broad detection of this highly variable virus genus.

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A NEW LATERAL FLOW DEVICE FOR SPECIFIC WNV DETECTION IN BIOLOGICAL SAMPLES


Keywords: West Nile Virus, Flavivirus, Lateral Flow Device

INTRODUCTION:
West Nile Virus (WNV) is a member of the Flaviviridae family, genus flavivirus which includes other emerging and re-emerging mosquito-borne zoonotic pathogens such as dengue, yellow fever, Tick Borne encephalitis and Japanese encephalitis viruses. To date 6-8 different lineages of WNV have been described. Since the entrance in 1999 in the USA this virus has spread rapidly across North of America. Most WNV infections are asymptomatic in humans and horses therefore laboratory tests are essential to detect infection due to WNV. Routine surveillance consists in serological test, however seropositivity needs to be carefully interpreted due the cross reactivity among flavivirus. WNV is a positive-strand RNA virus which encodes for three structural proteins (C, M, E) and seven non-structural proteins. The DIII of the E protein produce neutralizing antibodies and these antibodies are generally specific for each virus and do not cross react with other viruses. The present study shows the development of a lateral flow device (LFD) based on the use of monoclonal antibodies to domain III of E protein of WNV, able to differentiate between WNV antigen from other related Flavivirus antigen.

MATERIALS AND METHODS:
DIII of E protein was cloned from the RNA of WNV L1 strain NY’99, and expressed in E.coli and baculovirus systems. His and GST tags were introduced in order to allow the purification of the protein. This purified protein was used to immunize mice to produce monoclonal antibodies (Mabs). A panel of Mabs were obtained and one was selected for developing a LFD because its high affinity and specificity to WNV E protein. This Mab was used to print the test line in the nitrocellulose membrane and also for conjugation to colored latex particles. Samples were obtained from: a) cell cultures infected with different WNV lineages (L1, L2, L3 and L6) and other related Flaviviruses and b) brains of 7 experimentally infected mice with different WNV strains.

RESULTS:
To check the ability of this test to differentiate between WNV and related flaviviruses, the supernatant of cell cultures infected with different Flaviviruses were analyzed. The results indicated that the LFD detected all the 4 lineages of WNV tested (several strains of each lineage) and did not detect any of the other 14 flaviviruses. Analytical sensitivity was 10e4 TCID 50. A second validation was carried out using brains of 7 experimentally WNV-infected mice. The test detected viral antigen in all the brain extracts samples tested.

DISCUSSION AND CONCLUSIONS:
A LFD based on the use of a Mab specific for domain III of E protein of WNV has been developed being able to detect specifically WNV in infected cell cultures but no other Flaviviruses and showing analytical sensitivity close to 10e4 TCID 50. In addition the test could detect WNV antigen in brain extracts from WNV experimentally infected mice. WNV has recently re-emerged in Europe, leading to the increase and persistence of the outbreaks. Tools enabling rapid detection of new emerging flaviviruses and differentiation of important subgroups for diagnosis and surveillance are crucial for detecting virus introductions. The LFD provides a rapid and specific method to distinguish between WNV and other Flaviviruses, at least in samples from infected cell cultures. The utility of the assay is enhanced by its handling easiness. Bearing in mind that neither sophisticated equipment nor highly qualified personnel are necessary for the method and that the result is obtained in 10 minutes, this method could be an alternative to PCR for specific detection of WNV in these kind of samples. Further studies are needed to check the ability of the test to be used with other type of samples like oral swabs, feathers, and spleen or corporal fluids like urine or blood.

REFERENCES:
INTRODUCTION:
African Swine Fever (ASF) is a highly contagious disease of swine that produces great economic losses in the affected countries. Domestic pigs and European wild boars are susceptible, showing a wide range of clinical forms. Epidemiological studies have demonstrated that the entrance of ASF virus (ASFV) in ASF-free areas is primarily related to feeding pigs with contaminated garbage from international airports and seaports. This fact, together with the extensive commercial trade, puts ASFV-free countries at constant risk of having the disease introduced in their territory. In the absence of vaccine, control and eradication strategies are mainly based on rapid laboratory diagnosis of ASFV positive and carrier animals and on the enforcement of strict sanitary measures.

To this end, INGENASA in collaboration with the European Union Reference Laboratory (EURL) for ASFV (INIA-CISA), have been working in the development and standardization of rapid, easy and reliable diagnostic tools based on Lateral Flow Assay (LFA) and Real Time PCR (INgene® q PPA) for accurate detection of ASFV in serum, blood and tissue samples.

MATERIALS AND METHODS:
For real-time PCR, a primer set and a UPL-probe (Universal Probe Library/Roche) specific for ASFV detection were selected. An exogenous internal control using a primer set and a VIC-labeled hydrolysis Taq-Man-MGB probe was designed for porcine β-actin gene detection. Optimal reaction conditions were established to get a duplex real-time PCR test allowing a correct differential detection of ASFV and the internal control. On the other hand, a Lateral Flow Assay for antigen detection based on the use of MAbs against ASFV VP72 protein has been developed. Initially, a spike in test was performed by adding VP72 recombinant protein into blood. To test the applicability of these two assays to detect ASFV, a panel of experimental porcine sera and blood samples were used.

RESULTS:
Results of INgene® q PPA test were compared with those using the ASFV UPL-Real Time PCR previously described by Fernández-Pinero et al., 2013 in a validation study performed at the EURL for ASFV (INIA-CISA). Concerning LFA, results were compared with the ones obtained by a commercially available ELISA Sandwich (INGEZIM® PPA DAS), qPCR or hemadsorbing titre. Regarding the LFA for ASFV detection, the test showed the same sensitivity as the INGEZIM® PPA DAS when recombinant protein VP72 or inactivated culture virus were used. When the assay was performed with blood samples from experimentally infected animals, viral loads between 102-103 HAU could be detected.

DISCUSSION AND CONCLUSIONS:
From the validation study of INgene® q PPA assay, it can be concluded that there is an excellent agreement with the reference UPL-PCR method showing 98% sensitivity and 96,8% specificity and being able to detect all isolates checked (n=21). Moreover, the assay was able to detect the ASFV during chronic, subacute and acute ASF infections with similar sensitivity to those showed by the reference UPL-PCR method.

These results suggest that the developed assays can be useful tools for rapid and reliable detection of ASFV, not only at laboratory level (INgene® q PPA) but also at field level (LFA-ASFV), providing a useful tool in situations where laboratory support and skilled personnel are limited.

The research leading to the results has been partially funding by the EU, Seventh Research Framework Program FP7-KBBE-2207-2013 under grant number nº 311931 (ASFORCE).

REFERENCES:
RAPID INSULATED ISOTHERMAL RT-PCR ASSAY FOR THE DETECTION OF EQUINE ARTERITIS VIRUS NUCLEIC ACID IN EQUINE SEMEN


Keywords: equine viral arteritis, insulated isothermal PCR, real-time RT-PCR

INTRODUCTION:
Equine viral arteritis (EVA) is an important contagious disease in horses caused by equine arteritis virus (EAV). About 30-70% of the infected stallions become asymptomatic carriers and continue to shed virus in semen for a long period of time. Identification of the carrier stallion is therefore of critical epidemiological importance in the prevention and control of EAV infection. The POCKIT Xpress™ Portable PCR Platform has the potential to serve as a rapid, specific and sensitive tool for routine detection of pathogens, making it a great aid for disease surveillance and management. The POCKIT™ system incorporates fluorescent probe-based insulated isothermal polymerase chain reaction (iiPCR) technology (1, 2, 3), and it can provide test results automatically from nucleic acid samples within one hour.

MATERIALS AND METHODS:
A reverse transcription (RT)-iiPCR [POCKITTM] targeting open reading frame 7 (ORF7) was established in this study. The limit of detection of RT-iiPCR was determined using in vitro transcribed (IVT) EAV ORF7 RNA. Serial dilutions of tissue culture fluids containing EAV virulent Bucyrus strain (VBS) and KY 84 strain RNA (100 to 10-10 dilutions) were used to compare the sensitivity of RT-iiPCR with that of a previously described real-time RT-PCR (rRT-PCR) (4). Twenty six known EAV laboratory, field and modified live virus vaccine strains, and thirteen important equine pathogens were tested to evaluate the specificity of the established assay. Both assays were compared to virus isolation (gold standard) to evaluate their accuracy in detecting EAV in semen samples (n=118) collected from experimentally infected carrier stallions. Viral RNA from each semen sample was extracted with a column-based extraction method (PetNAD™ Nucleic Acid Co-prep kit) and a miniaturized automatic extraction method (taco™ mini Automatic Nucleic Acid Extraction System) and compared to MagMAX™-96 Viral RNA Isolation Kit.

RESULTS:
The limit of detection of RT-iiPCR was determined to be 10 copies of IVT RNA per reaction. The sensitivity of RT-iiPCR was 10-folds higher than the real-time RT-PCR (rRT-PCR). All EAV strains were tested positive by both assays. Both assays were highly specific and there was no cross reactivity with nucleic acids extracted from thirteen important equine pathogens. Compared to virus isolation, the relative sensitivity and specificity were 98.41% (62/63) and 100.00% (55/55), respectively, for the rRT-PCR, and 98.18% (54/55), respectively, for the RT-iiPCR. Accordingly, the accuracy was 99.15% (Cohen’s kappa value 0.98) and 99.15%, (Cohen’s kappa value 0.98) for the rRT-PCR and RT-iiPCR, respectively. The column-based extraction method and the miniaturized automatic extraction method were shown to extract EAV RNA with efficiency comparable to the reference method.

DISCUSSION AND CONCLUSIONS:
These results suggest that both rRT-PCR and RT-iiPCR are sensitive, specific and robust to identify EAV nucleic acid in semen samples with good accuracy. Furthermore, both extraction methods evaluated provided templates compatible with both PCR methods.

REFERENCES:
NEW INSIGHTS INTO ORAL FLUIDS AS A DIAGNOSIS PROCEDURE TO DETECT AND DETERMINE THE PREVALENCE OF PORCINE RESPIRATORY AND REPRODUCTIVE VIRUS SYNDROME (PRRSV) UNDER FIELD CONDITIONS

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Keywords: PRRS, oral fluids, RT-qPCR

INTRODUCTION:
Diagnostic tests are often used to assess the PRRSV infection status of pig herds. For routine settings, ELISA test methods and reversed transcriptase qPCR are used to determine antibody titers and detect antigen. Until now, reversed transcriptase qPCR (RT-qPCR) on serum/blood and tissue samples is the most used technique to detect PRRSV antigen for this disease. Recently, the detection of this virus in oral fluids is worldwide being used as an alternative technique. There is no information about the practical implementation of this technique under field conditions.

The main goal of this study was to establish clear recommendations in herd management for swine practitioners when oral fluids are used as a diagnosis procedure depending on the goal: detection of a disease versus prevalence determination with a focus on PRRSV.

MATERIALS AND METHODS:
Raw Data coming from 2 different experiments were included. In both cases, before starting the studies, oral fluids and blood/serum samples were collected and analyzed by RT-qPCR and ELISA to confirm PRRSV negative status.

Experiment 1: A challenge with a PRRSV European strain. After carrying out the challenge with the virus, a weekly sampling of 80 pigs was carried out during 3 months. Every week, individual blood samples were taken of all pigs included and oral fluids samples of the included pens.

Experiment 2: A PRRSV experimental infection with a North American strain was carried out in naïve pigs. Animals were sampled repeatedly over the 40 days post-infection, individual blood samples and oral fluids samples of each pen.

In both cases, sample extraction was carried out with magnetic beads sample extraction (MagMAX™ Pathogen RNA/DNA kit and MagVet Universal Isolation Kit 4x 96 tests) and RT-qPCR with LSI VetMAX™ PRRS EU/NA and TaqMan® NA and EU PRRSV Reagents.

Data analysis
Diagnostic sensitivity and specificity has been calculated using a free software (http://www.winepi.net/).

A logistic regression analysis was carried out in SPSS 15.0 (SPSS Inc., 1989–2006) to calculate the probability of virus detection in a pen by oral fluid (PCR positive test result) taking into account the prevalence of PRRSV in serum as independent variable. With this probability, basic epidemiologic information (Thrusfield, 1997) was used to correctly estimate the number of pens necessary to be sampled in order to detect a disease. A Spearman’s rank correlation statistics between the prevalence in serum and in oral fluids was carried out using SPSS 15.0 (SPSS Inc., 1989–2006) software to study the statistical dependence between these two variables.

RESULTS:
Diagnostic sensitivity is 87.5% (78.8%, 96.2%) and diagnostic specificity is 100.0% (100.0%, 100.0%) in experiment 1. Based on the data of experiment 1 and 2, the probability to detect PRRSV virus in oral fluids is significantly associated to the PRRSV prevalence in serum (p<0.0001) and the determination coefficient of the logistic regression analysis is 82%. The probability (0-1) to detect PRRSV in oral fluid samples was 0.15, 0.40, 0.72, 0.91 and 0.97 for a serum prevalence (%) of 10, 20, 30, 40 and 50, respectively. If the serum prevalence is higher than 50%, the probability to detect this virus in oral fluid samples is close to 1. The number of oral fluids samples on herd level to be taken in order to find, at least, 1 positive PRRSV oral fluid sample was 1, 1, 2, 2, 2, 3, 4, 8, 19 and 58 for a serum prevalence (%) of 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10, respectively. It is not feasible to estimate correctly the prevalence in serum from the prevalence calculated from oral fluid sampling when the prevalence at serum level is between 0 and 40-50%.

DISCUSSION AND CONCLUSIONS:
Oral fluid sample is a good tool to detect PRRSV at herd level but it is not suitable to determine the prevalence of this disease. Testing the sampling recommendation under field conditions is requested and these studies are being carried out.

REFERENCES:
ORAL PRESENTATIONS

OTHER ANIMAL DISEASES
EVALUATION OF TWO RAPID SCRAPIE ELISAS FOR RETRO-PHARYNGEAL LYMPH NODES (RLN) IN SHEEP

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Keywords: scrapie, elisa, lymphoid tissue, sheep

INTRODUCTION:
Rapid screening tests for scrapie have been comprehensively evaluated for use on brain samples, with high sensitivity and specificity1,2. Four rapid screening ELISAs showed good sensitivity, ranging from 83.1 to 94.2%, and specificity, ranging from 97.8% to 100%1,2,3. For the Bio-Rad TeSeE ELISA (ELISA-BR), the sensitivity for mesenteric lymph nodes was markedly lower (78.9%) than for brain tissue samples (99.6%), although it had an excellent specificity of 100%. In another study, using ELISA-BR on RLNs, sensitivity and specificity were 100%4. ELISA-BR has the advantage that it can be used for rapid testing of scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD). For this reason, it is the test method of choice in our laboratory. The discrepancies in published evaluation data for this ELISA on lymphoid tissues prompted us to evaluate it further.

MATERIALS AND METHODS:
A non-infected panel of RLN was collected from 737 sheep from New Zealand that had been culled, found in extremis or died. Brain stem samples were also collected from 131 of these sheep. The panel of infected samples comprised 218 and 117 RLN from confirmed scrapie cases that had originated in Europe and the USA, respectively. All samples were screened using two commercial, rapid, TSE ELISA kits, the ELISA-BR and in addition, the IDEXX HerdChek BSE-Scrapie AG Test (ELISA-ID).

RESULTS:
For the non-infected samples from New Zealand, the diagnostic specificity of both ELISA kits was 100%. When considering all infected samples, the diagnostic sensitivity was 70.4% for ELISA-BR and 91.6% for ELISA-ID. If scrapie became established in New Zealand, an estimated 596 cases would occur per year; of these 234 (39%) and 271 (46%) would be in sheep carrying ARQ/ARQ and ARQ/VRQ PrP genotypes, respectively. For the ARQ/ARQ genotype (n=195), the sensitivity was 66.2% for ELISA-BR and 90.8% for ELISA-ID, and for the ARQ/VRQ genotype (n=107), the sensitivity was 81.3% for ELISA-BR and 98.1% for ELISA-ID.

DISCUSSION AND CONCLUSIONS:
In this study, the ELISA-ID kit demonstrated a higher diagnostic sensitivity for detecting scrapie in samples of RLN from sheep carrying scrapie-susceptible PrP genotypes than the ELISA-BR kit at comparable diagnostic specificity. The diagnostic performance of the ELISA-ID kit using ovine RLN merits the consideration of including this assay in the national scrapie surveillance programme in New Zealand. Unlike the ELISA-BR, which can be used for scrapie, BSE and CWD, the ELISA-ID is recommended for testing of BSE and scrapie only.

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IMPLEMENTATION OF A NOVEL SERODIAGNOSTIC TEST TO MEASURE ASCARIS SUUM INFECTIONS IN FATTENING PIGS AND TO ASSESS THE POTENTIAL IMPACT OF THIS PARASITE ON FARM PRODUCTIVITY

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Keywords: Ascaris suum, novel serodiagnostic test, farm productivity

INTRODUCTION:
Ascaris suum is currently the only helminth that is still highly prevalent in intensive pig production systems, resulting in significant economic losses. However, due to the subclinical nature of the disease, ascariasis often remains undiagnosed, creating a lack of information regarding the worm-status of a farm, which ultimately makes it difficult to evaluate the applied deworming programs. The first aim of this study was to assess the prevalence of A. suum infections in fatteners throughout Europe by using a recently developed serodiagnostic assay that is based on the recognition of a parasite haemoglobin protein by antibodies of infected animals (Vlaminck et al., 2012). The second aim was to assess whether the ELISA could be used to measure potential associations between Ascaris infection levels and farm productivity.

MATERIALS AND METHODS:
For the seroprevalence study, blood samples from 10 individual fattening pigs were collected on 471 farms across several European countries and subsequently analysed on the Ascaris ELISA as previously described (Vlaminck et al., 2012). To assess the association between Ascaris and farm productivity, ELISA results of 20 fattening farms were compared with slaughterhouse data (such as percentage of affected livers, pleuritis and lung lesions) and farm performance parameters (such as feed conversion efficiency, days to market, daily weight gain, carcass quality and mortality).

RESULTS:
The outcome of the serological analysis indicated that A. suum is still highly prevalent in fattening farms across Europe, with more than 50% of the farms analyzed testing positive. Furthermore, the obtained results also provided a strong indication that the presence of A. suum on a farm could have a significant impact on farm economical parameters since strong correlations were detected between the ELISA results and different production parameters of a farm, like days to market or average daily weight gain.

DISCUSSION AND CONCLUSIONS:
The outcome of this study showed that the Ascaris ELISA is an easy-to-use and sensitive tool that can be used to both assess exposure of fattening pigs to A. suum, estimate the economic losses due to this parasite and monitor the efficacy of anthelmintic treatment programs. Currently, the first development and validation studies are being performed in collaboration with Boehringer Ingelheim Svanova to further optimize this assay for commercial use.

REFERENCES:
INTRODUCTION:
For the first time in Italy in 2013, thanks to the National Marine Mammals Stranding Network, it has been possible to manage cetaceans stranding with coordination and synergy. A complete traceability of reports, sampling, diagnostic examinations and sharing of the results was obtained. In detail between January and March 2013 a cetacean unusual mortality event (UME) was reported along the Tyrrhenian coast of Italy, characterized by an exceptional number of cetacean strandings (Casalone et al. 2014). The present study has the aim to show the diagnostic results of the stranded animals necropsies during the year 2013, included the UME.

MATERIALS AND METHODS:
Two hundred forty four stranded cetaceans were reported by the National Database on Cetacean Strandings in 2013 (Fig. 1), 122 of them stranded during the first 3 months of the year. Post-mortem investigations of 105 specimens (43,4%) were performed by various laboratories using a standard sampling protocol according to the body conservation code (Geraci & Loundsbury 2005).

Tissues were sampled, both frozen at −80°C for microbiological and biomolecular investigations and preserved in 10% neutral-buffered formalin for histopathological and immunohistochemical (IHC) analyses.

DISCUSSION AND CONCLUSIONS:
DMV is considered the most pathogenic and immunosuppressive viral agent for cetaceans, making the affected animals more susceptible to secondary infections. Despite the lack of a range of characteristic morphologic changes in the tissues, biomolecular analysis yielded clear evidence for DMV infections in 42 % of specimens tested during the UME, often associated to co-infections, supporting the hypothesis that the virus played an important role in this event. In the remaining part of the year, 33% of the cetaceans tested resulted positive for DVM: it leads to suppose that the virus still circulates along the Italian seacoasts. The detection of a case of Brucella ceti, along with the 3 cases recently published (Alba et al 2012; Garofolo et al 2014), highlights the presence of this dangerous zoonotic pathogen in the Italian sea. The other pathogens detected, such as Toxoplasma gondii and Herpesvirus, are not thought to play any primary role in the cause of death of the animals investigated.

In conclusion, this study highlights the importance to continue the application of a national sanitary surveillance system on cetacean stranded in order to monitor, in the long-term and large geographic scale, the aquatic environment and the animal and public health.

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INTRODUCTION:
Mycoplasma spp. belongs to Mollicutes class and is wall-less and gram-positive. Among the bacteria they have the smallest genome size. They are fastidious and difficult to in vitro culturing organisms (4). Mycoplasma bovis infection causes many significant problems in cattle herds in many countries all over the world. The most important diseases are the pneumonia, mastitis, arthritis, keratoconjunctivitis, otitis, meningitis, infertility, abortions and other disorders (1, 2, 3). The habitats of mycoplasmas are mainly joints and mammary glands, respiratory, alimentary and urogenital tracts, and even conjunctival sac (4).

MATERIALS AND METHODS:
The study presents the preliminary results concerning the prevalence of M. bovis infection in a south-eastern region of Poland mainly based on the detection of specific for the mycoplasma DNA, antigen and antibody. The study was performed on 258 samples (sera, swabs, milk) collected from cattle suffered from bronchopneumonia, mastitis and arthritis. The presence of anti-M. bovis antibodies was tested with the use of indirect ELISA kit for serodiagnosis of M. bovis in cattle blood sera and milk samples (n=164)(Bio-X Diagnostics, Belgium). In the cattle nasal and joint swabs (n=94) M. bovis antigen was detected with the use of Antigenic Pulmotest - sandwich ELISA (Bio-X Diagnostics, Belgium) and the presence of M. bovis specific DNA sequence was tested by PCR. The PCR was done with the use of 2 primers specific for M. bovis DNA, that were based on a uvrC gene. Primers were designed according to Subramaniam et al. (5).

RESULTS:
The results are summarised in Tab. 1. The presence of specific anti-M. bovis antibodies were detected in sera and milk samples. The observed incidence was within a range of + to ++++. The 28.57 % of positive results were received from milk samples and 20.37 % from sera samples. Therefore, the average result for these both kinds of samples is 23.17%. Positive results were more frequently obtained from the affected cattle with disease symptoms characterized M. bovis infections. Furthermore, the presence of specific antibodies against M. bovis was also reported in some PCR and Pulmotest-negative animal samples. Within the swabs detected the positive results were obtained in 17.02 % of them for PCR and 13.86 % for Pulmotest used in the study. Our results will allow better insights into the epidemiological background of the M. bovis infection, will expand the knowledge about pathogenic mechanisms, and are able to substantiate the M. bovis case definition in seropositive animals.

DISCUSSION AND CONCLUSIONS:
In conclusion, the study showed that in the affected cattle from south-eastern region of Poland the number of animals in which anti-M. bovis antibodies were found is higher than the number of positive ones for M. bovis antigen or where specific for M. bovis DNA was detected. This indicates that the presence of anti-M. bovis antibodies in the cattle sera or milk samples does not always mean the current infection. It can provide also about the previous infection. Overall, the results indicated that the use of few different methods maximizing the likelihood of pathogen detection.

The study was supported by the Polish National Science Centre grant No. 2013/09/N/NZ7/02158 entitled: "The prevalence and molecular characteristic of Mycoplasma bovis infections in cattle in Poland." (2014-2016).

REFERENCES:
CHEMILUMINESCENT MULTIPLEX ELISA FOR SIMULTANEOUS DETECTION OF MVV/CAE, CLA AND JOHNE’S IN GOATS AND SHEEP


Keywords: multiplex, antibodies, goats, CAE CLA, Johnes

INTRODUCTION:
Maedi-Visna (MV), Caprine Arthritis Encephalitis (CAE), Caseous Lymphadenitis (CLA), and Johne’s disease (JD) are important diseases in goats and sheep, causing significant morbidity and mortality and production losses. Diagnosis is usually made on the basis of detection of antibodies in blood or milk to the causative pathogens: Maedi–Visna virus/ Caprine Arthritis Encephalitis Virus, Corynebacterium pseudotuberculosis, and Mycobacterium paratuberculosis respectively. Currently, separate ELISAs for each disease are run which is time consuming and expensive. We describe the development of a multiplex test (MVD-Enferplex) which will detect antibodies specific for each of these three pathogens in a single test. The assay utilises antigen arrays printed on conventional ELISA plates, allowing a standard ELISA-type protocol to be followed.

MATERIALS AND METHODS:
Five antigens were used: recombinant MVV p25 core protein, MVV gp46 synthetic peptide (1), CLA recombinant phospholipase D, CLA recombinant CP40 (2, 3), and Johne’s PPA3 antigen (4). The antigens were printed as discrete spots onto the bottom of flat-bottom black-walled ELISA plates. Thereafter, standard ELISA procedures followed except that a chemiluminescent substrate was used. Commercial ELISAs where available were used to establish panels of positive and negative reference samples. Other samples were from herds/flocks with no long-term history of MVV/CAE, CLA or Johne’s disease.

RESULTS:
In goat positive reference sera, 125/125 (100%), 42/42 (100%) and 52/54 (99.2%) were detected as positive for CAE, CLA and Johne’s respectively in the multiplex test, while in negative reference sera, 462/466 (99.1%), 410/412 (99.5%) and 362/366 (98.9%) were negative respectively. In sheep positive reference sera, 54/55 (98.2%) and 60/63 (95.2%) were detected as positive for CAE and CLA respectively, while in negative reference sera, 114/117 (97.4%) and 84/85 (98.8%) were negative respectively. A commercial ELISA detected 15 pTB positive samples in an infected herd, while Enferplex detected 24 positives. Both ELISA and Enferplex registered 151 samples as negative. In goat positive reference bulk milks, 103/105 (98.1%) and 94/95 (99.0%) and were detected as positive for CAE and CLA respectively in the multiplex test, while in negative reference bulk milks, 139/141 (98.6%) and 39/39 (99.5%) were negative respectively. The Multiplex test is currently in use in the Norwegian TINE Healthier Goat Programme, which aims to eradicate these three diseases. The Programme has resulted in 40% increased milk yields, reduced SCC, and reduced burden from other pathogens.

DISCUSSION AND CONCLUSIONS:
This study shows that it is feasible to use arrays for simultaneous detection of three disparate (one viral, 2 bacterial) diseases by including one or more antigens from each pathogen. The individual components of the test have similar sensitivities and specificities to those of commercially available ELISA tests. Previously, this technology has been used to detect antibodies to Mycobacterium bovis in cattle and other species (5-7). There is scope to increase the number of antigens up to 25, allowing multiple antigens from a single pathogen or multiple separate pathogens to be targeted. Thus, a single test can now be used instead of 3 tests, providing significant cost savings. The application of the MVD-Enferplex test in the Norwegian Healthier Goat Programme attests to its successful routine use in the field. Such multiplex approaches open up new approaches to disease diagnostics, surveillance, and eradication.

REFERENCES:

CHEMILUMINESCENT MULTIPLEX ELISA FOR SIMULTANEOUS DETECTION OF MVV/CAE, CLA AND JOHNE’S IN GOATS AND SHEEP


Keywords: multiplex, antibodies, goats, CAE CLA, Johnes
INTRODUCTION:
Equine piroplasmosis, a life threatening tick-borne disease caused by B. caballi and T. equi, is subject to international movement restrictions. While antibodies can be lifelong, as also for the carrier state, the OIE prescribed tests are still serologically based, even if these may be negative at the beginning of infection [5]. Also, the definition of the carrier state, employing direct methods is especially important in endemic areas for justifying and verifying the treatment efficacy due to its potential toxicity. For this, a study was conducted for the adoption of molecular methods for the diagnosis of these infections by evaluating the performance of different PCR methods, traditional and Real Time (RT), on field samples for both types of infections.

MATERIALS AND METHODS:
103 whole blood samples of clinically suspect equids, collected within a research project of the CERME, were analysed using 4 different PCRs for each protozoan. Genomic DNA was extracted using Cador Pathogen 96 QIAcube HT Kit (Qiagen®). PCR protocols were conducted as described in literature or according to instructions. For T. equi (T): end point PCR (T1) and nested-PCR (T2), targeting equine merozoite antigen (EMA) complex gene (amplicons 268bp and 102bp respectively) [1,6]; RT PCR (T3) targeting the 18S gene (81bp) [4]; Path-T. equi Genesig ® (T4) targeting EMA 1 (about 120bp). For B. caballi (B): End point PCR (B1) and nested PCR (B2), targeting rhoptry associated protein complex gene (825bp and 430bp respectively) [1,3]; RT PCR (B3) targeting the 18S gene (95bp) [2]; Path-B. caballi (Genesig ®) (B4) targeting the 18s gene (about 100bp). The specificity of discordant results was verified by sequencing. The PCR detecting the greatest number of positives was chosen for assessing relative sensitivity (rSe) and relative specificity (rSp). Agreement among the PCRs was estimated for each protozoan.

RESULTS:
Number of positives per method are as follows: for B1 (4); B2 (8); B3 (4); B4 (2); for T1 (29); T2 (29); T3 (35); T4 (27). An overall agreement of 91.3% was observed for B1 and 90.3% for T1. Table 1 reports the number of samples in agreement for 2, 3 and 4 PCRs. As B3 and T3 detected the highest number of positives and the discordant samples were specific products, they were used as reference tests to estimate the rSe and rSp, reported in Table 2.

DISCUSSION AND CONCLUSIONS:
B3 and T3 were considered the best PCRs probably due to their primer efficiency and their short amplicons. Moreover, T3 primers were designed within a highly conserved region, and B3 employs an MGB probe consenting the use of shorter targets. The lower rSe of B1 and B2 could be due to the higher mutation frequency or degradation of their long targets, but recruitment of a major number of positives is necessary to verify this result. In general, a good overall agreement (>90%) for the PCRs of each parasite is observed. The major positivity in PCR for T. equi could be due to its reported marked higher parasitemia and prevalence than that of B. caballi [2,5]. Furthermore, from this preliminary study, when compared to the serological tests, the PCRs identified carriers among the seronegatives, as well as non-carriers among the seropositives. For T. equi, of the 36 PCR positive, 17 were seronegative and for B. caballi, all PCR positives were seronegative. In view of these results, B3 and T3 can be employed in routine diagnosis and developed as quantitative methods to assess correlation between parasitemia and the clinical phase of infection to aid the clinician, in deciding or verifying treatment. Moreover, it would be recommendable for international movement control to include PCR, in adjunct to sero-methods in use.

REFERENCES:
ORAL PRESENTATIONS

FOOD SAFETY AND ZOONOSES
INTRODUCTION:
Despite the proven efficacy as therapeutical remedies in a wide range of pathologies, glucocorticoids (GCs) are also illegally used as growth promoting agents in animal breeding. In particular in the last few years dexamethasone has been shown tempting characteristics in the field of illicit treatments in food producing animals, due to its high glucocorticoid potency associated to a fast kinetics, furthermore because of the synergic effect when used in association with prednisolone, estrogens and β-agonists. Moreover the widely debated endogenic origin of prednisolone promotes its use in anabolic protocols of treatment. Promising results from thymus histological screening tests could guarantee an enhancement of end user protection.

MATERIALS AND METHODS:
We examined the thymic atrophy grading and the expression profile of the immunohistochemical marker of apoptosis Cleaved Lamin A (Cell Signaling Technology) of 40 male veal calves that were divided into four different groups. Group A (10 calves), Group B (10 calves), Group C (10 calves) received at sixth month for 20 consecutive days respectively: 0.4 mg day-1 of dexamethasone, 0.2 mg of dexamethasone + 4 mg of prednisolone day-1 and 8 mg day-1 of prednisolone. Group K (10 calves) was kept as control. The central area of the thoracic thymus of each animal was sampled at slaughterhouse ten days after the last treatment and fixed in 4% buffered formaldehyde at room temperature for 2 days, routinely processed, embedded in paraffin wax, sectioned at 3–5 µm and subjected to the following staining techniques:
a) Hematoxyline and Eosine, to observe the grading of thymic atrophy by light microscopy. Two pathologists assigned to each sample a grading of thymic atrophy related to fat infiltration: 1 for mild, 2 for moderate and 3 for severe atrophy;
b) Immunohistochemistry applying an antibody for Cleaved Lamin A (1:100) was performed. The total number of apoptotic cells was counted in a blind manner in five high magnification (40x) microscopic fields by two pathologists.

Statistical analyses were performed to assess differences in grading of thymic atrophy between group B and group C compared to the control Group. Group A wasn’t subjected to evaluation of thymus atrophy as this parameter was previously studied in a larger group of dexamethasone treated calves (data not shown). Furthermore, differences in the apoptotic patterns were evaluated between each treated group and group K.

RESULTS:
a) Group B and K showed statistically significant differences for thymic atrophy (p=0.02). As grade 1 was attributed to every animal of group C and K, no differences between these two groups were seen.
b) The mean number of apoptotic cells was: Group A 57.36 (CI 95%: 41.56-73.15); Group B 76.08 (CI 95%: 55.21-96.94); Group C 112.02 (CI 95%: 89.30-134.73) ad Group K 164.46 (CI 95%: 140.23-188.68). Cleaved Lamin A marker resulted a significant predictor of dexamethasone treatment alone (p=0.000) and in association with prednisolone (p=0.000), and it is also significantly associated with prednisolone treatment alone (p=0.002).

DISCUSSION AND CONCLUSIONS:
We can conclude that the higher glucocorticoid potency of dexamethasone, even if in association with prednisolone, affects to greater extent thymus morphology. Moreover Cleaved Lamin A marker could represent an efficient monitoring tool to distinguish between treated and control calves and may strengthen the traditional histological analysis of the target organ thymus to detect anabolic treatments with GCs.

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021

GONE FISHING! MAGNETIC CAPTURE AND REAL TIME PCR (MC-PCR) OF ECHINOCOCCUS
MULTILOCULARIS DNA IN RED FOX (VULPES VULPES) FAECAL SAMPLES


Keywords: Echinococcus multilocularis, red fox, diagnostic method

INTRODUCTION:
Following the first finding of Echinococcus multilocularis in Sweden in 2011 [1], 2985 red foxes (Vulpes vulpes) were analysed by the segmental sedimentation and counting technique. This is a labour intensive method and requires handling of the whole carcass of the fox, resulting in a costly analysis. In an effort to reduce the cost of labour and sample handling, an alternative method has been developed. A semi-automated magnetic capture probe DNA “fishing” extraction method and real time hydrolysis probe polymerase chain reaction assay (MC-PCR) was developed for detection of E. multilocularis in faecal samples. The MC-PCR presented has been used in the Swedish E. multilocularis monitoring program for 2012-2013 on more than 2000 faecal samples.

MATERIALS AND METHODS:
We describe a new semi automated MC-PCR method for the detection of E. multilocularis DNA in 3 ml faecal samples from red fox. The diagnostic sensitivity was determined by validating the new method against the sedimentation and counting technique [2] in foxes collected in Switzerland where E. multilocularis is highly endemic.

RESULTS:
Of 177 foxes analysed by the sedimentation and counting technique, E. multilocularis was detected in 93 animals. Eighty-two (88%) of these were positive in the MC-PCR. In foxes with more than 100 worms, the MC-PCR was positive in 44 out of 46 (95.7%) cases. The two MC-PCR negative samples originated from foxes with only immature worms. In foxes with 100 worms or less, (n=47), 38 (80.9%) were positive in the MC-PCR. The diagnostic specificity of the MC-PCR was evaluated using fox scats collected within the Swedish screening. Of 2158 samples analysed, two were positive. This implies that the specificity is at least 99.9%.

DISCUSSION AND CONCLUSIONS:
In this study we have described a novel approach for enrichment and extraction of E. multilocularis DNA from fox faeces. The capture probe based enrichment of E. multilocularis DNA is specific, minimizes limitations due to inhibitory substances and can be performed on large sample volumes. With automatisation, this method is a useful tool in large scale screening programmes and is currently used in a nationwide surveillance program in Sweden. The MC-PCR proved to have a high sensitivity and a very high specificity making it well suited for surveillance programs, in particular in areas with low incidence of infection. The test is partially automated but is also possible to perform manually if desired.

REFERENCES:
INTRODUCTION: Brucella suis biovar 2 is a consistent phylogenetic group in the B. suis clade [1]. Although is recognized as one of the agents of the porcine brucellosis, this bacteria seems host adapted to wild boars and hares, and it is geographically restricted only to Europe [2]. In Europe the description of others B. suis biovars is extremely rare, thus porcine brucellosis is mostly caused by the B. suis biovar 2 [2]. Several reports showed that hares and wild boars populations are widely affected throughout Europe [3]. New swine breeding systems, with the animals reared outdoor, expose the domestic pigs to the feral population creating a more effective transmission of porcine brucellosis. Despite few reports are published, in Italy brucellosis in wild boars seems to be endemic [4]. The aim of the study was to genetically characterize B. suis biovar 2 from Italy isolated in the last decade. We expected to understand the phylogenetic connection between the Italian and the European strains using MLVA methodology.

MATERIALS AND METHODS: We genotyped 98 B. suis from porcine brucellosis cases from the following regions of Italy: Piemonte, Abruzzo, Emilia Romagna, Lazio and Sardinia. Samples were processed using multiplex PCR and capillary electrophoresis on an ABI 3500 instrument with POP 7 polymer for all the strains [5]. No missing alleles were observed in the MLVA-16 panel. The genotyping data of the 98 strains were compared to genotypes from 261 isolates available in the MLVA database. Minimum-spanning and UPGMA trees were constructed using PHYLOViZ 1.0. and PAUP* 4.0b.

RESULTS: MLVA-16 distinguished 57 genotypes from the 98 isolates of B. suis biovar 2, 44 of which were represented by single isolate (Fig. 1). Spatial information revealed that in some instances genotypes were isolated from restricted geographical areas. The phylogeographic pattern of 98B. suis isolates from Italy was compared to 261 MLVA profiles from the international database using MLVA-11. We found 12 different MLAV-11 genotypes, with the genotypes 44, 57 and 39 most commonly found, while the remaining ones were single locus variants of these 3 genotypes.

DISCUSSION AND CONCLUSIONS: The phylogeographic assessment was done analyzing our MLVA profiles together with publicly available profiles using the 11 loci panel. The minimum spanning tree showed 2 main clusters; one was specific to the Iberian peninsula, and the other spread throughout continental Europe. The Italian strains fell in the European clade in association with isolates from France, Germany and Switzerland. Conversely the MLVA-16 clustering revealed that in Italy is acting a high diverse B. suis biovar 2 population. The cluster analysis showed 3 main clusters with the cluster I mostly recovered in Piemonte, the cluster II almost spread over the Appennini chain and the cluster III, as the basal group, characteristic of Sardinia. In conclusion, MLVA was able to construct the genetic structure in Italy with the presence of some clusters geographically restricted. The global connection with the other European strains could be explained because of the modern trade of wild boars that occurred in the recent past. In the next future whole genome sequencing of the B. suis biovar 2 strains will be executed to find out specific Italian DNA signatures able to better characterize our own population.

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APPLICATION OF THE ENFERPLEX CAPRINE TB TEST TO A BREAKDOWN HERD IN THE UK

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Keywords: tuberculosis, antibodies, multiplex, goat

INTRODUCTION:
Mycobacterium bovis causes tuberculosis in cattle and a wide variety of non-bovine species. Diagnosis is usually made by application of tuberculin skin testing, post-mortem identification of lesions and culture of M. bovis. Enfer Scientific have developed a multiplex array test for antibodies that detects responses to 6 or more M. bovis antigens in a variety of species (1-6). The sensitivity and specificity of the assay was found previously to be 95% and 100% respectively (4). Here, we present data showing the use of the Enferplex Caprine TB test in an infected dairy goat herd.

MATERIALS AND METHODS:
The M. bovis antigens used for printing were MPB70 peptide preparation, PPD-B, recombinant MPB70, MPB83, CFP-10, and ESAT-6 (1-6). The antigens were printed as discreet spots onto the bottom of flat-bottom black ELISA plates. Standard ELISA procedures were followed thereafter except a chemiluminescent substrate was used. A sample was deemed to be positive if the chemiluminescent signals were above the set cut-offs for 2 or more antigens. A rapid TB assay (DPP CervidTB test, Chembio) was used on selected samples. ZN stains to detect acid-fast bacteria were performed on selected milk samples. Positive (n=34) and negative (n=73) goat sera were obtained from Irish herds (4). Serum samples were obtained from goats in Norway that had (n=74) or had not (n=18) been vaccinated against Mycobacterium paratuberculosis (MAP).

RESULTS:
A herd of 500 dairy goats (all vaccinated against MAP) was supplemented by 200 dairy goats from another dairy farm. The majority of the purchased herd were found to be skin test reactors and all 200 slaughtered within 4 months of purchase. A large proportion had visible lesions at post-mortem. Skin testing of the original herd 3 months later showed 14 reactors and these were removed. The skin test negative animals were tested for antibody in serum (n=447) and milk (n=275). In serum, 351/447 (78.5%) were positive, with 279/447 (62.4%) reacting against 4 or more of the antigens. The DPP rapid test used on 2 of the 6-antigen positive samples and 2 of the negative samples confirmed the Enferplex results. In milk, 257/275 (93.5%) were positive, with 241/275 (87.6%) reacting against 4-6 antigens. The correlation between serum and milk was high for each of the 6 antigens (Spearman’s R=0.79 to R=0.94; all P < 0.0001). Preliminary work showed that 2/6 milks were positive for acid fast bacteria, and 5/8 animals antibody positive for 5 or 6 antigens had visible lesions in the mammary gland. Samples from Johnes’ vaccinated and unvaccinated goats were all negative in the Enferplex test, indicating that vaccination against Johnes does not induce cross-reacting antibodies.

DISCUSSION AND CONCLUSIONS:
Previous work in goats has suggested that the skin test is an efficient diagnostic tool for detecting TB infection (7). However, here we show that a very high proportion of skin test negative animals had antibody responses to M. bovis antigens in both serum and milk. Johnes’ vaccination was ruled out as an explanation for the positive TB antibody responses. The results show quite clearly that skin test missed a large number of TB infected animals and these can be revealed by antibody testing. This result mirrors results obtained in skin test negative cattle where a very high proportion of the Enferplex BovineTB test antibody positive animals had lesions (3). The high correlation between serum and milk antibody responses indicates that milk could be used for screening purposes.

The Enferplex Caprine TB antibody test can detect infection missed by skin testing and the results suggest that milk could be used for screening purposes. The multiplex open up a new approaches to diagnostics and surveillance of TB in goats.

REFERENCES:
ORAL PRESENTATIONS

QUALITY ASSURANCE AND AUTOMATION
INTRODUCTION:
The fit of purpose of a diagnostic test can be assessed with a cross sectional study. Estimates of sensitivity, specificity, prevalence, and positive and negative predictive values (PPV/NPV) can be assessed, as well as the respective 95% confidence intervals (95CI). Unfortunately, cross sectional study can be difficult to implement and case/control studies are often preferred when assessing the performance of a diagnostic test. The sensitivity and specificity observed are then extrapolated to calculate predictive values given different prevalence. This approach provides good point estimates of PPV/NPV but does not allow an easy calculation of the 95CI.

MATERIALS AND METHODS:
A simulation strategy is proposed to estimate the confidence intervals of PPV/NPV. From the sensitivity and specificity estimates, the bayesian 95CI are calculated. These confidence intervals present the advantage of distributions and therefore can be used to sample from. The sampling outputs are computed with a given prevalence value in order to establish a posterior distribution of the predictive values. The respective 95CI are then established. The simulation is repeated for all prevalence values between 0 and 1 with increments of 0.01. All computations are realized in R 3.0.1. The simulation is illustrated using two hypothetical tests (Test 1: Se=99%, Sp=99%; Test 2: Se=95%, Sp=100%) and is evaluated in two populations of different size (Pop. a: 100 infected, 100 healthy; Pop. b: 100 infected, 1000 healthy).

RESULTS:
Graphical outputs illustrate PPV/NPV and their respective 95CIs in function of the prevalence, sensitivity, and specificity. Test 1 and test 2 are compared and despite the difference between the positive predictive values at low prevalence, large overlapping of their confidence intervals illustrate the importance of considering both the value and the precision of the value before inferring decision upon fit for purpose of the test. Additional comparison illustrates the impact of sample size on the predictive values’ confidence intervals.

DISCUSSION AND CONCLUSIONS:
The empirical method hereby described presents a convenient alternative to obtain 95% confidence intervals of predictive values extrapolated from sensitivity and specificity estimates from case control studies. This method can be use to compare predictive value before selecting a test over another in a particular population. Alternatively, the simulation model could be reversed and used to infer ideal sample size for obtaining the precision targeted for the PPV/NPV.

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A POINT-OF-NEED PCR DETECTION PLATFORM BASED ON POCKITTM INSULATED ISOTHERMAL POLYMERASE CHAIN REACTION FOR RAPID AND SENSITIVE DIAGNOSIS OF CANINE DISTEMPER VIRUS

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Keywords: canine distemper virus, insulated isothermal PCR, point-of-need diagnosis

INTRODUCTION:
Canine distemper virus (CDV) is the etiological agent of an important disease in canids and a wide range of carnivores. POCKITTM Nucleic Acid Analyzer is a field-deployable device capable of generating automatically interpreted iiPCR results from extracted nucleic acid within one hour (1-3). In this study, reverse transcription-insulated isothermal PCR (RT-iiPCR) was developed to facilitate point-of-need diagnosis of CDV infection.

MATERIALS AND METHODS:
CDV RT-iiPCR reagent targeting the nucleoprotein (N) gene was established and prepared in a lyophilized form. This reagent was used in conjunction with the POCKIT Analyzer. Assay limit of detection with a 95% (LOD95%) probability was evaluated using in-vitro transcribed RNA. Sensitivity of the CDV RT-iiPCR assay was compared to a reference real-time RT-PCR (qPCR) assay (4) using serial dilutions of the Onderstepoort strain. Bordetella bronchiseptica, canine parvovirus, canine herpesvirus, canine adenovirus, canine influenza virus (subtype H3N8), canine parainfluenza virus, and canine respiratory coronavirus were tested to evaluate assay specificity. The accuracy of the CDV RT-iiPCR for detection of CDV in clinical samples was determined by comparing results with the reference qPCR assay using 110 clinical samples collected from dogs, raccoons, and foxes.

RESULTS:
LOD95% was 11 copies of in-vitro transcribed RNA per reaction. Sensitivity of the CDV RT-iiPCR was comparable to that of the reference qPCR assay. No cross reactivity with the other canine pathogens tested was found. The RT-iiPCR had 100% sensitivity and 100.00% specificity compared to the reference assay in detecting CDV in clinical samples.

DISCUSSION AND CONCLUSIONS:
The results indicated an excellent correlation between RT-iiPCR and a reference qPCR method. Working in a lyophilized format, the established RT-iiPCR has potential utility for rapid and easy point-of-need detection of CDV infection in animals, especially in resource-limited facilities.

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026
THE NETWORK OF ITALIAN LABORATORIES FOR ONCOLOGY IN VETERINARY MEDICINE: HARMONIZING DIAGNOSIS AND DATA SHARING

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Keywords: cancer, harmonisation, database

INTRODUCTION:
Laboratories for veterinary oncology in Italy belongs to different organizations: there are histopathology laboratories in Universities, Veterinary Medical Research institutes (IIZZSS) and private laboratories as well. Therefore in Italy there is a high potential to enhance the depth of knowledge in veterinary oncology, but there are often difficulties in sharing data, due to the lack of harmonisation in data collection. Aim of our work is to describe a network activated among veterinary histopathology laboratories (NILOV) to harmonize diagnosis and data sharing, staring from IIZZSS. Involved laboratories should guarantee not only the quality of diagnosis verified by regular participation in ring tests, but also the quality of the data by means of common data collection procedures to fill the NILOV database. Moreover NILOV will facilitate the exchange of information about available cases in each IIZZSS to realize ad hoc studies in veterinary oncology.

MATERIALS AND METHODS:
Ring test: 5 set of 36 haematoxylin-eosin stained slides were blinded and sent to the participants (23 histologist, 11 labs). Results were expressed as Benign (B), Malignant (M) or Non-neoplastic (N). The agreement among readers was assessed by calculating Cohen’s k-overall and Cohen’s k-combined and their 95% confidence intervals (CI) The NILOV database: Involved histopathologists agreed on adopting the WHO1 classification and on using international codes for topography (ICD-10) and diagnosis (ICD-O). The national data collection system is supplied, on regular basis, by each IIZZSS through a shared record layout and a web upload procedure. After a check on data consistency, data are collected in a national database. A descriptive analysis of the data collected during the first 6 months of activity is carried out.

RESULTS:
Ring test: Cohen’s k-overall everybody against everybody and Cohen’s k-overall to majority diagnosis are showed in Figure 1. The k-combined is 0.72 (95% CI 0.71 - 0.74).

DISCUSSION AND CONCLUSIONS:
The agreement among participants was good according to the definition reported by Altman2, even if the k-overall had a lower point estimation compared to the previous Ring Test3, presumably for the participation of new junior pathologists. The implementation of a common database collecting diagnosis from laboratories for veterinary oncology had to balance the need of information useful to supply the SI with information already collected by each IIZZSS. Data extraction from a SI to meet our record layout requires high skills in informatics and a certain amount of time when firstly set up, but has the potential to guarantee a constant data flow. The adoption of ICD codes, while not immediately straightforward, gives a common language among different electronic archives. Moreover we are planning to extend our network to every histopathology laboratory (university, private) willing to share data on regular basis and to have access to the database. Our work was supported by the Ministry of Health grant IZSPLV 10/11 RC.

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027
DIFFERENTIAL DIAGNOSTICS OF THE INFECTIOUS AGENTS CAUSING ABORTION IN BOVINES: INTEREST OF A MULTIPLEX PCR TECHNIQUE WITHIN A STANDARDIZED APPROACH

Colin S.[1], Guatteo R.[4],[8], Nicollet P.[2], Le Drean E.[5], Ninio C.[3], Maingourd C.[6], Joly A.[7]


Keywords: abortion, bovine, diagnostic, pcr, harmonize

INTRODUCTION:
The abortions and particularly repeat abortions are among the most common health problems and the most economically troubles in the cattle herd. In spite of the risks to animal health, but also to human health because of the zoonotic nature of many abortion agents for cattle (Brucella, Coxiella burnetii, Chlamydophila, Salmonella, Listeria…), is still deplored an underreporting of abortions. In addition, the majority of those involved in the diagnosis report a low clearance rate of 25% to 30% only, in the best cases. The recently acquired knowledges on some pathogens added to the development of more sensitive methods of diagnosis (PCR) open interesting opportunities in analysis of repeated abortions in cattle. The objective of this study is to describe infectious pathogens involved in abortions among cattle in three distinct French areas (Ille et Vilaine, Morbihan, Deux-Sèvres). For that they used a multi-agents PCR tool and the study was done during 14 months (from December, 2011 to January, 2013).

MATERIALS AND METHODS:
To do it, 925 cattle abortions in these three areas have been analyzed. The cases were only bovine abortions, for which we had at least one sample from: placenta, vaginal swab, or abortion. For each abortion, the available sample was analyzed using the LSI VetMAXTM Screening Pack - Ruminant Abortion - SARP-(Thermo Fisher Scientific) for the simultaneous detection of 8 abortion agents (Coxiella burnetii, Chlamydophila spp, Listeria monocytogenes, Salmonella spp., Campylobacter fetus, Anaplasm phagocytophilum, BHV4 and pathogenic Leptospira). They chose these pathogens because they are potentially expected in the placenta during the abortion, that at excluded BVD and Neospora. All this abortion agents have a DNA genome that allowed doing one sample extraction. A sample was considered positive for a target if Ct value <45. Moreover, some potential additional results (example: antibodies detection for BVD, Q fever and Neosporosis) were available. The positive PCR results were analyzed to allow the identification of eventual seasons, areas, samples and stage of gestation effects and also compared with the bacteriology for Salmonella spp and Listeria monocytogenes to verify the consistency of results.

RESULTS:
33% of samples were positive and the main agent identified by Real time PCR is unsurprisingly Coxiella burnetii (almost 18% of cases). To maximize specificity of the diagnostic, a quantitative threshold of 104 bacteria / ml of analyzed substrate was proposed to assign with “certainty” the abortion to Q fever. Ehrlichiosis is also found often, justifying its diagnostic, especially during vector activity period. Finally, serological results associated, with poor availability, confirm the important role of Neospora caninum and BVD virus, with Q fever, as 3 major abortion pathogens in cattle.

DISCUSSION AND CONCLUSIONS:
This study confirms the benefit of basic diagnostic, with a systematic research in first intention of Q fever, Neospora and BVD in a national initiative to harmonize the abortion diagnosis. Indeed, by combining PCR and serological results, a pathogen is strongly suspected as the abortion responsible in 6 times out of 10. PCR tools for the simultaneous detection of many pathogens also open interesting perspectives to increase the sensitivity of diagnostic procedures.

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3rd European EAVLD Congress - Pisa (Italy), October 12-15, 2014
ORAL PRESENTATIONS

MOLECULAR DIAGNOSTICS AND EPIDEMIOLOGY
GENETIC VARIATION AMONG AFRICAN SWINE FEVER GENOTYPE II VIRUSES, EASTERN AND CENTRAL EUROPE


Keywords: African swine fever, genotype II, genetic variability

INTRODUCTION:
African swine fever (ASF) is a devastating disease of domestic and wild suids caused by the ASF virus (ASFV). ASF is endemic in sub-Saharan countries and in Sardinia and has become more prevalent in Russia and the Caucasus region since its spread from eastern Africa to Georgia in 2007. The ongoing spread of ASFV to adjacent eastern European countries, such as Ukraine and Belarus, and the uncontrolled situation in Russia have placed the bordering areas of the EU at high risk for the introduction of ASFV. In January and February 2014, the first cases of ASF were reported in the EU in 4 wild boars in areas of Lithuania and Poland bordering with Belarus. In May 2014, four new wild boar cases were detected in Poland in the same affected area. To further our knowledge of the epidemiology and spread of ASFV, we sequenced the ASFVs isolated in Poland and Lithuania using international standardized procedures (1) and by the analysis of an additional ASFV genome marker region characterized by the presence of tandem repeat sequences (TRSs).

MATERIALS AND METHODS:
ASFV-positive clinical samples from the 8 infected wild boar were sent to the EU reference laboratory for ASF (CISA-INIA, Spain) for confirming testing and genetic characterization. After ASFV confirmation, initial genetic characterization was performed using standardized genotyping procedures including: i) C-terminal end of the p72 gene, ii) full sequence of the p54 gene, and ii) central variable region (CVR) within the B602L gene (1,2,3). 21 genotype II ASFVs isolated from wild and domestic pigs in Russia and the Caucasus region (2007-2013) were included in the study (4). To determine the origin and to map the spread of closely related ASFV isolates circulating in Eastern Europe a new set of primers were designed to amplify a 356-bp fragment located between the I73R and I329L genes characterized by the presence of tandem repeat sequences (TRSs).

RESULTS:
The 2014 Lithuania and Poland ASFVs clustered within p72 genotype II and showed 100% nucleotide identity with all compared ASFV isolates from Eastern Europe across p72 gene and p54 gene sequenced. We obtained same result in the CVR revealing 10 copies of TRS 100% identical and unique to those of the ASFV circulating in the Caucasus regions since 2007. However the amplification and further sequencing of the intergenic region between the I73R and I329L genes revealed size difference among the Poland, Lithuania, Belarus and Ukraine ASFV isolates caused by the insertion of an additional TRS (GGAATATATA) absent in the remaining viruses from eastern Europe.

DISCUSSION AND CONCLUSIONS:
The analysis of the 3 independent regions included in the classical genotyping showed that sequences for ASFV isolates from Lithuania and Poland were 100% homologous with those for ASFVs from Eastern Europe. Although the CVR has proven useful for resolving epidemiologic complexities at the genotype, country, and region levels, additional genome markers are required to discriminate among circulating virus isolates. The approach described here focused on analysis of the intergenic region between the I73R and I329L genes showed that the viruses from Poland and Lithuania had a TRS insertion identical to that present in ASFV isolates from Belarus and Ukraine.

These molecular data, together with the epidemiologic findings, confirmed that the ASFVs detected in Poland and Lithuania most probably originated from Belarus.

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SUITABILITY OF FAECES SAMPLES IN A NON-INVASIVE SAMPLING STRATEGY TO MONITOR AFRICAN SWINE FEVER (ASF) VIRUS AND CLASSICAL SWINE FEVER (CSF) VIRUS IN WILD BOAR

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Keywords: African swine fever, Classical swine fever, monitoring, faeces, wild boar

INTRODUCTION:
Presence of ASF virus (ASFV) and CSF virus (CSFV) in wild boar is of concern because of their possible role as disease reservoirs from which transmission can occur to domestic pigs. Therefore, the design of effective monitoring programs, both for early warning, and to determine the ongoing epidemiological situation in an infected population are important aspects. Testing blood samples requires invasive sampling strategies like hunting or capture of wild boar. Besides a bias towards healthy animals, such strategies are also linked to further spread of the virus. Non-invasive sampling strategies would increase the reliability of monitoring of ASFV and CSFV in wild boar populations, without the negative side effects. To evaluate the potential of faeces samples as a basis for non-invasive sampling strategies in wild boar, three factors are crucial: 1) excretion dynamics of the virus in faeces, 2) stability of viral DNA or RNA in faeces (for PCR-diagnostics), and 3) access to faeces samples in the field. This study addresses the first two points.

MATERIALS AND METHODS:
Domestic pigs were used as a model for wild boar. Pigs were infected with ASFV (n=40) or CSFV (n=15) with strains of different virulence. Viraemia and virus excretion in faeces were monitored in time by PCR. Furthermore, the effect of time and temperature on the detection of ASFV DNA and CSFV RNA in faeces were analysed by storing faeces samples from infected pigs at 5°C, 12°C, 20°C, or 30°C. Stored faeces were sampled at regular intervals and analysed by PCR.

RESULTS:
For ASFV, in the acute phase (0-21 dpi) viral DNA was detected in faeces 50-80% of the days in which ASFV was detected in blood. This percentage was going down to below 10% for the subacute/chronic phase. For CSFV, in the acute phase (0-30 dpi) viral RNA was detected in faeces 26-80% (low, resp. highly virulent strain) of the days in which virus was detected in blood. During the chronic phase (30-44 dpi), this percentage went up to 100%. ASFV DNA was quite stable in faeces. Half-live values ranged from more than 2 years at temperature up to 12°C, to roughly 15 days at temperatures of 30°C. CSFV was less stable than ASFV. Half live values ranged from 30-43 days at temperatures up to 12°C, to 1,6 days at temperatures of 20°C or higher.

DISCUSSION AND CONCLUSIONS:
Collection of faecal pellets is being used to determine for instance the size of wild boar populations (1) and should be possible with relatively low effort compared to hunting or capture. During the acute phase, for moderately to highly virulent strains, both ASFV and CSFV are detectable in faeces in a high percentage of pigs that are also viraemic. Besides that, especially ASFV DNA is very stable in faeces. During the time period that faeces can be expected to remain present in the field, ASFV can still be detected with a high confidence. CSFV is less stable, but on the other hand, virus levels in faeces are in general higher than for ASFV (2,3), so also here, it can be expected that virus can be detected for quite a long time in faecal pellets from the field. High temperatures may, however, affect the detection of CSFV more than the detection of ASFV. During the chronic phase, ASFV and CSFV excretion dynamics are completely different. From ASFV infected and viraemic pigs, only very few still shed virus through the faeces. From CSFV infected and viraemic pigs, on the other hand, all will shed high amounts of virus through the faeces during the whole chronic period. In general it can be concluded that sampling wild boar faeces offers a good perspective for non-invasive sampling strategies for ongoing monitoring in infected populations, especially for ASFV, but likely also CSFV. Field tests in regions where ASF or CSF occurs in wild boar would be helpful to further develop these surveillance strategies, through which ultimately also a better insight can be gained in the role of wild boar in the ASF and CSF epidemiology.

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FURTHER EVALUATION OF THE EFFECTIVENESS OF ORAL IMMUNISATION OF WILD BOAR AGAINST CLASSICAL SWINE FEVER VIRUS BY THE CHIMERIC VACCINE CP7_E2ALF

Feliziani F.[1], Blome S.[2], Petrini S.[1], Iscaro C.[1], Convito L.[3], De Mia G.M.[1]


Keywords: CSF, oral vaccination, wild boar

INTRODUCTION:
Wild boars are an important reservoir of Classical swine fever virus. Oral immunisation has been effectively applied in the past to control this disease based on the conventionally attenuated live vaccine strain "C"; unfortunately, this does not allow differentiation between infected or vaccinated animals. Nowadays, the construction of a new marker vaccine even suitable for oral application in wild boar population was reported (Beer et al., 2007). This candidate vaccine is the chimeric Pestivirus "CP7_E2alf" (Reimann et al., 2004). Several experimental investigations (Koening et al., 2011; Leifer et al., 2009), have shown the safety and efficacy of this vaccine for oral or intramuscular vaccination of domestic pigs and wild boar, but none of these studies was carried out with a bait formulation. In this context two oral vaccination campaigns of wild boar were carried out in Italy, with the aim to verify the capability of the CP7_E2alf bait vaccine to induce an effective immunological response in field conditions.

MATERIALS AND METHODS:
Two areas were selected for the field trials in the Umbria Region (Italy). The animals were vaccinated with a live attenuated marker vaccine based on the recently constructed chimeric pestivirus CP7_E2alf. The immunisation procedure included 2 distinct vaccination campaigns: the first one was started in September 2011 consisting in a single vaccination; the second one was based on 2 vaccinations with 1-week interval carried out in September 2012. The campaigns were performed about 30 days before the annual hunting activities when samples for serological investigations were collected. Furthermore, 1 year after the second vaccination campaign (Autumn 2013), additional serological tests were performed to evaluate the immunological status of wild boar population in the selected areas. For antibody detection conventional virus neutralization test (VNT) was performed according to the diagnostic manual of the European Commission using the reference CSF virus "Alfort-187".

RESULTS:
After the first vaccination campaign (2011), a total of 168 wild boars were shot; serology showed 59 positive samples (35.1%). One year later, after the double vaccination campaign (2012), 81 wild boars were shot. Among them, 27 animals developed CSFV antibodies with a positivity rate of 33.3%. Interestingly, 23 wild boars were found antibody positive out of a total of 49 animals shot in the first 4 beatings (46.9%). Finally, 4 out of 56 samples derived from the last campaign (2013) were detected as positive (7.14%).

<table>
<thead>
<tr>
<th>Hunting activities</th>
<th>Collected samples</th>
<th>Response + VNT</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>First campaign</td>
<td>2011</td>
<td>168</td>
<td>59</td>
</tr>
<tr>
<td>Single vaccination</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Second campaign</td>
<td>2012</td>
<td>81</td>
<td>27</td>
</tr>
<tr>
<td>Double vaccination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Last campaign</td>
<td>2013</td>
<td>56</td>
<td>4</td>
</tr>
<tr>
<td>No vaccination</td>
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</tr>
</tbody>
</table>

Table 1: VNT test results based on samples derived from the considered hunting campaigns

DISCUSSION AND CONCLUSIONS:
The present study demonstrates that the CP7_E2alf bait vaccination is able to induce a significant immunization in wild boar populations. In fact, the observed seroprevalence, was comparable with findings previously obtained during similar oral vaccination campaigns by application of the C-strain vaccine. Particularly interesting was the evidence that, after one year from the last vaccination campaign, it was still possible to detect the presence of vaccinal antibodies in the target wild boar population. In conclusion, this study confirms the CP7_E2alf oral vaccination as an effective tool now available for the control of CSF in wild boar populations.

REFERENCES:
INTRODUCTION:
PRRSV is the most important cause of reproductive failure and pneumonia in pigs. Since the 90’s global spread and extensive genmic, antigenic and clinical differences were described in isolates of both EU and NA types (1). As PRRSV negative herds suffer from massive clinical disorders and economic losses after introduction of a new strain, high efforts are being made by farmers and veterinarians to avoid this or to verify the source of virus. Knowledge of recent PRRSV genetic diversity is essential for the control of the disease, to guarantee the negative status of the herds and to learn about the regional spread in a molecular way.

MATERIALS AND METHODS:
330 samples were sent from April 2012 till June 2013 by vets practitioners and collaborating labs of the OÖ-TGD. The samples were taken from 78 farms with endemic PRRSV status or acute outbreak situation. First screening of PRRSV types were done by a commercial qRT-PCR (2). Positive samples was sequenced by using ORF5 specific EU or NA primers (3,4) and phylogenetical analysis was done with Bionumerics Software(1). In those holdings with high similar PRRSV sequences, a intensive farm analysis was performed based on the farm type, animal movement, distance between farm locations, disease status and veterinarian practices.

RESULTS:
In total, 194 PRRSV sequences were detected in 191 samples from 70 farms. In 188 samples a unique PRRSV type was found and in 3 samples both PRRSV types could be identified, whether field and vaccine strains together, or only vaccine strains. All EU strains analysed were subtype 1. Within EU-vaccine strains (26,8%), one cluster included piglets samples from 7 farms sent after vaccination with Porcilis® PRRS vaccine strain. The second cluster was linked to a natural outbreak in a boar stud with a vaccine-like strain 99% similar to Porcilis® PRRSV, with transmission by semen and consequent abortions in inseminated sows from 9 different farms. Field strains were found in 70.6% of the samples and shared low genetic identity (86%), with 38 different subgroups. PRRSV unique field strains were found in 31 from the 38 subgroups, whereas 7 strains were found to cooccour in more than 2 farms. All 5 sequences from the NA type in 4 farms were related to the Ingelvac® PRRS MLV vaccine (94% identity). Vaccination with NA type is not allowed in Austria. In all NA type cases, piglets were directly introduced from Germany, or farms were in immediate vicinity to a slaughter also for German pigs, where virus transmission through aerosol or indirect vectors was considered.

DISCUSSION AND CONCLUSIONS:
The outbreak in a boar stud caused by a PRRSV vaccine-like strain, and a high number of submitted emerging abortions explained the high percentage of vaccine strain sequences. PRRSV unique strains were found in 82% of the farms, whereas the rest infected at least 2 to 6 different farms. The genetic regional diversity was higher than expected, since there are a lot of family farms in the region of Upper Austria. Reintroduction of PRRSV field strains into free herds during the project, and also transmission from farm to farm through animal trade between endemic infected farms were seen. Also farm-to-farm and farm-to-slaughter neighborhoods, transport vehicles or in some unclear cases the role of the veterinary practitioner can be considered as cause of PRRSV transmission. The introduction of new strains from other regions and countries, instead of local spread of endemic strains probably explains the regional diversity. Although the epidemiological links between some farms with the same PRRSV strain was not always determined, PRRSV sequencing is an important and valuable diagnostic tool for practitioners and official swine institutions and nowadays is further being used in the control strategy of the region.

REFERENCES:
FIRST OCCURRENCE OF AN EMERGENT PCV2B VARIANT IN ITALY

Faccini S.[1], Barbieri I. [1], Franzini G. [1], Rosignoli C.[1], Moreno A.[2], Morganti P.[4], Nigrelli A.D.[1]


Keywords: PCV2, variant, genotype, pig

INTRODUCTION:
Porcine circovirus type 2 is highly prevalent in pig population worldwide and is associated with several clinical manifestations collectively named PCV disease (PCVD) or PCV acquired disease (PCVAD). Three different genotypes are recognized for this virus: PCV2a, PCV2b, and PCV2c. In 2010 a PCV2b variant (vPCV2b), initially classified as PCV2d, has been described in China [1]. Highly similar strains were subsequently identified in the United States, Serbia, and Brazil [3-5]. This study reports the occurrence of this emergent PCV2b variant in Italy.

MATERIALS AND METHODS:
In March 2014, lymph nodes from two pigs of 50Kg with gastric ulcers were submitted to the IZSLER laboratories for diagnostic investigation. The farm was a farrow to finish with about 150 productive sows. The herd was routinely vaccinated against PCV2. A PRRSv break had been active in the farm since the end of February. Mortality was about 5%: 3.5% linked to respiratory symptoms and 1.4% to gastric ulcers. PCV2 detection and quantification was performed as previously described [2]. PRRSV was detected by TaqMan NA and EU PRRSV kit (Ambion, USA). Full-length genome of PCV2 was amplified by a previously described method [4]. Sequencing was performed on both strands by BigDye Terminator Cycle Sequencing kit v1.1 on 3500xl genetic analyzer (Life Technologies). Sequences were analyzed using Lasergene software (DNASTar, USA). Phylogenetic analysis was performed using Neighbor-Joining method with K2P model (MEGA 6).

RESULTS:
A PCV2 load of about 9 Log(10) genome copies/g was estimated in both lymph nodes. Both samples were also positive for PRRSv Type 1. The full genome of the two strains was composed of 1,767 nucleotides. ORF1 was 945nt long and encoded a protein of 314 amino acids (aa); ORF2 was 705nt long and encoded a protein of 234aa instead of 233aa as in classic PCV2. Sequence analysis showed that IZSLER PCV2 strains shared 100% nucleotide identity and had a high level of identity (>99.5%) with vPCV2b strains previously isolated in other countries [1,3-5]. Phylogenetic analysis confirmed that IZSLER strains were closely related to the emerging vPCV2b (fig.1). Further sequence analysis revealed one nucleotide substitution (T1471C) leading to aa change in ORF1 (I84V) not previously reported and the presence of an additional K residue, at position 234 of ORF2 (K234), already detected in other vPCV2b.

DISCUSSION AND CONCLUSIONS:
In recent years the emergence of a new variant of PCV2b has been described in many countries. The strains here reported represent the first demonstration of the occurrence of this emergent vPCV2b in Italy. Differently from what described for previously isolated vPCV2b strains [1,3-5], clinical signs in the herd were not attributable to a typical severe PCVAD outbreak so there are not enough elements to affirm that the present isolation occurred in a case of vaccine failure. This report confirms the increasing diffusion of vPCV2b worldwide. Acknowledgements: The work was partially founded by Italian Ministry of Health research project PR2011015.

REFERENCES:

Figure 1: Linearized neighbor-joining tree based on the comparison of full genome sequences of IZSLER vPCV2b strains (●), prototype strains of PCV2a (♦), PCV2b (■), and PCV2c (▲) genotypes and other PCV2 strains isolated all over the world retrieved from GenBank.
MOLECULAR INVESTIGATION OF ANTHRAX IN ZAMBIA

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Keywords: Bacillus anthracis, epidemiology, Zambia

INTRODUCTION:
Anthrax is an old and neglected zoonosis that continues to raise concerns in Southern Africa. Although outbreaks of this disease have occasionally been reported from different parts of Zambia [1], the number of anthrax cases has recently increased and also new areas are reporting the disease [2]. In Zambia, clostridia infections [3] and anthrax outbreaks [4] are higher in the Western Province and Luangwa valley [1,4] than the rest of the country. Recently in the 2012, a large outbreak was reported for the first time in the Lower Zambezi where it was reported that more than 100 Hippopotamus amphibius amphibious hippocotomuses died [5]. In the study presented, 15 VNTRs panel and Canonical SNPs (CanSNPs) were used to analyse genetic relationships within animal anthrax in Zambia and to identify the possible global epidemiological correlations.

MATERIALS AND METHODS:
Twenty-one slides from dead animals with suspected anthrax were analyzed. From each slide DNAs extraction and semi selective cultures were executed. The suspected colonies were tested with a specific B. anthracis PCR assay (6). Finally the DNAs were subjected to MLVA with 15 VNTRs methodology, and CanSNPs as previously described (7). The results were compared to the 12 recognized worldwide sub-lineages and sub-groups (7).

RESULTS:
In total 21 slides were analysed. The bacteriological test permitted to isolate live B. anthracis from the material collected from three slides, while all the others were bacteriologically negative. The PCR, performed on DNA extracted from all slides and colonies, confirmed positivity for B. anthracis. The MLVA with 15 VNTRs showed different genotypes. The CanSNPs test demonstrated that all the strains belonged to the group A.Br.005/006, with the exception of two strains that belong to A.Br.001/002.

DISCUSSION AND CONCLUSIONS:
Despite anthrax being prevalent in Zambia, very few reports are documented. In some neighbouring countries several papers describe anthrax as a common disease for livestock and wildlife. For example, Zimbabwe and South Africa are recognized as territories with a high prevalence, with the presence of both the major lineages A and B (7). On the other hand, it was recently discovered that Namibia only has genetical groups within the lineage A (8). Here we report that in Zambia the anthrax outbreaks are caused by the two major clades: A.Br.005/006 and A.Br.001/002 in the lineage A. The group A.Br.005/006 is considered the ancient group within the A lineage, nevertheless its presence in Zambia as well the other southern African countries demonstrates that the first selection of this group probably occurred in Africa. Conversely ABr.001/002 is believed a more recent group probably selected in Asia and reintroduced in Africa either through introduction of domesticated livestock from Asia or by modern trades within the Commonwealth countries. To the best of our knowledge, here we first report the phylogenetic connection of the Zambian strains in a global phylogeny. In conclusion, the molecular characterization of isolates from anthrax outbreaks in Zambia has permitted to reveal a genetic structure in agreement with previous studies from neighbouring countries. Further, studies will be implemented in the near future on how to better manage anthrax outbreaks and define the risk maps of Zambia.

REFERENCES:
INTRODUCTION:
Five herds of Charolais cattle in Burgundy are facing recent serious health problems affecting young calves (severe enteritis) and adult females (lateral metri-peritonitis). The only common pathogen isolated is the BoHV4 virus, found either in the lungs or in genital swabs. In parallel, a high seroprevalence of anti-BoHV4 antibodies (80% on average) was highlighted among each herd. The involvement of BoHV4 was then considered, even if this virus is usually described to only exacerbate the inflammation initiated by bacteria. A working group comprising the breeders’ association, the veterinary technical group, the LDA71 and Biosellal was formed to answer three questions:

- Are the Charolais culard cattle more susceptible to BoHV4?
- Does the BoHV4 strain circulating in these 5 herds belong to a peculiar genotype?(1)
- Is the herd’s environment responsible of a severe reactivation of BoHV4?

MATERIALS AND METHODS:
Seroprevalence survey was conducted on 19 culard herds, 22 neighbouring herds and 8 witnesses non-culard herds. 20 cows per herd were analyzed with ELISA ID Screen® BHV-4 Indirect (IDvet). A full genome sequencing of 2 virulent strains (146 kb) was performed on MiSeq platform (Illumina) by the company Biosellal together with the sequencing of 2 strains with more classical expression isolated in the 1990s in french Brittany. Briefly, after purification of nucleic acids using the QiAmp mini kit (Qiagen), 50 ng were tagmented with Nextera®DNA kit (Illumina) to obtain 500 bp fragments on average. The 4 strains were sequenced simultaneously paired-end on Standard Flow cell (Illumina) with a depth of 200X. The reads generated were assembled de novo using software CLCBio and the sequences were compared with one another and with the two reference strains: AF318573.1 and JN133502.1.

12 cows from two herds of interest were sold in 2 epidemiologically un-related flocks. Conversely, some hardier animals were brought into these two herds of interest. The clinical status of these animals after parturition was followed.

RESULTS:
The average seroprevalence of antibodies against BoHV4 in culard herds is 12.63%, while this of the control is 4.38%. The prevalence of neighbouring herds is much more variable: 0%, 4.5%, 20% and 25%. The full genome sequencing reveals a number of points of single mutations between the strains of Burgundy and French Brittany. Animals leaving the 2 herds of interest have still underground-purulent peritonitis, scabies, but after two months, the animals regain a good condition. Conversely, hardier animals (Aubrac breed) introduced in these 2 herds express in the second season of calving some cases of scabies and purulent metritis.

DISCUSSION AND CONCLUSIONS:
The seroprevalence survey suggests a link between the culard phenotype and prevalence of antibodies against BoHV4. As there are no neutralizing antibodies, total antibodies are a marker of viral spread. Culard animals are known to be more susceptible to opportunistic infections. This immunosuppression may facilitate BoHV4 multiplication and dissemination within a herd. On the other side, often high prevalence (over 20%) were observed in the neighbouring farms. These data reveal a risk of rapid spreading of the virus when seroprevalence rates become very high (above 80%). The study of the virulence of the BoHV4 strain therefore becomes essential in order to anticipate the risk of dissemination of clinical outbreaks in our region. BoHV4 virus genome is a double-stranded DNA, mutations during replication are very rare (2). Full genome sequencing is necessary to be certain to detect some genetic markers. We found a few point mutations. As mechanisms of pathogenicity are poorly known, it is difficult to correlate these mutations with virulence (1)(2). However, we now have genetic markers to monitor the spread of the Burgundy strain.

The general microbial herd pressure also seems to play a major role in exacerbating the symptoms.

REFERENCES:
ORAL PRESENTATIONS

NOVEL TECHNOLOGIES IN DIAGNOSTICS
**INTRODUCTION:**
Salmonella is still one of the most widespread foodborne bacterial diseases in man; pork has been considered the source of about 70% of human cases of salmonellosis in Italy [1]. On that account, there is an urgent need for new methods for Salmonella control in pigs. The intestinal microbiota is an efficient barrier against pathogen colonization and plays a role in the progression of salmonellosis. The aim of this study was to investigate the relation between Salmonella Typhimurium virulence and gut microbiota in infected pigs.

**MATERIALS AND METHODS:**
Virulent S. Typhimurium ATCC14028 and its isogenic attenuated mutant strain, S. Typhimurium ΔznuABC[2] were used throughout the study. Thirty-one weaned piglets, 20-25 days old, were enrolled in the experiment. Animals were divided in a group of 9 (Group A) and two groups of 11 (Groups B and C) piglets. Group A was used as control, Groups B and C were orally infected with 2×10⁹ CFU of S. Typhimurium znuABC (Group B) or 2×10⁹ CFU of S. Typhimurium ATCC 14028 (Group C). Faecal samples were collected at different time points (T0, challenge; T2 two days and T4, 12 days after infection) from each piglet, and total DNA was extracted using the QIAamp DNA Stool Mini Kit. Extracted DNA was used to amplify by PCR the hypervariable V3-V4 regions of the 16S rRNA [3]. PCR amplicons underwent sequencing library prep according to the Nextera XT DNA Sample Preparation Kit (Illumina, US). All the libraries were normalized and pooled by 24 prior to sequencing on Illumina MiSeq using a 2x250 paired-end setting. The Lederhosen pipeline (based on UCLUST software and green genes v 13.5 16S database) was used to create the operational taxonomic unit (OTU) table for each sample. Kruskal-Wallis Test was used to test significant differences among the groups. Benjamini-Hochberg False Discovery Rate (FDR) method was applied to correct for multiple testing. Statistical significance was set at P<0.05.

**RESULTS:**
Piglets infected either with fully virulent or attenuated S. Typhimurium started to shed bacteria through faeces the day after the experimental infection, with different patterns. Virulent S. Typhimurium reached the peak of excretion at 2 days after infection and a similar amount of bacteria were shed in faeces throughout the period of observation. Conversely, the attenuated S. Typhimurium strain, after the peak at 2 days after infection, showed a decline in bacterial shedding. Metagenomics analyses revealed no significant differences among the three groups at T0. At T2, 7 phyla, 112 families and 404 genera resulted differentially present among the three groups. Notably, the hierarchical clustering of the most represented genera (abundance ratio greater than 0.1) displayed a perfect clusterization of each single sample into its belonging study group (Figure 1). At T4, 15 phyla, 143 families and 719 genera, respectively, differed across the groups. The hierarchical clustering of the most represented genera displayed a very good clusterization of each single sample into its belonging study group (Figure 2).

**DISCUSSION AND CONCLUSIONS:**
The gut flora of group A pigs changed along the experiment, showing a significant reduction of the family Prevotellaceae, which is the most abundant in pig intestine, and Clostridiales. This change was not observed in pigs belonging to groups B and C, suggesting an effect of Salmonella on gut flora natural development. At T2 and T4, pigs belonging to different groups clustered together; this difference was more evident at T4, when the excretion of Salmonella was significantly lower in group B compared to group C. Pigs belonging to group C had a flora richer in Prevotella, Eubacterium and Lactobacillus, and poorer in Ruminococcus. In conclusion, Salmonella infection caused a shift of porcine gut microbiota composition, whose extent was correlated to the virulence of the strain.

**REFERENCES:**
A NOVEL PARVOVIRUS FROM HORSE WITH POLYSYNOVITIS


CSIRO Australian Animal Health Laboratory ~ Geelong ~ Australia

Keywords: parvovirus, horse, next generation sequencing (NGS), polysynovitis, quantitative reverse transcription PCR (qRT-PCR)

INTRODUCTION:
In June/July 2012, a disease event occurred in thoroughbred weanlings in a breeding Stud, in the North East of New South Wales, Australia. Clinical symptoms in affected animals included synovitis, with swollen joints and acute lameness. The pathological examination demonstrated massive infiltration of neutrophils in joint tap fluid. Samples from a euthanized foal were sent to CSIRO Australian Animal Health Laboratory (AAHL) for disease investigation. No pathogens were detected following initial virus isolation attempt and PCR using pan-family primers for alphavirus, flavivirus and herpesvirus. To identify the cause of the disease, we analysed blood, lymph node and joint fluid from the affected horse using next generation sequencing (NGS) technology.

MATERIALS AND METHODS:
Whole blood, lymph node and joint fluid from an affected horse were used for laboratory testing. Virus isolation was conducted on lymph node and joint fluid using equine embryonic kidney cells and Vero cells for three consecutive passages. Generic PCR assays for alphavirus, flavivirus and herpesvirus were applied on nucleic acid extracted from all samples. The original samples were subsequently purified by ultracentrifugation and extracted nucleic acid was used for sequencing library preparation. NGS was conducted by using 454 GS FLX system (Roche) and MiSeq system (Illumina), respectively. A real-time quantitative reverse transcription PCR (qRT-PCR) assay was later developed for the specific detection of newly identified virus based on NGS results.

RESULTS:
No viral pathogen was isolated following 3 passages on each of the two cell lines.
There was no virus detected from samples on all three PCR assays for alphavirus, flavivirus, and herpesvirus.
The two libraries prepared from blood and lymph node generated 51,820 and 32,451 sequence reads, respectively, following 454 sequencing. The raw sequence reads were assembled into 574 and 679 large contigs, respectively, using De novo assembly. The sequences were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLASTN analysis at the nucleotide level showed no sequence identity to any of the known viruses in GenBank. A protein-based BLASTX search resulted in 13 contigs showing best protein sequence identity to chipmunk parvovirus and various degree of sequence identity to several other members of genus Erythroparvovirus, in the family Parvoviridae, including human parvovirus B19. The samples were re-sequenced using Illumina MiSeq system. Sequence gaps were filled using Sanger sequencing. The full-length genome sequence (5,538 bp) of the virus was obtained. Genetic analysis demonstrated that the virus is distinct from all known parvoviruses. The viral genome has 55.7%, 52.1%, and 50.5% nucleotide identity to chipmunk parvovirus, bovine parvovirus 3, human parvovirus B19, respectively.
A qRT-PCR developed based on the newly identified virus was utilised to test additional horses on the farm where the virus was first identified. The virus was detected from blood samples from three additional horses that had similar clinical symptoms.

DISCUSSION AND CONCLUSIONS:
A novel parvovirus has been identified from horses with polysynovitis using NGS. The novel virus represents a distinct genetic lineage from all existing parvoviruses. The virus exhibited highest sequence identity with a recently detected and characterised novel parvovirus, chipmunk parvovirus. To our knowledge, this is the first demonstration of parvovirus in horses with disease syndrome. The presence of the virus in site-appropriate samples from affected individuals and the similarity of clinical findings to those of humans infected with parvovirus B19 suggest that the virus is likely the causative agent of the disease. Further investigation into epidemiology and pathogenicity of this novel virus is on-going and will be reported in future.

REFERENCES:
NEUROTROPIC ASTROVIRUS IN EUROPEAN CATTLE WITH NON-SUPPURATIVE ENCEPHALITIS


Neurocenter, Division of Neurological Sciences, Vetsuisse Faculty, University of Berne ~ Berne ~ Switzerland, Interfaculty Bioinformatics Unit, University of Berne ~ Berne ~ Switzerland, Institute of Genetics, Vetsuisse Faculty, University of Berne ~ Berne ~ Switzerland

Keywords: metagenomic, astrovirus, viral encephalitis, cattle

INTRODUCTION:
Encephalitis is a frequently diagnosed condition in cattle with neurological disease. Many of the affected animals present with a non-suppurative inflammatory reaction pattern in the brain, i.e., with marked neuronal necrosis, gliosis and mononuclear lymphocyte infiltration (Theil et al., 1998). While this pattern supports a viral etiology, the causative pathogen remains unknown in a large proportion of the cases.

MATERIALS AND METHODS:
Samples included frozen unfixed tissues and formalin-fixed paraffin-embedded tissue blocks of the medulla oblongata of 25 cows presented clinical signs of CNS disease. Three out of those 25 samples were subjected for Next Generation Sequencing analysis whereas the rest 22 were tested by reverse transcription-PCR and in situ hybridization. The viral genome was confirmed by Sanger sequencing. The full-length capsid protein amino acid sequence of the astrovirus obtained in this study together with sequences of representative Astroviridae were used for phylogenetic analysis. The same analysis was also applied to additional PCR positive samples in order to estimate the viral diversity.

RESULTS:
Using viral metagenomics, we identified an astrovirus (BoAstV-CH13) in the brain of a cow with non-suppurative encephalitis. Additionally, BoAstV RNA was detected by reverse transcription-PCR and in situ hybridization in about one-fourth (5/22) of cattle with non suppurative encephalitis of unknown etiology. Viral RNA was primarily found in the neurons and at the site of pathology. Phylogenetically, BoAstV-CH13 was closely related to rare astrovirus isolates from encephalitis cases in animals and a human patient. Moreover, comparison among the different neurotropic isolates identified in our samples revealed a high similarity at level of the capsid protein.

DISCUSSION AND CONCLUSIONS:
Our findings provide plausible evidence for a causal relationship between BoAstV-CH13 and non-suppurative encephalitis in cattle. However, most of the non-suppurative encephalitis cases were BoAstV-CH13 negative suggesting that other pathogens are also implicated in this condition. The astrovirus strains detected in our cases seem to be phylogenetically very close and also similar to a recently presented isolate which was detected in neurologically affected cattle in USA (Li et al., 2013). Future research needs to be directed towards the pathogenic mechanisms, epidemiology and potential cross species transmission of these neurotropic astroviruses.

REFERENCES:
INTRODUCTION:
Aleutian Disease (AD), the worldwide most important disease in mink production, is caused by Aleutian Disease Virus (ADV) (1), a linear ssDNA-virus from the family parvoviridae. Infection results in excessive deposition of immune complexes severely affecting animal health and welfare. In addition, fur quality and fertility is reduced, imposing financial loss to the farmers. There is no efficient cure or vaccine against AD, and therefore a national control program was implemented in Denmark in 1976 (2). Danish law regulates the program, which has the aim to eradicate AD by identifying infected farms and preventing spread of infection e.g. by stamping out. Despite the negative effects on animals and industry, transmission routes of ADV are poorly understood. Little is known about viral diversity since previous molecular biological studies have focused on smaller regions of the genome, and just few isolates have been sequenced (3, 4, 5, 6).
A better understanding of the complete genome and its diversity will hopefully make it possible to differentiate strains and thereby identify isolates during outbreaks, and thus examine transmission-routes. The aim of this study is to sequence the ADV genome using Next Generation Sequencing.

MATERIALS AND METHODS:
Five mink confirmed positive for ADV (by CIEP and PCR) were included. All animals came from farms in Jutland sampled in 2012 or 2013. Viral DNA was extracted from serum using the QiAmp MinElute Virus Spin Kit (Qiagen, Germany) according to the manufacturers instructions. Primers for a long-range PCR assay were designed to amplify the genome in one fragment of 4,389 nt. (not yet published). PCR-products of correct size were visualized on a 1% agarose gel stained with ethidiumbromide. Then they were purified using QiAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturers instructions, quantified, diluted in TE-buffer and sent to DMAC (Denmark) for NGS using the Ion Torrent PGM System (Life Technologies) on a 318-chip according to the manufactures protocols. Raw data available in fastq-format underwent quality control, removal of primer sequences, trimming and filtering (Cutadapt version 1.4.1 and Prinseq Lite 20.4) before being mapped to the full-length ADV genome available in NCBI, a non-virulent isolate (ADV-G, accession number NC_001662 (Bloom et al. 1988)) (Samtools version 1.19). Nucleotide alignments were constructed in Geneious R7.1.5.

RESULTS:
PCR-amplification of viral DNA was confirmed by visualization of bands with the expected size (4,389 base-pairs). Approximately 99% of the reads mapped to the full-length ADV sequence available in NCBI, with the average coverage per nucleotide being 2000 reads (fig. 1).

DISCUSSION AND CONCLUSIONS:
In the present study a protocol for sequencing near full-length sequences of the ADV genome was developed. Five viral strains were isolated, PCR-amplified and deep sequenced. One of the limitations of the sequencing technology used lies in the ion-semiconductor having difficulties reading homopolymeric regions, such as between position 2,470-2,520 in the ADV-G genome. However uncertainty can be overcome by confirming data using other technologies, e.g. Sanger sequencing as demonstrated here. In order to investigate quasi-species/intra-individual variation, variation within and between farms, more full-length ADV sequences are needed. The results from the present study indicate there is a potential for improving AD outbreak investigation due to increased knowledge to the viral genome.

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GENOME-BASED PHYLOGENETIC ANALYSIS OF O26 VEROCYTOTOXIN-PRODUCING ESCHERICHIA COLI

Michelacci V. [1], Grande L. [1], Tozzoli R. [1], Maugliani A. [1], Dallman T. [2], Jenkins C. [2], Caprioli A. [1], Scavia G. [1], Morabito S. [1]


Keywords: VTEC, O26, Genomics

INTRODUCTION:
Verocytotoxin (VT)-producing Escherichia coli are zoonotic pathogens causing human disease worldwide. A restricted number of VTEC serogroups have been associated with severe infections such as the haemolytic uremic syndrome (HUS), with O26 representing the second most commonly isolated VTEC serogroup from HUS patients after O157. A VTEC O26 clone belonging to sequence type (ST) 29 and producing VT2 has been recently described and it has been hypothesized that its emergence led to the replacement a ST21 VT1-producing clone common up to mid-90s (1).

In Italy, during summer 2013, a cluster of 19 HUS cases with evidence of VTEC O26 infection was identified. We analysed the whole genome sequences (WGS) of eight VTEC O26 isolated from the 2013 cases and compared them with the other VTEC O26 WGS available in GenBank.

MATERIALS AND METHODS:
Eight VTEC O26 strains (EF456, ED902, ED909, ED911-915) isolated in 2013 and two additional VTEC O26 strains isolated in Italy in 1989 (ED17) and 2010 (ED766) were fully sequenced with the Illumina MiSeq 200bp paired end protocol. The sequences were analysed through the SNP-tree, MLST and VirulenceFinder tools at the DTU CGE webserver (2). WGS from three human and five animal VTEC O26 from USA and one from Japan of unknown source, available in GenBank, were added for comparison.

RESULTS:
The WGS of the Italian strains were assembled in about 500 contigs each. All the Italian strains from 2013 and the ED766 produced the VT2a toxin subtype, while the strain ED17 possessed the VT1a-coding genes. Four of the Italian strains isolated in 2013 and ED766 were ST29, while all the other Italian strains belonged to ST21, similarly to all the O26 strains whose sequences were obtained from the GenBank. A dendrogram was built on the basis of the 2,274 single nucleotide polymorphisms (SNPs) identified among all the tested sequences. Three main clusters could be identified, regardless the origin and sources of the tested strains.

Five strains from the 2013 Italian cases grouped into the same cluster (ED902, ED915, EF456, ED909 and ED911), but their distances indicated that they belonged to two distinct outbreaks (Groups C1 and C2, Figure 1), confirmed by the MLST typing. The remaining three Italian strains were assigned to the other groups (figure 1) indicating the lack of genomic correlation.

DISCUSSION AND CONCLUSIONS:
The analysis of the SNPs scattered along the entire genomes, revealed the VTEC O26 have a general variability not segregating with the ST. In fact, ST21 and ST29 strains clustered in the same groups (groups B and C, Figure 1). Additionally, the genetic distances indicated that at least two outbreaks might have occurred in Italy in 2013, together with a few sporadic cases of infections. The distribution of the ST among the VTEC O26 isolates is of interest. We observed that all the ST29 strains were from human cases of infection, while ST21 included both human and animal isolates. This finding is in line with the reported rare isolation of ST29 strains from animal sources (3), bringing into question the zoonotic origin for these VTEC O26. According to the “source-sink” model of pathogens’ evolution (4), the ST29 sub-population might have evolved from an ST21, or an ancestor in common with the latter hailing from the same “source”, cattle, by lingering into a transient “sink” habitat. Such a sink in turn could have been either another host or the environment and might have represented the source of the cases observed in Italy in 2013. Further work is needed to elucidate the dynamics of emergence of the ST29 clone.

REFERENCES:
3- Bletz S. et al. 2013; Genome Biol Evol; 5(10):1807-16
4- Sokurenko E. et al. 2006; Nat Rev Microbiol; 4(7):548-55
INTRODUCTION:
Allergen-specific IgE detection tests have been settled as a reliable and patient-friendly diagnostic alternative to skin tests in veterinary practice. They can support the designing of allergy immunotherapy and elimination diet therapy. Most available serological tests for the detection of type 1 sensitizations in canine sera are based on the determination of sIgE, using a panel of immobilized allergens and further detection of the immune-interaction with labeled anti-IgE antibodies. The scope of microarrays for high-throughput and multiplex analysis has expanded impressively in the last years. However, their use as routine in vitro diagnostics is hampered not only by technical and operational challenges in manufacturing but by their expensive laboratory facilities needed. To overcome these issues and to close microarray immunoassays platforms to veterinary practice, we are developing an allergen-sIgE detection immune based assay for canine sera using compact disk technology. This system exploits the benefits of using unmodified commercial disks (DVDs and Blu-ray disks) as supports and the ordinary disc drive as reader. This technology has been shown cost-effective, reliable and highly sensitive methodology in several fields (1, 2).

MATERIALS AND METHODS:
A panel of mouse mAbs against dog IgE (Bethyl Lab) of which clones 11H6 and 14C12 were further characterized for detection and capturing antibodies respectively were used. A total of randomly selected 192 canine sera were collected from Madrid Municipal Animal Shelter. 16 canine sera, previously sIgE tested by commercial kits, were kindly provided by Laboratorio Veterinario Garfia (Córdoba, Spain). ELISA and microarray immunoassays were developed in indirect format in which allergen extracts were coated or printed, respectively. Spotting on epoxy-coated glass slides and on Blu-ray and DVDs were performed as described previously (1, 2, 3). Allergen extracts were provided by INMUNAL (Madrid, Spain). Allergen-sIgE binding was semi-quantitatively measured by using HRP-conjugated 11H6 mAb as detector and TMB (SDT) as enzymatic substrate. Spot signal intensity was measured by CLAIR microarray reader (Sensovation). The signals acquired by the disc drive were digitalized and transformed into an image for further quantification as previously described (1, 2).

RESULTS:
192 canine sera were analyzed by in-house indirect ELISA against 26 allergen extracts. The highest prevalence of allergen sensitization was against the mite (9%) and the grass pollen (8%) groups whereas the prevalence against tree pollens and weed pollens were 7% and 6%, respectively. None of the 192 sera were sensitized against any of the four mold extracts assayed and 4% of sera had a positive reaction to cat flea extract. The concordance rate between ELISA and microarray immunoassays on epoxy-coated glass chips was 89%. These assays were used as analytical standards for developing allergen-sIgE detection immunoassays on Blu-ray or DVD disks using commercial disk players as readers (Fig 1).

DISCUSSION AND CONCLUSIONS:
1. We have set up an ELISA and microarray immunoassays on both glass slides and optical compact disks (DVD and Blu-ray) for allergen-sIgE detection in canine sera.
2. Compact disk technology is a reliable and sensitive methodology for allergen-sIgE detection in canine sera, showing similar results in terms of sensitivity, selectivity and reproducibility to those obtained by ELISA.

REFERENCES:
POSTER PRESENTATIONS
ANIMAL DISEASES, BACTERIAL
A MULTIPLEXED IMMUNOASSAY FOR DETECTION OF ANTIBODIES TO ACTINOBACILLUS PLEUROPNEUMONIAE IN PIGS

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Keywords: Multiplexed immunoassay, Actinobacillus pleuropneumoniae, Lipopolysaccharide

INTRODUCTION:
The bacterium Actinobacillus pleuropneumoniae (App) is the causative agent of porcine pleuropneumoniae, a contagious and severe respiratory disease in pigs. Based on capsular antigens, 15 App serovars have been described, and the prevalence and morbidity of these serovars vary with geographic regions (1). In Denmark, the most important serovars are considered to be App 1, 2, 5, 6, 7, 10 and 12. As part of the Danish surveillance program for App, the Danish Veterinary Institute uses ELISAs and complement fixation tests (CFT) to test for porcine anti-App antibodies (2-7). In an effort to improve our diagnostic tools, we are currently developing a novel indirect fluorescent microsphere immunoassay that can facilitate simultaneous detection of antibodies towards multiple App serovars within a single serum sample volume. The multiplex immunoassay is based on Luminex technology (8) and has several benefits compared to ELISA and CFT, including reduced serum sample volumes, lowered amount of labor and faster acquisition of results.

MATERIALS AND METHODS:
The multiplexed assay employs up to 80 batches of microscopic magnetic beads that differ in fluorescence. In our assay, seven batches of beads were successfully coupled with capsular App antigens that were previously purified for use in ELISA and CFT. These antigens included lipopolysaccharide (LPS) extracted from cultures of six App serovars (App 1, 2, 6, 7, 10 and 12) or a mixture of LPS and capsular polysaccharide extracted from App 5. The coupled beads were plexed and incubated with sera from experimentally infected pigs as well as from naturally infected and non-infected pigs. Bound antibody was detected with layers of biotinylated anti-pig antibodies and fluorescently labelled streptavidin. Samples were analyzed in a Bioplex 200 reader (9) and results were read as median fluorescence intensity (MFI).

RESULTS:
The specificity and sensitivity of the multiplex immunoassay were similar to that of our ELISAs. Since we have not previously succeeded in establishing an App 1 ELISA, we currently use CFT to detect infection with this serovar. However, App 1 LPS was successfully coupled to beads and included in the multiplex immunoassay. Antigen-specific reactivities measured in a monoplex format were attained when combining beads coupled with App 1, 2, 5, 6, 7, 10 and 12 in a multiplex format, indicating limited cross-reactivity. In addition, antigen-coupled beads maintained stable interaction with serum antibodies when analyzed over a period of four months. Longer storage periods have not yet been tested.

DISCUSSION AND CONCLUSIONS:
The multiplex assay as designed is a sensitive and specific method for detection of porcine antibodies to App, and shows good overall agreement with our well-established ELISAs. The multiplex assay is rapid and simple and has the potential of being an important addition to current immunoassays for detection of infection with App as well as additional infectious agents in pigs or other production animals.

REFERENCES:
8. Luminex Corporation. Austin, TX, USA.
INTRODUCTION:
Mycoplasma meleagridis (MM) is widespread in turkey flocks and could also establish natural infection in chickens (Mardassi et al., 2007, Béjaoui Khiari et al., 2011). A number of antigens appear to be shared between MM and the more important poultry mycoplasmas, as Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS), resulting in cross-reactivities, but there are few molecular data concerning the MM species. Although some studies have revealed antigenic heterogeneity between strains, genes encoding MM immunodominant antigens have not yet been isolated and characterized (Mardassi et al., 2007).

The aim of this work was to identify and initially characterize MM proteins that could be used as potential candidates for a more reliable diagnosis by using 2D electrophoresis analysis combined with immunoenzymatic and mass spectrophotometry assays.

MATERIALS AND METHODS:
A MM field sample, isolated from a turkey cloacal swab, identified by DGGE-PCR and immunoperoxidase staining was used. A preliminary 1D WB of whole cell proteins (5 μg protein/lane) was tested against two commercial MM Positive Controls (MM+), eight different sera from MM naturally infected turkeys, MG Positive Control (MG+), MS Positive Control (MS+) and a negative control (C-).

MM cells were prepared as described previously by Regula et al. (2000) and the sample was precipitated using the Methanol/Chloroform/Water Protein Precipitation. Once the protein concentration was determined by using Bradford protein assay, it was diluted to a suitable concentration for the successive separation steps.

Seven cm strips over the pH range of 3–10 (GE Healthcare) were subjected to isoelectric focusing (IEF), equilibrated and transferred onto 12 % SDS-polyacrylamide gels. Of the total gels, five (protein concentration of 60 μg) were electro transferred to PVDF membranes for separately WB analyses using two different positives naturally infected serum, a C-, a MG+ and a MS+ serum. In the other two gels, total proteins were visualized or by Blue Coomassie staining or by Silver Staining.

Gel images were acquired with a calibrated scanner (ImageScanner III, GE Healthcare), and immunoreactive spots were excised and identified using ESI-TOF mass spectrometer (Bruker).

The MM genome was sequenced by Illumina MiSeq sequencing technology, resulting in 11 unordered contigs and using Geneious software, all possible Open Reading Frames (ORFs) from the sequence were determined.

RESULTS:
In the 2D-Western Blot analysis, several immunospots were observed in both MM positive serum, no cross reaction with the MS+ and faint cross reaction in the samples C- and MG+ of around 40 Kd. Immunoreactive spots present only in 2D-WB probed with MM positive sera were excised and identified by mass spectrometry.

After sequencing the whole MM genome and obtaining its ORFs, the MS/MS spectra were searched against a custom database obtained from the NGS sequencing, and also against Blastp to find homologies with known bacterial proteins. The in silico analysis allowed us to identify a set of seven immunoreactives proteins, in which two seemed more suitable for MM diagnosis proposes.

DISCUSSION AND CONCLUSIONS:
The combination of high resolution protein separation by 2DE and mass spectrometry has proven to be an essential tool for proteomics, and in our study were applied in order to identify MM proteins that could be used as potential candidates for its diagnosis (Piras et al., 2012).

The first protein, matches the well characterized P80 lipoprotein from M. agalactiae (MA), which is an important antigen for the MA diagnostic. The second belongs to the family of Ornithine carbamoyltransferase, who is involved in the biosynthesis and/or degradation of arginine. Both proteins have a low similarity with the others avian mycoplasmas and seems to represent good candidates for a MM serological tests.

REFERENCES:
Béjaoui Khiari, A et al 2011 Isolation of M.meleagridis from chickens. Avian Dis 55:8–12
INTRODUCTION:
Mycobacterium bovis causes TB in Cervidae and can be a constraint on production and cause welfare problems while also creating TB reservoirs. Currently skin testing is used for diagnosis of TB in deer; however reports of test accuracy concerns highlight the need for alternative diagnostic tests for TB. Here we report on a serological multiplex ELISA that simultaneously detects responses to multiple M. bovis antigens. The Enferplex TB test is available for cattle, goats, alpacas, badgers and wild boar (1-6). Here we present data on the use of the Enferplex Cervid TB test.

MATERIALS AND METHODS:
A set of 10 recombinant proteins (ESAT6/CFP10/MPB70/MPB83/Rv3616c) and their peptides were used (5). The 10 antigens were spotted into each well of a microplate. The assay was carried out as previously described (1) with optimisation for Cervidae. Serum samples were diluted 1/500 and incubated at 25oC for 1 hour. After washing, peroxidase-labelled Protein G antibody was added and incubated at 25oC for 30 min. After washing substrate was added and the chemiluminescent signal read. Data was extracted as relative light units using a digital camera and analysed in an excel macro. Optimal cut-offs were set for each antigen using defined positive and negative samples. Over 700 samples from various sub/species of Cervidae from Canada and USA (red deer/elk hybrids, elk, reindeer, Axis Sitka, white-tailed and fallow deer) were included in this study, from naturally infected, confirmed negative and experimental challenge studies.

RESULTS:
The specificity of the test was 98.3% (95% CI: 96.8-99.8) with an overall sensitivity in natural and experimental infections of 86.8% (95% CI: 79.2-94.4). In a population of infected deer (M. bovis culture positive) which were negative in the skin test the multiplex assay detected 88.9% (95% CI: 77.1-100). In an experimental infection of Red deer/Elk hybrids (1.5x10^3 cfu M. bovis intratonsillar) serological responses were detected by day 30 in 1/10 animals progressing to 6/10 at day 86 and 10/10 by day 192.

DISCUSSION AND CONCLUSIONS:
Recent reports highlight the need for a new approach to TB diagnosis in Cervidae due to the overall unsuitability of currently available tests and confounding factors associated with them. Here we report on a novel multiplexed immunoassay which has high sensitivity and specificity in various Cervid species. A serology-based test could be important in monitoring potential wildlife populations for TB infection. The potential shown of the multiplex assay to detect infection where the skin test has not, highlights the potential of using a test that targets different immune responses to assist TB control in Cervidae. Results of this work show the possibility for the use of this novel blood based test for Cervidae. Potentially there is the option for the multiplex to be used for standalone screening or alongside tuberculin based tests in an eradication programme. Tackling the complex nature of TB using more than one diagnostic test with different biological basis could be the key to successful control. Further studies are needed with the multiplex assay on other Cervid species and to confirm findings. This work has shown the Enferplex Cervid TB ELISA has high sensitivity and specificity and can detect infection missed by skin testing. It may provide a useful tool for diagnosis and surveillance. The use of a multiplex assay has the potential of creating a more effective diagnostic test system for screening animals and the early detection of TB disease for disease management and eradication.

REFERENCES:
5. O’Brien et al (submitted to CVI)
INTRODUCTION:
Pathogenic mechanisms underlying subclinical mastitis caused by coagulase negative staphylococci (CNS) in sheep are poorly understood. In recent years, several factors have been identified as responsible of CNS virulence, mainly regarding the ability to form biofilms (1). Biofilms consist of an extracellular matrix composed by polymeric substances (2) that incorporate bacteria and facilitate tissue adhesion and infection. The main component of the extracellular matrix of most CNS is the polysaccharide intercellular adhesin (PIA). Together with the microbial surface components recognizing adhesive matrix molecules (MSCRAMM), it represents the main factor involved in biofilm formation in staphylococci. Biosynthesis of PIA is realized by the ica locus genes (3), whereas adhesive microbial surface components by the genes bap (3), aap (4,5), fbe (5), embP (6), and atlE (7). Factors involved in virulence are also the mecA gene (8) and the transposable elements IS257 and IS256 (9,12). The aim of the present study is to investigate the presence and the expression of virulence determinants in CNS isolated from sheep milk.

MATERIALS AND METHODS:
Aseptically collected sheep milk samples were inoculated in 10% ovine blood agar and suspected colonies subcultured and identified. Three hundred and 29 CNS were analyzed by a multiplex-PCR targeting ica genes and then by single PCRs for the virulence-associated genes aap, fbe, atlE, embP, mecA, IS257, and IS256 (9). The in vitro production of biofilm were investigated by the Congo red agar test (10) and by adhesion assays on abiotic surfaces of polystyrene (11,12). The reference strains ATCC 35983 and ATCC 14990, ica+ and ica- respectively, were included as controls.

RESULTS:
Ten isolates, 9 S. epidermidis and 1 S. chromogenes, resulted positive for ica genes; icaB was lacking in 1 S. epidermidis. All ica carriers and the 2 reference strains tested positive for genes encoding the surface proteins with adhesive function, with the exception of 1 isolate of S. epidermidis missing the aap gene. All isolates showed the IS257 element, whereas IS256 was detected in only 1 isolate of S. epidermidis. No strain resulted mecA carrier. Phenotypic production of biofilm was consistent with the presence of genes, except for one ica positive S. chromogenes that did not produce biofilm in vitro.

DISCUSSION AND CONCLUSIONS:
In our investigation, ica markers are not always linked to the ability of strains to form biofilm in vitro. In fact, only 3% of the isolates tested positive for ica genes. Probably because the PIA is not essential for biofilm production in our strains. Since differences in biofilm extracellular matrix were highlighted in CNS strains (13,14), it is likely that other extracellular factors could play a role in PIA-indipendent biofilm formation and in ovine mammary gland infection.

REFERENCES:
INTRODUCTION:
Neonatal diarrhoea leads to significant economic loss in the pig-rearing industry. Clostridium (C.) difficile, a gram-positive anaerobic growing rod-shaped bacterium, considered as an important causative agent of antibiotic-associated diarrhea in humans, is currently described as a possibly underestimated pathogen responsible for diarrhoea in piglets. As a part of the intestinal microbiota of pigs, C. difficile can proliferate extremely in case of dysbiosis. Enterotoxin A, Cytotoxin B and the Binary Toxin are virulence factors, expressed by toxigenic strains. Once bond to receptors on their target cells, the toxins are internalized and destruct the cytoskeleton and tight-junctions by deactivation of signal-transduction molecules. Increased vascular permeability and liquid-release into the intestinal lumen and tissue is the result, which manifests clinically as diarrhoea with 1 to 7 day-old piglets due to lesions in the gross intestine. The occurrence of toxigenic C. difficile strains in piglets with diarrhoea as well as the corresponding histologic and immunohistochemical findings are examined and evaluated under regard of other intestinal pathogens.

MATERIALS AND METHODS:
Examinations were carried out with suckling pigs with diarrhoea and a control-group without diarrhoea. In pathological dissections samples were taken for complete bacteriological, virological, histological and immunohistochemical examinations regarding intestinal pathogens and virulence factors. C. difficile isolates were further characterised concerning antimicrobial susceptibility and toxin-genes. For this different conventional and real-time PCR protocols were tested.

RESULTS:
161 piglets from 48 different farms in Germany have been examined. C. difficile was found in 67 % of all samples. 85,4 % of the farms were positive for C. difficile. In 43 cases C. difficile was the only pathogen found. Until now all C. difficile isolates were tested as toxigenic. Diversity of toxin-patterns were similar to those described in literature.

High-grade to middle-grade oedema of the mesocolon, and pasty to runny, yellow intestinal contents were found frequently. 83,1 % of the cases with mesocolonic oedema were positive for C. difficile. Histologically, an acute, multifocal to diffuse, erosive colitis was detectable in most samples. So called “volcano lesions” with focal exudation of mucous, fibrin and neutrophils into the intestinal lumen could be found in 3 cases. Those cases were positive for C. difficile and negative for other pathogens. In immunohistochemical examinations receptor-bond toxin could not yet be colocalized with histologic lesions in the colon.

DISCUSSION AND CONCLUSIONS:
Toxigenic C. difficile were found in most of the suckling piglets examined. For an aetiological diagnosis, it is important to also regard other intestinal pathogens and histological identification of their typical lesions. Mesocolonic oedema seems to be indicative, but not pathognomonic for C. difficile. Volcano lesions on the other hand might be pathognomonic. There will further examinations to strengthen this hypothesis and to check the significance of immunohistology.

REFERENCES:
ARE DERMATOPENTOR RETICULATUS AND IxODES RICINUS THE REAL RESERVOIR OF FRANCISELLA TULARENSIS?

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Dipartimento di Biologia Animale, Università degli Studi di Pavia ~ Pavia ~ Italy, Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano ~ Milano ~ Italy

Keywords: Ixodes ricinus, transovarial transmission, Francisella tularensis

INTRODUCTION:
Ixodes ricinus and Dermacentor reticulatus (Acari: Ixodidae) are a small hard ticks able to transmit a large variety of pathogens to humans and animals. These ticks have been described as a potential vectors of Francisella tularensis, a highly contagious zoonosis for a wide number of mammals, reptiles and birds [1]. Transtadial transmission of this bacterium from larva to adult has been demonstrated under laboratory conditions. However, transovarial transmission is still debated [2]. The aim of this study was to evaluate the possible transovarial transmission of F. tularensis in these two ticks.

MATERIALS AND METHODS:
Four guinea pigs were infected with 0.5 ml of F. tularensis type B (1000 cfu/ml). After 2 days, 25 females and 35 males of I. ricinus and 25 female and 35 males of D. reticulatus were placed on 4 guinea pigs. The experiment was done in six replicates for a total of 150 I. ricinus females and 150 females of D. reticulatus. After 6 days from the infestation, 24 engorged females of both species were dissected and salivary glands, midgut and ovary were collected and analyzed by real-time PCR target 23 kDa, transmission electron microscopy (TEM) and fluorescence in situ hybridization (FISH). The remaining females were maintained in incubator at 26°C and 90 RU to allow oviposition. Eggs were recovered and the ticks analyzed by PCR, TEM and FISH. Pool of 50 eggs were analyzed by PCR, culture and bioassay. Approximately 600 eggs were homogenized, suspended in saline solution and injected sc in 2 mice. Other groups of eggs were used to obtain larvae and nymphs. Three hundred larvae and 300 nymphs were used to infect 4 mice. A similar quantity of larvae and nymphs was tested by culture and PCR.

RESULTS:
All engorged ticks were positive by PCR (midgut), TEM (ovary) (Fig. 1), FISH (ovary and salivary glands) and culture (midgut and ovary). The PCR carried out on eggs was positive but the culture and bioassay in mice were negative. The same result was obtained on larvae: PCR positive and culture and bioassay negative. The nymphs after blood meal on mice were negative by PCR and culture.

DISCUSSION AND CONCLUSIONS:
TEM and FISH showed the successful infection of the ovary by F. tularensis and PCR showed the presence of bacterial DNA in eggs larvae. However, the negative results obtained by culture of eggs, larvae and nymphs and especially the negativity of bioassay, indicate the passage of non-viable bacteria from ticks to eggs, larvae and nymphs. In conclusion, the data suggest the failure of transovarial transmission of F. tularensis in eggs of I. ricinus and D. reticulatus.

REFERENCES:

Figure 1. TEM image of previtellogenic ovary of I. ricinus. Note the presence of residual bodies (cr) in a large vacuole of phagocytosis (arrow).
IS THE INNATE IMMUNITY AFFECTED BY MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN CATTLE? AN ITALIAN CASE-CONTROL STUDY

Mazzone P. [1], Arrigoni N. [2], Amadori M. [3], Bernardi R. [4], Cornelli S. [1], Di Paolo A. [5], Costarelli S. [1], Cruciani D. [1], Felici A. [1], Maresca C. [1], Papa P. [1], Moscati L. [1]


Keywords: Paratuberculosis, Innate Immunity, Albumin

INTRODUCTION:

The innate immune system is the oldest defence mechanism against microorganisms and provides the first line of defence. Animals survive many infectious challenges in the absence of pre-existing specific (adaptive) immunity because of the existence of a separate set of protective mechanisms that do not depend on specific antigenic recognition (innate immunity) [1]. Immunity against mycobacteria is characterized by a very complex series of events, designed to control the infection. The first line of defence is the uptake and processing of pathogen by macrophages, followed by the initiation of cell-mediated immune responses. Mycobacteria can survive and replicate within macrophages by inhibiting the maturation of the phagolysosome [2]. The secretion of pro-inflammatory cytokines such as IFN-γ is involved in the containment of mycobacterial infections. This also takes place in infection sustained by Mycobacterium avium subsp. paratuberculosis (MAP), the etiological agent of Paratuberculosis (PTB). In PTB pathogenesis the animals become infected at a young age, through the ingestion of MAP from colostrum, feces or contaminated fodder, but the disease does not take until 2-3 years of age, due to cell-mediated and innate immunity.

Aiming at better understanding the pathogenesis of PTB through a case-control study, we investigated some parameters of innate immunity relevant to chronic infections in MAP-infected cattle.

MATERIALS AND METHODS:

In a preliminary phase on 12 PTB-positive animals from a PTB-positive herd, we assessed the biochemical profile to identify significant parameters of innate immunity that could be involved in MAP infection. Subsequently, all the 90 cattle from the original herd were included in the study and tested for PTB antibody (ELISA-ID Vet) and for: Serum lysozyme and bactericidal activity, measured by lyso-plate assay [4] and by the method of Dorn, et al. 1980, respectively; the haemolytic complement assay [6] carried out in microtitre plates; different Acute Phase Proteins monitored by a commercial kit [5]. Finally Serum Total Proteins and Albumin were evaluated by cellulose acetate electrophoresis. Feces from all animals included in the study were collected for MAP isolation [7].

The association between PTB infection and innate immunity parameters was evaluated in terms of Odds Ratio (C.I. 95%) and linear regression and Spearman’s rho were performed for significant associations. P-values <0.05 were considered statistically significant. Analyses were performed using Excel and Stata 11.2 software package.

RESULTS:

Out of 90 animals, 27 were PTB ELISA-positive, and 63 negative; 15 animals were positive for MAP culture isolation. Infected animals showed a statistically significant association only with albumin values among the biochemical parameters under study (Tab. 1 and Fig.1). The OR albumin/PTB ELISA and albumin/MAP Culture were significant with a P-value respectively of 0.035 and 0.043. The other parameters were outside the normal range [8] and although not significant, did have a strong association with clinical PTB.

DISCUSSION AND CONCLUSIONS:

Results of this pilot study highlight the correlation between albumin abnormal values (negative acute phase response) and PTB clinical signs. The low number of samples did not allow us to highlight additional links. Our results suggest that further correlations could be shown between the abnormal parameters and MAP infection, in further case-control studies on a higher number of animals in MAP-infected herds. Work funded by the Italian Ministry of Health, research project code RC IZSUM 04/2011.

REFERENCES:

7. OIE Terrestrial Manual 2008
8. Amadori M. et al. (2002) La valutazione del benessere nella specie bovina; Fondazione Iniziative Zooprofilattiche e Zootecniche

Table 1: Odds Ratio innate immunity for PARATB risk (C.I. 95%) correlation with PTB ELISA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OR</th>
<th>C.I. 95%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement</td>
<td>0.9</td>
<td>0.205 - 2.217</td>
<td>0.818</td>
</tr>
<tr>
<td>Lysosome</td>
<td>0.5</td>
<td>0.190 - 1.747</td>
<td>0.148</td>
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<tr>
<td>Proteins</td>
<td>0.642</td>
<td>0.317 - 2.24</td>
<td>0.731</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.711</td>
<td>1.074 - 6.844</td>
<td>0.036</td>
</tr>
<tr>
<td>Alpha-globulin</td>
<td>1.375</td>
<td>0.556 - 3.402</td>
<td>0.491</td>
</tr>
<tr>
<td>Beta-globulin</td>
<td>0.262</td>
<td>0.041 - 1.689</td>
<td>0.158</td>
</tr>
<tr>
<td>Gamma-globulin</td>
<td>1.75</td>
<td>0.614 - 4.988</td>
<td>0.295</td>
</tr>
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</table>

Figure 1: Regression Line and Correlation IndexSpearman. Albumin Normal Range Values: 27-43 g/l. PTB ELISA Ratio Sample/Positive values >70: Positive
MOLECULAR SEROTYPING AND VIRULENCE PROFILING OF YERSINIA PSEUDOTUBERCULOSIS FROM WILD AND DOMESTIC ANIMALS IN ITALY

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Keywords: Yersinia pseudotuberculosis, zoonoses, molecular serotyping

INTRODUCTION:
Yersinia pseudotuberculosis (YP) is a zoonotic bacterium that causes infections in both wild and domestic animals. Human infections are most often acquired through the ingestion of contaminated food or water by faeces of infected animals. YP has been isolated from meat, fresh produce and milk but its presence is frequently unapparent due to detection difficulties. This fact suggests that, at present, YP contribution to disease might be under-estimated. Detection is an especially important concern because YP can readily proliferate at refrigeration temperature, that is often used as common methods to food preservation.

Pathogenicity in YP depends on three main factors: the virulence plasmid pYV, the chromosomal high-pathogenicity island HPI and the YP superantigenic toxins YPMs. 21 O-serotypes have also been identified on the basis of the composition of O-antigen gene clusters, between hemH and gsk.

The aims of this work were to investigate the distribution of virulence factors among 90 YP strains isolated from domestic and wild animals in Italy, and to study the serogroup prevalence and distribution.

MATERIALS AND METHODS:
Ninety strains of YP originated from 9 different Italian regions, the years of isolation ranged from 1993 to 2013. The isolated were from septicaemia in the hare (61) mastite or abortion in sheep (11), feaces of wild boars (3) and from birds (7), deer (1), rabbit (1),cottontail rabbit (2), goat (1), roe (1) and pig (1). Some of them were cultured following a cold selective enrichment in PBS medium for 7/14 days and then sub-cultured onto selective CIN agar plate at 30°C for 24/48 h, others were collected from culture collections from the IIZZSS. Virulence genes inv and pYV (virF, yopB, YopH) were detected by a multiplex PCR (1). The primers used for analysing YPMs (YpmA, YpmB, YpmC) and HPI (irp2, IS100, yptE, asnT,-int, psn, ybtP-ybtQ, int) were described previously (2). Nine sets of primers targeting different regions of the O-antigen gene cluster of YP were used in a multiplex PCR(3).

RESULTS:
The isolates were confirmed as YP by PCR direct to the inv gene. pYV was detected in 64% of the samples and was always present in strains isolated from birds and wild boars. HPI was always present, in its complete or truncated form, in isolates from sheep, wild boars and birds. All strains tested negative for YPMs, except for two atypical wild boars strains which harboured ypmc. The majority of the isolates belonged to O:1a and O:1b serotypes, followed by O:2a. Two isolates from hares showed a 0:12/13 and 0:5a profile. Two isolates from wild boars showed an atypical O:3 profile with wbyk+ gene,confirmed by sequence (1).

REFERENCES:

DISCUSSION AND CONCLUSIONS:
Biomolecular methods have been applied to the serotypes analysis and to the virulence profiling of 90 YP strains, isolated from wild and domestic animals in Italy. Current work is underway to assess the feasibility of applying direct molecular methods to faeces and tissue sample, reducing the time for bacterial identification and allowing for greater efficiency, sensitivity and specificity. The molecular approach can be employed to gain a better understanding of the patterns of infection in animals, to analyse the transmission dynamics and risk assessment in public health.

REFERENCES:

Fig 1: Origin and numbers of YP strains from different Italian regions

Fig 2: Molecular characterization of YP strains.
APPLICATION OF AN ELISA FOR THE CONTROL OF CASEOUS LYMPHADENITIS IN SMALL RUMINANTS IN AUSTRIA

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Keywords: Caseous lymphadenitis, ELISA, small ruminants

INTRODUCTION: Caseous Lymphadents (CLA) caused by Corynebacterium pseudotuberculosis (C. pseudotuberculosis) has become a common disease in small ruminants throughout the world (STING et al., 2012). As in Austria and most of the European countries published prevalence data do not exist, CLA prevalence cannot be estimated precisely. C. pseudotuberculosis infections are associated with the formation of pyogranulomas. The external form is characterized by abscession of superficial lymphnodes, whereas animals affected by the visceral form show abscesses in internal lymph nodes and other organs. Both forms can coexist within the same animal. Because of the chronic and often subclinical character of this infectious disease, control and management measures are difficult to carry out (BAIRD and FONTAINE, 2007). Since there exists no CLA control program throughout Austria, a voluntary control program for Upper Austrian sheep and goat farms was established. The aim of this program is to identify infected animals and herds followed by eradication of these animals.

MATERIALS AND METHODS: In 2010 and 2011 a pilot study concerning serological diagnostic tests for CLA was carried out by the Institute for Veterinary Disease Control Linz (AGES IVET Linz), the Upper Austrian Veterinary Health Service and the Upper Austrian Chamber of Agriculture. A commercially available ELISA und a PLD specific Western Blot were implemented and validated at AGES Linz. Blood samples of sheep and goat from all over Austria were examined for CLA at the Institute. In 2012 a voluntary control program for Upper Austrian sheep and goat farms was established. The requirements are similar to the MAEDI/VISNA CAE control program. In both programs blood samples have to be tested by ELISA (Enzyme Linked Immunosorbent Assay) within 12 to 24 months in accordance with a sampling scheme at AGES Linz. The samples are tested by ID Screen CLA Indirect ELISA (ID Vet, Montpellier, France) according to the instructions provided by the manufacturer. The test is based on the humoral response to PLD (Phospholipase D) exotoxin. PLD has been identified as a potent exotoxin in C. pseudotuberculosis and is a key virulence factor in the development of CLA.

RESULTS: There is a steady rise in the number of tested animals from 2010 to 2013. In 2010 totally 921 blood samples from sheep and 1068 samples from goats were investigated, whereas in 2013 the number of investigations already was 1971 in sheep and 2192 in goats. In 2010 2.8% of sheep and 37.3% of the goats were tested ELISA positive. In 2013 4.1% of sheep and 14.9% of goats were identified as positive.

DISCUSSION AND CONCLUSIONS: CLA in sheep and goats gained in importance in the last years. Increasing herd size, increasing national and international trade of livestock and the underestimation in the last years (STING et al., 2012) are probable reasons for that. The Upper Austrian control program intends to protect uninfected animals and give participants a certain competitive advantage. Table 1 shows that in Austria goats are more often affected than sheep. The number of positive goats declined from 2010 to 2013. This can be explained by the fact that some positive tested herds left the program. The ID Screen indirect is a suitable tool for the detection of C. pseudotuberculosis infected animals.

REFERENCES:
INTRODUCTION:
Mexico produces 42.8% of goat milk in America with 155 million liters reported in 2005 (SAGARPA). There are several risk factors that influence the type and frequency of isolation on mastitis-causing organisms, such as milking hygiene, location, management, lactation stage and weather conditions (Min 2007). Despite the economic loss that mastitis causes in dairy goats in Mexico, there is little information on the epidemiological status in our country. This study aims to establish the bacterial groups present in milk goats with clinical and subclinical mastitis, and whether there are genus differences in milk bacterial isolates according to intensive or semi-intensive production systems.

MATERIALS AND METHODS:
Sampling was carried out covering a whole period of lactation; milk samples were collected from two farms located in Queretaro, Mexico. The first farm with an intensive system but mainly with a dual purpose-oriented to milk production with specialized breeds French Alpine, Toggenburg and Saanen. The second farm with the same breeds in intensive and mixed production systems. Samples were obtained from the two glands of each goat. From d 15 post-partum samples were taken every 45 d along with California Mastitis Test (CMT), to 240 d of lactation in females who achieved such milk persistence. 30 µl of milk were placed in a blood agar plate and McConkey agar; both plates were incubated at 37 C for 24 h under aerobic conditions, and if no growth observed were again incubated for 48 h to discard bacterial growth. Each isolate was identified by genus and in some cases even to the species level. For identification of the coagulase-negative Staphylococcus (SCN) API Staph system (V4.1) from Biomerieux laboratory was used.

RESULTS:
The results were analyzed using the JMP version 8.0 (SAS Institute Inc.) for Windows, using Chi square where appropriate Fishers exact test and logistic regression analysis. The main bacterial genera most frequently found were coagulase negative Staphylococcus (SCN) 62/97 (63.9%), Streptococcus uberis 10/97 (10.3%) and Staphylococcus aureus 5/97 (5.1%). The results observed in the logistic regression model shows that in the isolates, significant effects were found among the system and lactation period between 60 and 105 (P ≤0.01), 105 and 150 (P≤0.01) and between 150 and 195 de postpartum (P≤0.01).

DISCUSSION AND CONCLUSIONS:
In order to meet the bacterial genus associated with goat mastitis, microbiological analysis was performed on all samples obtained from the two farms in both production systems, where the most frequently bacterial genus isolated were coagulase-negative Staphylococcus, Streptococcus uberis and followed by S. aureus. According to studies conducted in different European countries like Spain, France, Italy, England and Scotland the SCN are the main causative agents of clinical and subclinical mastitis, followed by S. aureus, being S. uberis secondary pathogen; however, S. uberis is a pathogen that can be isolated from skin of the mammary gland and other anatomical sites, as well as floor and bed. The number of bacterial isolates obtained was higher in the intensive system of both farms only at 15 and 60 d post-partum, i.e at the start of lactation, consistent with other work (Bergonier, 2003) where the highest incidence of subclinical mastitis was found at the beginning of milking. According to statistical analysis, there were no differences between the two major bacterial genera isolated in intensive and semi-intensive production systems; only S. aureus was associated with the intensive production system.

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PCR DETECTION AND DIFFERENTIATION OF MYCOPLASMA GALLISEPTICUM FIELD SAMPLES FROM 6/85

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Keywords: Mycoplasma gallisepticum, Typing, PCR

INTRODUCTION:
Mycoplasma gallisepticum (Mg) is a pathogenic agent, which causes important financial losses each year. Mg causes a Chronic Respiratory Disease (CRD) in chickens and sinusitis in turkeys. At present, there is a great interest in typing vaccines and wild type strains of M. gallisepticum (Mg). Types of vaccines to be differentiated vary depending on the country and the vaccination programs. The goal of these study was the identification and differentiation of the 6/85 vaccine strain. Up to now, the study of an unique gene has not been enough to carry out the characterization of Mycoplasma strains. Therefore it cannot be considered a final characterization method of the 6/85 vaccine strain. Currently other significant genes such as RNA16S, Lp, gapA and pvpA and mgc2 [1-5] are being studied. In order to differentiate field isolates from the 6/85 vaccine, INGENASA has worked on the development of two PCRs based on the Lipoprotein [6] and the cytoadhesing protein genes [7].

MATERIALS AND METHODS:
We designed a pair of primers for each PCR as follows: - the Lp primers were selected to have high sensitivity and specificity to detect the M. gallisepticum in field samples without previous growing. Additionally, they allowed differentiating same patterns by probes and RFLP digestion and sequencing.- the mgc2 primers were selected close to a deletion in the 6/85 mgc2 DNA with a size suitable to be differentiated by agarose gel migration. The Mg characterization method used at present is based on the analysis by automatic sequencing of Lp and by fragment length analysis of mgc2 genes, as a complementary way for detection and differentiation of the 6/85 vaccine.

RESULTS:
Using these methods we have found: a) wild-type strains showing the same genetic sequences as the 6/85 vaccine strain in the Lp gene, 99% homology, and different sequence on the mgc2 gene and b) other strains identical to the 6/85 vaccine strain in all the tests carried out, including the analysis of the gene mgc2.

DISCUSSION AND CONCLUSIONS:
Results obtained indicated that, concerning Spanish situation, the length analysis of the 6/85 gene fragment described is the best option to differentiate vaccinated animals from field isolates. Therefore, this method could be useful to follow up the situation on farms. Additionally these two PCR assays could be used to sequence and to find the closest sequences by blast analysis against the NCBI database.

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MOLECULAR IDENTIFICATION OF SPECIES WITHIN THE MYCOBACTERIUM TUBERCULOSIS COMPLEX BY HIGH RESOLUTION MELTING ANALYSIS

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Keywords: MTBC, HRM, Validation

INTRODUCTION:

Members of Mycobacterium tuberculosis complex (MTBC) are the causative agents of tuberculosis in humans and animals. MTBC includes M. tuberculosis, M. bovis, M. caprae, M. bovis Bacillus Calmette and Guérin (BCG), M. africanum, M. microtuberculi, M. canettii and M. pinnipedi. The MTBC is characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences (1). Several molecular techniques were designed to rapidly differentiate MTBC species. Among them, region of difference (RD) analysis (2), gyrB-restriction fragment length polymorphism (gyrB-RFLP) analysis (3) and Spoligotyping (4) are the most widely applied. The aim of the present study was to develop and validate a Real-Time Polymerase Chain Reaction (RT-PCR) assay coupled with High Resolution Melting curve (HRM) analysis for simultaneous detection and identification of MTBC species in a single-tube reaction. The HRM method has been successfully used to discriminate single nucleotide polymorphisms (SNPs). We evaluated the discriminatory power of this analysis to differentiate common MTBC isolates, based on gyrB gene sequence polymorphisms. Two amplifications were developed and validated in this study. The first reaction allows distinction of M. tuberculosis, M. bovis and M. microtuberculi; while the second differentiate M. bovis from M. caprae. This method cannot differentiate M. tuberculosis from M. africanum, M. canettii, M. pinnipedi and M. bovis BCG. Analytical sensitivity, specificity, repeatability and reproducibility of the two reactions were determined.

MATERIALS AND METHODS:

The Real Time-PCR was optimized to amplify a fragment of 135 and 150 bp of the gyrB gene, for the first and second reaction respectively, in the presence of the EvaGreen fluorescent dye. A total of 553 MTBC isolates, previously identified using traditional methods as gyrB-RFLP and Spoligotyping, were analyzed by HRM.

RESULTS:

The assay was evaluated by testing reference strains and positive field isolates for common MTBC. The MTBC species are clearly distinguished in both reactions. The limit of detection of the two reactions, determined in 5 replicates of each dilutions (2x10^3-1 genomic copies/µl) of the two MTBC titrated isolates (M. caprae and M. bovis BCG) was 1 genomic copy/µl. No positive signals were detected by analyzing 54 isolates of atypical mycobacteria (identified by sequencing) of 30 different species. Inter-assay reproducibility, determined by analyzing the DNA from each reference strains in duplicate in 10 different days was total. Also intra-run analysis successfully detected the presence of MTBC in three positive samples for each species analyzed in duplicate. The range, mean, standard deviation (SD) and coefficient of variation (%CV) of the melting-temperature (Tm) were calculated and reported in Table 1. The performance of the HRM assay was compared to gyrB-RFLP and spoligotyping methods. All the samples were classed correctly by the HRM analysis.

DISCUSSION AND CONCLUSIONS:

The present study developed and validated an HRM analysis for rapid and specific detection and simultaneous identification of MTBC members. The results showed that HRM analysis is comparable to gyrB-RFLP and spoligotyping. This assay is a reliable, sensitive, reproducible and cost-effective method.

REFERENCES:

INTRODUCTION:
Evira has for years participated in the HELCOM monitoring program to assess population structure, size and growth, reproduction and breeding, contaminant burden and health status of seals in the Baltic Sea area. Every year several animals have been examined for nutritional condition and for parasitic and bacteriological infections. In the year 2013 we detected for the first time brucellosis in one of these samples.

MATERIALS AND METHODS:
An adult male grey seal (Halichoerus grypus) was found dead in a bow net in Bothnian Bay. It was autopsied and bacteriological examination was performed on the sample from a liver abscess. Content of the abscess was cultivated on blood agar plate and two FAA (fastidious anaerobe agar) plates. The plates were incubated at 37 °C in aerobic, anaerobic and microaerophilic atmosphere, respectively, and observed for bacterial growth after 1, 2 and 4 d.

RESULTS:
The seal was in normal body condition. The death was caused by drowning. Signs of inflammation were observed in liver and bile ducts. The bile ducts were strongly dilated, the duct wall was thick and fibrotic and the ducts contained masses of flukes (Pseudamphistomum truncatum). Roundworms were observed in the intestine. Several abscesses and small granulomatous lesions were detected in the liver. Small translucent colonies were observed on blood agar and FAA agar after 4 d in the liver abscess sample. The growth was better in microaerophilic atmosphere. Gram-negative small coccobacilli were observed in gram-stain. Oxidase and catalase reactions and urea test were positive. Stamp staining showed small red-staining coccobacilli.
Identification was continued in biosafety level 3 laboratory. The bacterium grew typically on selective Farrell medium and gave a result as Brucella sp. in the PCR method for Brucella on genus level. On the basis of these results the bacterium was confirmed to belong to genus Brucella, most probably to species Brucella pinnipedialis.

DISCUSSION AND CONCLUSIONS:
The potential hosts of brucellosis in aquatic environments include several marine mammal species, including both cetaceans (dolphins, porpoises and whales) and pinnipeds (sea lions, walrus and true seals). There are two marine Brucella species, B. pinnipedialis affecting pinnipeds and B. ceti affecting cetaceans. Brucella pinnipedialis has been detected in six seal species in the North Atlantic Ocean, North Sea and Pacific Ocean. The isolations have been made in Northern Ireland, Scotland, Germany, New England, Canada and California, USA. However, we have not found any previous reports on the occurrence of marine Brucella species in the Baltic Sea area. Most isolates of B. pinnipedialis have been recovered from clinically healthy animals, as in the present study. The growth of Brucella was slow and clearly more abundant when the plates were incubated in microaerophilic atmosphere. These features should be kept in mind when samples from marine mammals are examined for pathogenic bacteria. Brucellosis is a zoonosis and the identification should be performed in biosafety level 3 laboratory.

REFERENCES:
INTRODUCTION:
In the Netherlands, screening for tick-borne pathogens is performed using real time PCR on DNA extracted from ticks, followed by sequencing of positive samples, thereby disallowing identification in case of double-infections. Until recently, testing was performed with several reverse line blots (RLBs), each containing ~10 different probes. Advantages of RLBs are their capabilities to differentiate between bacterial species and to detect double infections, disadvantages are that RLBs are fastidious, elaborate, and time-consuming.

The aim of this work was to ‘test drive’ bead-based suspension arrays for the rapid detection and identification of multiple Borrelia species in ticks. Bead-based suspension arrays are suitable for multiplex detection. For this technology, fluorescent nanoparticles (beads) are used to build multiplex assays, simply by using a mix of different bead sets that each carry specific probes, which can be analyzed on the flow cytometry-based xMAP Luminex platform. A new bead-based suspension array for detection and identification of Borrelia species was developed and tested by investigating the prevalence of Borrelia species in ~1000 ticks collected from dogs and vegetation.

MATERIALS AND METHODS:
Six published probes (sensu lato [n=3], sensu stricto, afzelii and garinii) (1) were selected. As preliminary experiments with the original 31 nt long valaisiana probe resulted in nonspecific reactions, two new valaisiana probes (16-20 nt) were designed. Using standard chemistry, aminated probes were covalently linked to carboxylated Luminex beads (2), resulting in bead sets with general and specific probes for the 23S-5S intergenic spacer region (IGS) of Borrelia. Coupling of probes was verified by hybridizing complementary biotinylated oligonucleotides in presence of a mixture of 1000 beads per bead set, followed by labelling with streptavidin-phycoerythrin. A minimum of 100 beads per bead set was investigated using a dedicated flow cytometer (Luminex 200). Adult ticks were collected from dogs (n=388) and vegetation (n=619) in the corresponding area. DNA was isolated using the Qiagen DNeasy blood and tissue kit for engorging ticks from dogs, or boiling in ammonium hydroxide for flat ticks from vegetation. Each DNA extract was subjected to a universal PCR for the Borrelia 23S-5S IGS (1). Resulting biotinylated amplicons were used to detect and identify Borrelia species through hybridization to beads, essentially as described above.

RESULTS:
Out of 1007 ticks, 182 (18.2%) were found to carry Borrelia: 9.5% (n=95) carry one Borrelia, 5.3% (n=53) carry two or more Borrelia species, and 3.4% carry unspecified Borrelia as they generate a signal on one or more of the sensu lato probes exclusively. The most prevalent encountered species was B. afzelii (n=101), followed by B. burgdorferi sensu stricto (66), B. garinii (n=36), and B. valaisiana (n=14). First analyses of the data in relation to dogs/vegetation suggest that the prevalence in vegetation is not reflected in ticks extracted from dogs.

DISCUSSION AND CONCLUSIONS:
To demonstrate the viability of bead-based suspension arrays for relatively rapid and easy detection of multiple infections, a multiplex assay was developed for the detection and identification of Borrelia species. About 18% of the ticks were found to carry Borrelia, in agreement with earlier findings (3). Currently, confirmation of these results by next generation sequencing is in progress.

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INTRODUCTION:
Paenibacillus larvae, a Gram-positive endospore-forming bacterium, is the causative agent of American foulbrood, the most severe and widespread disease affecting the brood of Apis mellifera. The search of P. larvae spores in samples taken from the hive (honey, bees or debris) is a useful tool to identify infected colonies. A method for the detection of P. larvae on debris collected on the bottom of the hive has been recently developed in the Czech Republic (Titera and Haklova 2003). In that method is used toluene to extract the spores from the debris and this is a critical point, furthermore it has long run times.

In 2007, it was introduced a variant of this method which make use of Tween 80 (TM) instead of toluene and appears to be significantly more effective in detecting P. larvae spores (Bzdil 2007). Also this technique is laborious and this limits the possibilities of use, especially when many samples must be examined at the same time as often occurs in practice.

We have developed a method based on distilled water extraction of spores (WM). The execution of this method is simpler and analysis time is shorter if compared to the TM.

The aim of this work was to describe this method and to compare the results obtained with WM and TM on the same samples.

MATERIALS AND METHODS:
We examined 30 samples of beehive debris collected in 2013 at the bottom of 30 hives belonging to 12 apiaries. Each sample was analyzed for the detection of P. larvae spores with WM and TM.

WM was carried out as follows: a) put 1 g of debris in a 15 ml Falcon containing 9 ml of sterile distilled water; b) vigorously shake by hand for 30 seconds; c) heat in a water bath at 85°-90 °C for 15 minutes; d) filter immediately after the heat treatment through a sterile gauze; e) plate 500 microliters of the filtered sample onto 5 plates (100µl/plate) of MYPGP agar supplemented with nalidixic acid and pipemidic acid; f) incubate the agar plates at 37 °C in an atmosphere with 10% CO2; g) examine the plates after 3 days for the first reading and after 8 days for the final reading.

TM was performed as described by Bzdil (Bzdil 2007). Results were expressed in CFU/g for both methods.

We used Cohen’s kappa coefficient (k) to compare the results between WM and TM. In this step, results were classified as presence/absence. Then we used the Wilcoxon signed-rank test (V) and Spearman’s rank correlation (ρ) to compare colony counts.

RESULTS:
In 13 out of 30 samples colonies did not grew on plate neither with WM nor with TM. In 16 samples colonies grew with both methods, and in 1 sample they grew only with TM. The agreement between the methods was high (k=0.933). In the 17 samples where colonies grew we compared colony counts. Analysis suggested that there was no difference between the two methods (V=112, p-value=0.098; ρ =0.947).

DISCUSSION AND CONCLUSIONS:
The analysis performed shows no evidence of difference between the two methods, both from quantitative and qualitative viewpoint. Whereas the results obtained with the WM and the TM method not show significant differences, the use of the WM compared to TM presents practical advantages: it is less expensive, it is easier to perform and the sample preparation time is shorter (about 20 minutes with WM, about 4 hours with TM). These characteristics are important, especially when you need to examine large numbers of samples.

REFERENCES:


SEROLOGICAL DIAGNOSIS OF CHLAMYDIA INFECTION IN CATTLE

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Keywords: ELISA, Complement Fixation Test, chlamydial antigen

INTRODUCTION:
Chlamydia infection in cattle is widespread in many countries including Russia [1,2]. Chlamydia are also a potential threat to human health. Investigations to find the best diagnostics of this infection are performed by researchers of many countries [3,4]. The most accessible means of farm monitoring for chlamydia in Russia are serological methods, of which the Complement fixation test (CFT) with group-specific chlamydial antigen remains the main in veterinary medicine [1,2]. Unfortunately this method has several disadvantages associated with low sensitivity, duration of test performance, etc. The aim of our investigations was to test a new antigen for the diagnosis of chlamydial infection in cattle by ELISA, to create on its basis a commercial diagnostic kit, followed by its testing and implementation into veterinary practice.

MATERIALS AND METHODS:
1708 cattle blood sera from 33 farms of Russian Federation infected with chlamydia were investigated. CFT was performed according to the standard technique using “Set of antigens and sera for serological diagnosis of farm animal chlamydiosis” manufactured in Federal Center for Toxicological, Radiation and Biological Safety (FCTRBS), Kazan, Russia. The results were assessed visually by the phenomenon of hemolysis of erythrocytes. Complete absence of erythrocyte hemolysis was evaluated as a positive reaction (4 crosses), and complete hemolysis, respectively, as a negative reaction. Indirect ELISA was performed using the “ELISA kit for diagnosis of cattle chlamydia infection” manufactured in FCTRBS, Kazan, Russia. Interpretation of ELISA results was conducted on spectrophotometer Bio-Rad Model 680 at a wavelength 490.

RESULTS:
In the 33 investigated farms clinical signs of chlamydia, such as abortion, stillbirth and birth of weak calves, pneumonia, enteritis in calves, as well as joint diseases were observed. 156/1708 (9.1%) animals reacted positive to chlamidiosis in the CFT. The titers of specific complement-fixing antibodies ranged 1:5 – 1:40 which correspond to the immune background of infected farms. 341/1708 (19.9%) animals reacted positive in the ELISA, with specific antibody titers ranged 1:200 – 1:3,200.

DISCUSSION AND CONCLUSIONS:
In comparative tests, the ELISA results showed a good correlation with the baseline test – CFT. The value of antibody titers in ELISA depended directly on the intensity of clinical signs of the disease in animals. In animals with acute course of chlamydiosis antibody titers were significantly higher than in animals with a latent stage of infection. In some cases, the results of serological tests confirmed in direct Immunofluorescence assay and by isolation of the pathogen in chicken embryos. These studies revealed that the ELISA has a higher sensitivity compared to the CFT. Twice more positive results were detected in the ELISA than in the CFT, which indicates the high efficiency of suggested test system.

REFERENCES:
BACTERIAL AND HISTOLOGICAL FINDINGS IN AUSTRIAN PIGLETS WITH DIARRHEA


Keywords: Clostridium difficile, diarrhea, pathohistology

INTRODUCTION:
From April 2012 until March 2013 19 Styrian holdings with severe diarrhea in piglets were examined according to a project protocol. This poster shows the bacterial results and histological findings. See also the poster Richter et al. regarding virologic findings. Suckling and weaner pigs were selected by responsible veterinarians for analysis. One to three live piglets per holding (in total 55) and up to 6 faecal samples (in total 85) of other pigs were sent to the institute. Animals were euthanized and immediately processed for sampling. Samples of stomach, duodenum, jejunum, ileum, colon and rectum were obtained and prepared for bacteriological, histological, ultrastructural and PCR analysis.

MATERIALS AND METHODS:
Bacteriological examination of ileum, colon and faecal swabs included aerobic culture for detection of Escherichia coli, anaerobic culture for detection of Clostridium perfringens, and direct anaerobic culture as well as culture after enrichment to find C. difficile. C. perfringens and C. difficile were further typed with PCR toxin typing and ribotyping. Mucosal swabs of caeca were investigated with phase contrast microscopy for detection of Brachyspira spp., and further analyzed by means of PCR.

RESULTS:
C. difficile was present in 5 holdings, 10 (18%) pigs and 10 (12%) swabs. Results of molecular subtyping are: PCR – Ribotype (RT) AI – 12 (4 pig isolates, 2 swab isolates), RT 005 (1 pig, 1 swab), RT 078 (2 pig, 2 swab), RT 598 (4 pig, 4 swab), RT 241 (1 swab). C. perfringens Toxin Type A was present in 18 holdings, 39 (71%) pigs and 61 (72%) swabs; hemolytic E. coli we detected in 12 holdings, 27 (49%) pigs and 29 (34%) swabs, Brachyspira sp. was found in 7 holdings and 18 (33%) pigs, none of them was B. hyodysenteriae or pilisicoli, in two cases B. intermedia/innocens were identified. 5 Pigs (9%) of 3 holdings were infected with parasites of Eimeriidae, and 1 (2%) pig with Trichuris suis. Seven holdings showed bacterial infections with two or more type of bacteria, three holdings were additionally infected with parasites and most holdings had confections with viruses (Corona-, Rota-, Calici- and Circovirus).

Histological analyses helped to clarify the etiological relevance of the detected microbial agents. Tissue lesions typical for bacterial infections were apical necrosis of the intestinal villi, ulcerations, crypt abscesses, neutrophil granulocytes and detritus in the activated lymphoglandular complexes. Bacterial lesions were more often found in neonatal pigs.

DISCUSSION AND CONCLUSIONS:
In 69% of all piglets viruses were found, in 100% bacteria. In 31% only bacteria were detected. This study showed that especially Cl. perfringens Type A is widely spread in Styria. Histological findings showed that bacterial infections were related to pathomorphological reactions of the intestines.

REFERENCES:
INTRODUCTION:
Q fever is an infectious disease caused by Coxiella burnetii. Diagnosis of Q fever based on clinical symptoms is unattainable; thus, different laboratory techniques are used to detect the infection. Routine diagnosis of Q fever in aborted ruminants is generally performed by the detection of bacteria in stained smears or impressions of placentas, combined with serological investigations of adult animals using ELISA, CFT or IFA (2). Serological methods are of limited validity because they often fail to detect C. burnetii shedding animals and shown different sensitivities. The aim of the study was to compare the diagnostic potential of ELISA, CFT, conventional PCR, real time PCR and cell culture.

MATERIALS AND METHODS:
Serological test (ELISA, CFT) were carried out on 2,251 serum samples from ruminants originating from different regions of Poland, collected in the year 2007-2011. Moreover, 668 placentas, 1,277 vaginal swabs and 306 specimens of the internal organs of aborted foetuses were examined by PCR (IS1111 gene was targeting) and cell culture (Vero cells line was used). Pearson’s chi-square test was used to compare the results obtained by ELISA, CFT, PCR, real time PCR and isolation in cell culture. Correlation coefficients were calculated for all the methods used. The following guidelines for interpreting the degree of correlations were used: r=0-0.09: no or negligible; r=0.1-0.29: week relationship; r=0.3-0.49: moderate; r=0.5-0.69: strong; r=0.7-0.99 = very strong; r=1: full.

RESULTS:
The χ2 test confirmed that in most cases the results obtained by means of the different methods were correlated with each other (P<0.05). The highest correlation coefficients (r=0.76-0.87) were observed in the case of real time PCR and conventional PCR. ELISA and CFT were moderately correlated (r=0.43-0.45). When the comparison was made between the results of tests run on samples from swabs and aborted foetuses, the r values between ELISA and CFT were lower than those between ELISA and PCRs. A negligible, or weak to moderate relationship was mostly observed when the method of cell culture isolation was compared with all the other analytical techniques investigated.

DISCUSSION AND CONCLUSIONS:
Laboratory diagnosis of Q fever should be based on the interpretation of results obtained by different kind of methods both detecting the serological response as well as the presence of pathogen. In addition, the choice of proper laboratory methods depends on numerous factors e.g. case definition, type of availability material etc. To sum up the values and results obtained, it can be concluded that the statistical analysis of data from comparison of the five diagnostic methods has shown that serological, molecular and culture methods can be used in practice for diagnosis Q fever. However, their diagnostic potential and the level of correlation between them was variable and it is necessary to use several methods simultaneously, preferably ELISA for serological studies and PCR for pathogen detection (1).

REFERENCES:
DEVELOPMENT OF A MULTI-SCREENING FAST REAL-TIME PCR FOR THE DETECTION OF ABORTIVE INFECTIOUS AGENTS IN RUMINANTS

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Keywords: abortive agents, multi-screening, Fast Real-time PCR

INTRODUCTION:
Many infectious agents cause abortion in ruminants and their importance is related with economic losses for farmers and with a consistent risk for human health(1). Abortive agents induce similar symptoms in ruminants, making diagnosis difficult. Detection of these pathogens is usually made by histological examination (for parasites), bacteriological isolation, cell culture isolation and serological test, everyone with some limits. Respect to Toxoplasma and Neospora, tissue cysts are difficult to distinguish from each other, histologically. Pathogen isolation for abortion diagnosis is complicated, time consuming, hazardous and sometimes inconclusive. Lastly, the use of serological tests have the potential disadvantage of indicating post-exposure rather than persistence of infection(1,2).

Diagnosis based on molecular assay can help to easily detect the specific organism involved. The aim of the study was to develop a multi-screening by singleplex Fast Real-time PCR assays with the same amplification profile that provides a powerful analytical tool for rapid, safer and more specific detection of Coxiella burnetii, Leptospira spp., Toxoplasma gondii, Neospora caninum and Chlamydia (3) (previously developed).

MATERIALS AND METHODS:
DNA was extracted by commercial kit from cell cultures (for Leptospira spp.), from organ homogenates (fetuses, fetal annexes) and vaginal swabs for the other pathogens. The specificity of primers/probe set was checked experimentally by testing each agent against one another. The targets were: IS1111 for Coxiella burnetii (4), Nc5 for Neospora caninum (5), LipL32 for Leptospira spp. (6), B1 gene for Toxoplasma gondii (primers/probe newly designed) and 23SrRNA for Chlamydia (3).

Real-time PCR was run in a total volume of 20 µl with 10 µl 2x TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG, 2 µl DNA, while primers and probes were used at adapted concentrations for each target. The PCR was performed on 7500HT Fast Real-Time PCR System using the following cycling conditions: 95°C for 20 s, 40 cycles with 95°C for 5 s and 60°C for 30 s.

For LOD (Limit of Detection) evaluation, expressed as genomic copies (g.c.), each target was amplified with a customized PCR protocol and each fragment purified by a commercial kit. After biofotometer quantification, each amplicon was 2-fold serially diluted in salmon sperm DNA-supplemented TE.

RESULTS:
No cross-reaction was shown between each target. The LOD was 5 g.c. for Coxiella Burnetii, Toxoplasma Gondii and Chlamydia spp. while for Leptospira spp. and Neospora caninum was 30 g.c..

DISCUSSION AND CONCLUSIONS:
In summary, the multi-screening approach with Fast mode Real-time PCR to detect in the same assay 5 abortive infectious agents in ruminants, seems efficient, time-effective and it improves analytical results interpretation.

Further development of the multi-screening panel will be done by introducing Brucella spp. in the assay and validation of the whole method will be performed.

REFERENCES:
INTRODUCTION:
C. perfringens is a Gram-positive, anaerobic and sporulating bacterium. It is widespread in the environment and although it is common in the intestinal flora of animals and humans, it can cause various diseases in animals and humans. C. perfringens strains are classified into five categories: A, B, C, D and E, according to the production of one or several of the four major toxins (α, β, ε, ι). Besides producing one or more of these toxins, some C. perfringens strains produce additional “minor” toxins, one of which is the β2 toxin. The cpb2 gene has a plasmid localization and present two allelic forms; the first one, defined as “consensus”, has a porcine origin and the other, named “atypical”, encodes for a non-porcine β2 toxin. Its role in the clostridial disease is not clear, but its presence seems related to the severity of the gastrointestinal form of disease. The aim of this study was to standardize the purification method of a deleted recombinant atypical β2 toxin obtained by baculovirus expression and the evaluation of the toxicity of the purified product in mice.

MATERIALS AND METHODS:
Expression and extraction of recombinant β2 protein: Sf21 cells were infected with recombinant baculovirus, expressing the N-terminal deleted β2 form, and incubated at 27 °C. Three days post-infection the cells were harvested and treated with lysis buffer (Phosphate Buffer Saline plus UREA 8M, Triton 1% and DTT 5 mM). After sonication, the cellular lysates were centrifuged at 16,000X g for 10 min. The supernatants were recovered and stored at -20°C until use.
Ni++ affinity chromatography: the supernatant containing deleted β2 toxin was purified in accordance to the manufacture protocol (SIGMA). Analysis of recombinant protein: purified and not purified deleted recombinant β2 were resolved by SDS-PAGE electrophoresis and analysed by brilliant Blue Comassie staining gel and Western blot using the anti-His C-Term HRP antibody.
Dialysis and quantification: the purified protein was dialyzed at 4°C against PBS buffer using a slyde-A-lyzer G2 dialysis cassettes 3,5K MCWO (Thermo Scientific) and quantified by Bradford method (BIO-RAD).
Test of toxicity: different concentrations of protein were tested in mice inoculating the toxin intraperitoneally and the animals were kept under clinical observation for one week.
RESULTS:
During the standardization of purification procedure two different methods of elution were tested. In the first step the bound recombinant protein was eluted using an elution buffer containing 300 mM of imidazole. In the second procedure, the deleted protein was eluted using the buffer at different pH. As evidenced by staining of the gel with Comassie, the highest recovery for the purified protein, was obtained using an elution buffer at pH 4.5 (Fig 1).

DISCUSSION AND CONCLUSIONS:
In this study a procedure for the purification of intracellular deleted recombinant β2 toxin was developed. The results have shown the possibility of obtaining large quantities of product with a high degree of purity. In addition, the deleted atypical form was found to be non-toxic for the mice. This feature opens to a potential application of the purified recombinant protein as immuno-reagents for the development of diagnostic ELISA method and/or the use as immunogen in anti clostridial vaccines.

REFERENCES:
LEPTOSPIROSIS IN EURASIAN BEAVER (CASTOR FIBER) IN SWITZERLAND

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Keywords: leptospirosis, Eurasian beaver, Real-time PCR

INTRODUCTION:
Leptospirosis is an emerging zoonotic disease with worldwide distribution and has been detected in more than 180 animal species (Guerra 2009). It is caused by various pathogenic serovars of the spirochetes Leptospira spp. Due to its wide variety of unspecific clinical signs - ranging from mild flu-like- to multiple organ failure-associated signs - it is assumed to be substantially underdiagnosed. The spreading of leptospirosis is essentially due to its ability to persist in the proximal renal tubules of carrier animals. Maintenance hosts usually remain clinically asymptomatic, but are epidemiologically relevant by becoming chronic shedders of the agent via urine into the environment (Haake et al. 2000; Rojas et al. 2010). Indirect transmission is common and may occur through exposure to contaminated water sources, soil and food (Greene et al. 2012). Leptospirosis was diagnosed in two free ranging Eurasian beavers (Castor fiber) showing severe lung hemorrhages consistent with the disease, by Real-time PCR.

MATERIALS AND METHODS:
The urine sample was centrifuged at 4°C and 10’000 rpm for 10 min and the pellet resuspended in lysate buffer and inactivated 1 h at 56°C followed by 1 min at 97°C. Tissue samples were homogenized on an IKA ULTRA-TURRAX® tube drive (IKA®-Werke GmbH, Staufen, Germany) and DNA was extracted using the QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany). Sample duplicates were analysed by Real-time PCR using primers and probe described by Villumsen and colleagues (2012) that target the antigenic lipoprotein LipL32. The reactions were carried out on a 7500 Real Time PCR System (Applied Biosystems), amplification consisted of 45 cycles and for interpretation a threshold of 0.06 and the default baseline setting (3 to 15) were chosen.

RESULTS:
All the organs tested yielded positive amplification curves with CT values ranging from 29.5 to 34.3. The mean CT value for the urine sample was 38.1.

DISCUSSION AND CONCLUSIONS:
Rats were first recognized as an important source of leptospirosis (Ido et al. 1917); there are several reports of rodents as maintenance hosts of pathogenic Leptospira spp. (Collares-Pereira et al. 2000; Michel et al. 2001; Richardson et al. 2003; Goodman et al. 2012). The Real-time PCR proved useful for screening urine and organ samples of beavers for leptospirosis. Eurasian beavers in Switzerland are likely to constitute a reservoir for this disease; however, its prevalence is unknown. Further studies are underway to determine the epidemiological role of Eurasian beaver in the maintenance of leptospirosis in Switzerland.

REFERENCES:
INTRODUCTION:
Psittacosis is a zoonosis caused by the intracellular bacterium Chlamydia psittaci and birds are the main source of infection for humans. In the Netherlands between 25 to 85 human cases are notified annually, although this is probably an underestimation (1). In pet birds recent prevalence data on C. psittaci are lacking and limited information is available on wild birds. The latest study in Dutch pet birds was published in 1989. About 27 percent of the investigated pet shops and 40 percent of budgerigar breeding facilities tested positive for the bacterium (2). In a study in wild birds in 2006 faecal droppings from feral pigeons were investigated and a prevalence of 7.9 percent was found (3). To improve assessment of the potential sources birds pose for human infection in the Netherlands more data on C. psittaci in pet and wild birds are needed.

MATERIALS AND METHODS:
From 2009 to 2013 CVI received 6474 cloacal and faecal samples, mainly from pet birds clinically suspected of psittacosis or for source detection of human cases. Additionally, between 2010 and 2012 cloacal swabs were collected from 660 wild birds submitted to identify the cause of death. All samples were tested by realtime PCR, targeting the ompA gene of the Chlamydia genome.

RESULTS:
Overall 4 percent of the 6474 pet bird samples tested positive. Data were also analysed per bird order, although in only 20 percent of the submissions information was provided on the species of bird. The analysis per bird order indicated a higher prevalence in Columbiformes. In wild birds 3 percent of the 660 samples tested positive.

DISCUSSION AND CONCLUSIONS:
The 4 percent prevalence for C. psittaci in pet birds is probably an overestimation for the population as most samples were submitted for confirmation of clinical psittacosis. For wild birds the prevalence of 3 percent might also be biased as birds were submitted for post-mortem examination. However the data show C. psittaci is present in pet and wild birds and might indicate higher prevalences in some bird orders, for example Columbiformes. Additional research is needed to elucidate the true prevalence in different pet and wild bird species, leading to better insight in potential sources for human psittacosis. Currently, molecular typing of positive samples is in progress.

REFERENCES:
INTRODUCTION:
In the last 10 years, in many European countries, reports of outbreaks of botulism in cattle are significantly increased. In most of these episodes several animals were affected by the disease with great economic losses for the farms involved (1). Botulinum neurotoxins (BoNTs), especially antigenic types C and D and their mosaic variant, are responsible for botulism in cattle (2). Typical clinical manifestations of the disease are muscular weakness and flaccid paralysis. Even though the symptoms are highly evocative of the disease, for diagnosis of botulism is necessary the detection and typing of BoNTs with mouse bioassay (3). However, the use of live animals raises evident welfare considerations. Some studies indicate that spores of C. botulinum are often present in gastrointestinal tract of cattle with clinically suspect or confirmed cases of botulism (4, 5). Nevertheless, the lack of data concerning the prevalence of the spores of C. botulinum in the digestive tract of healthy animals, does not allow assessing the diagnostic value of these findings. Aim of the present study was to determine the prevalence of BoNT-producing clostridia in intestinal contents of dairy and beef cattle at slaughter.

MATERIALS AND METHODS:
From June to November 2012, samples of cecal contents from 200 dairy cows and 200 beef cattle, randomly selected, were collected at an abattoir located in the northern part of Italy. The dairy cattle were from 174 farms while the beef cattle were from 33 fattening units. Dairy and fattening farms were located respectively in 19 and 13 different provinces. According to a protocol kindly provided by the National Reference Centre for Botulism (I.S.S., Rome, Italy), two multiplex Real-Time PCR were performed to detect the presence of types A, B, C, D, E and F BoNT genes in heat shock and pre-enrichment culture of samples.

RESULTS:
Only 4 of the 400 samples of intestinal content of slaughtered cattle analyzed gave Real-Time PCR positive results, indicating a prevalence of 1% (i.e. 95% confidence interval: 0.02 - 1.98%). Only type B BoNT-producing clostridia being found, no other toxin types were detected. Despite the complex matrix tested, no sample showed inhibition of amplification. The 4 positive intestinal contents were sampled from dairy cows coming from 4 herds located in 4 different provinces. C. botulinum was isolated from 2 of the 4 Real-Time PCR positive samples. Both cultures were confirmed to contain the type B toxin gene by Real-Time PCR.

DISCUSSION AND CONCLUSIONS:
To the best of our knowledge, this is the first time that a population of healthy cattle at slaughter has been investigated in Italy for the presence of BoNT-producing clostridia in the digestive tract. The results of this study show that the presence of neurotoxin-producing clostridia in intestinal content in dairy cows and beef cattle population is very low. Furthermore, no samples, among those analyzed, were positive for C. botulinum C and D, the toxin types most frequently recovered in gastrointestinal tract of animals with botulism. In accordance with these results, detection of C. botulinum type C or D in the gastrointestinal content of cattle should be considered an evidence supporting the clinical diagnosis of botulism.

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REFERENCES:
INTRODUCTION:
Among the drug susceptibility tests performed on solid media, the proportion method is the most widely used and is considered the reference system for M. tuberculosis (2). However, when adopted to slow growing strains, such as M. bovis, it couldn’t produce results on time. In addition, this test cannot be applied to pyrazinamidase activity test (PZAase test). The presence or absence of PZAase activity is used as a test to differentiate between M. bovis and M. bovis subsp. caprae (1).

The aim of this study was to evaluate the liquid VersaTREK (VT) pyrazinamide (PZA) susceptibility test as an alternative method able to rule out the major errors reported with others broth-based systems (3) and to check if the results obtained with the VT method support the use of a reduced incubation time (from 35 to 13 days).

MATERIALS AND METHODS:
The susceptibility of sixteen M. bovis and five M. bovis subsp. caprae strains versus first – line anti-tuberculosis drugs (Isoniazide -INH, Rifampicin - RIF, Streptomycin - STR and Ethambutol - EMB) was measured using the VT method. PZAase test was conducted with the aim to differentiate M. bovis to M. bovis subsp. caprae.

RESULTS:
Approximately one third (5/16) of M. bovis strains tested were sensitive to all drugs. Among the resistant strains of M. bovis, one showed resistance to STR at the lower concentration (2.0 µg/ml) and two to INH at the lower concentration (0.1µg/ml). The strains of M. bovis subsp. caprae, instead, were all susceptible to all of the antibiotics at all of the concentrations tested. All M. bovis strains (16/16) were found to be PZA-resistant, while all M. bovis subsp. caprae (5/5) were PZA-susceptible.

DISCUSSION AND CONCLUSIONS:
The VT system proved to be simple to perform and was shown to significantly reduce the incubation time required for the execution of the test. It also does not require the presence of bacterial growth on solid media, as it directly detects the strain previously inoculated into the liquid VT medium.

Results observed on resistant strains need to be confirmed, once standardized the inoculum, with those obtained with the proportion method, still representing the gold standard; moreover you must remember that in the majority of cases, growth of these strains on solid media, could arise by the selective growth of small numbers of resistant mutant bacilli present in the bacterial population before it comes into contact with the drug.

To get more accurate cases, we still need to increase the number of strains to be tested.

With regards to the PZA test, this has proved 100% reliable.

In conclusion, we found evidence of drug resistance circulation in M. bovis strains of bovine origin. These results are most likely to be due to a selective pressure caused by the routine use of antibiotics in veterinary medicine of repetitive antibacterial treatment both legal (streptomycin, cattle respiratory disease complex) and illegal (isoniazid) on not in vivo detected M. bovis infected animals.

REFERENCES:
INTRODUCTION:

Cattle infection with Mycobacterium bovis causes a strong cell-mediated immunoresponse that decreases as the disease proceeds and the bacterial count increases; humoral immunity shows an increase in antibodies in more advanced stages of the disease.

Using indirect ELISA in addition to the commonly used diagnostic tests based on cell-mediated response (1) can increase the overall sensitivity and reduce false positives rate due to animals in advanced stages of the disease (2).

The IZSPLV in Torino, has conducted a study on serum samples from bovines at different stages of the disease, using an ELISA test (IDEXX M. bovis Ab Test) for detection of Mycobacterium bovis antibodies.

MATERIALS AND METHODS:

For evaluation of sensitivity of the ELISA test serum, samples from 90 slaughtered bovines positive to M. bovis bacteriological test were used. For all animals, gamma-interferon and skin test results were known.

Based on pathological status of the animals, samples have been classified in 4 different classes:

1. Advanced Lesions (AL)
2. Complete Primary Complex (CPC);
3. Incomplete Primary Complex (IPC);
4. No Visible Lesion (NVL) (with positive result to bacterial culture test).

To evaluate the “booster effect”, samples taken between 3 and 14 days after skin test from 13 different animals were tested with ELISA.

For specificity evaluation of the ELISA test, 269 bovine serum samples from 4 different TB-free herds have been tested.

RESULTS:

Sensitivity
1. Advanced Lesions (AL): 8 out of 18 samples were ELISA positive and y-ifn test positive but 2 of them were skin test negative.
2. Complete Primary Complex (CPC): 6 out of 19 were ELISA positive and also y-ifn test positive, and 2 were negative to skin test.
3. Incomplete Primary Complex (IPC): 2 out of 18 samples were ELISA positive, 1 was y-ifn test and skin test positive, the other was non-discriminating with y-ifn test and skin test positive.
4. No Visible Lesion (NVL): one out of 20 animals was ELISA positive. This animal was also positive to y-ifn and skin test. In this group of animals, all have been detected as positive by at least one of the in vivo tests.

In order to evaluate the possible “booster effect” of the skin test on in vivo tests, 13 animals have been tested. Samples were taken 3 to 14 days after skin test. In 3 different cases samples were positive in ELISA: they were all from animals with NVL and sampling at 14 days after skin test. 2 of these samples were negative both to y-ifn and skin test while the third sample was unqualified with y-ifn and suspect with skin test.

Specificity
Only 2 of the 269 bovine serum samples from TB-free herds tested were ELISA test positive, showing a specificity of 99.26%.

DISCUSSION AND CONCLUSIONS:

Results obtained indicate that the sensitivity of the ELISA test is higher in animals with advanced lesions, while it is lower in animals in acute early phase of the disease. Although the number of samples is limited, it seems like the booster effect allows for detecting positive animals by ELISA test.

Specificity of the ELISA test was shown to be very high.

Based on the data obtained in this study, we can conclude that in case of confirmed outbreak, the use of the ELISA test could help detecting positive animals that would not be otherwise identified with the current methods used.

REFERENCES:

PRÜLLER S. [1], FRÖMKE C. [2], KLEIN G. [1], KREIENBROCK L. [2], KEHRENBerg C. [1]

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Keywords: Bordetella bronchiseptica, standardised protocol, growth curves

INTRODUCTION:
Bordetella bronchiseptica causes infections of the respiratory tract in swine and companion animals and also in immunocompromised humans. Furthermore, the pathogen is involved in atrophic rhinitis in swine.1-2 For a diagnostic-based antimicrobial therapy and, thus, to reduce the risk of antibiotic resistance development, it is important to determine the susceptibility status of this pathogen. Currently, there is a standardised protocol for non-fastidious, rapidly replicating bacteria available to perform broth microdilution susceptibility testing according to the CLSI-document VET01-A4.3 Due to the relatively slow growth of B. bronchiseptica compared to rapidly replicating bacteria like E. coli, it is necessary to prove, if the same testing conditions could apply for B. bronchiseptica in order to harmonise the broth microdilution susceptibility testing of B. bronchiseptica.7

MATERIALS AND METHODS:
Bacterial isolates: One reference and one type strain plus two field isolates of B. bronchiseptica were chosen to analyse growth curves in four different media. In addition, ten isolates with different PFGE patterns were used for the evaluation of broth microdilution susceptibility testing. The field isolates originate from different geographical regions and farms in Germany and were collected between 2010 and 2012 from diseased and healthy swine. Growth curves: Growth curves of four B. bronchiseptica isolates were performed in three independent experiments by measuring optical density and by culture-based enumeration in the four media: cation-supplemented Mueller-Hinton Broth (CAMHB) with and without 2 % lysed horse blood, Brain-Heart Infusion and Caso broth. The sampling of the cultures took place every two hours until 24 hours and thereafter every six hours until 48 hours. Statistical analysis of each test strain in each medium was performed to determine the applicability of the test media. Susceptibility testing: Reference and type strains as well as eight field isolates of B. bronchiseptica were used to test the suitability of a broth microdilution susceptibility testing method according to CLSI guidelines. Susceptibility tests were performed for 20 antimicrobial agents in five replicates. The data were collected after 20 and 24 h incubation and statistically analysed.

RESULTS:
Although statistical analysis showed differences in growth for some isolate-media combinations, the microbiological cell enumeration demonstrated a sufficient growth of B. bronchiseptica in CAMHB. Statistical analysis of susceptibility testing in five replicates revealed that a longer 24 h incubation time resulted in significantly more homogeneous minimum inhibitory concentrations (MIC), when compared to the shorter 20 h incubation. The MIC values from 8 out of ten isolates showed more homogeneous MIC values after 24 hours compared to 20 hours incubation time. Ampicillin, amoxicillin/clavulanic acid, ceftiofur, chloramphenicol, enrofloxacin, florfenicol, penicillin, tetracyclin, tiamulin and trimethoprim/sulfamethoxazole gave the most homogeneous MIC values after 24 hours.

DISCUSSION AND CONCLUSIONS:
The broth microdilution susceptibility testing method given in CLSI-document VET01-A4 is suitable for B. bronchiseptica with the recommendation of a longer incubation time.6 Reading of MIC values after 24 h leads to more homogeneous MICs and could lead to more consistent results in interlaboratory comparisons. For other fastidious pathogens such as Campylobacter spp. or Histophilus somni, modified incubation times are defined. Therefore, an extension to 24 h incubation for B. bronchiseptica would be beneficial to preserve more homogeneous MICs.

REFERENCES:
INTRODUCTION:
The causative agent of the Q fever zoonosis is Coxiella burnetii a Gram-negative obligate intracellular bacterium. Cattle, goats and sheep are the primary reservoirs although a variety of animal species may be infected. C. burnetii is extremely hard and resistant to heat and it was demonstrated that a single infective cell can initiate an infection. C. burnetii is transmitted to humans most often by aerosol contaminated by parturient products, urine or feces of infected animals. Nowadays there is no conclusive evidence that human consumption of milk and milk products containing C. burnetii resulted in clinical Q fever. Isolation of the bacterium is not so easy and usable for routine diagnosis in veterinary medicine because it is laborious, time consuming and requires confined level 3 laboratories due to the zoonotic nature of the agent. Moreover, actually there is not an official method to detect C. burnetii: it is important to identify a rapid and not expensive assay for the detection of the agent at laboratory level.

The aim of this study is to compare the performances of two PCR-based protocols for the identification of C. burnetii: a qualitative real-time PCR commercial kit (LSI VetMAXTM Coxiella burnetii-Life Technologies) and an End-Point PCR assay proposed by Berri et al. (1).

MATERIALS AND METHODS:
The target gene of both methods is the transposon-like IS1111; in the real-time protocol it is co-amplified with an endogenous Internal Positive Control (IPC) common to cattle, sheep and goat. The end-point PCR was carried out in a final volume of 20 µl containing 10 µl of HotStartTaq Plus Mix Kit (QIAGEN), 5.2 µl of DNA-free water, 4 µl of DNA and 0.2 µM of each primer. The thermal cycle was performed under the following conditions: five cycles consisting of denaturation at 94°C for 30 s, annealing at 64-60°C for 30 s (the temperature was decreased by 1°C between consecutive steps) and the extension at 72°C for 1 min. After that 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 50 s. 12 µl of PCR product were analyzed by Agarose gel electrophoresis on 1.5% Agarose gel and 10,000x Gel Red, visualized under UV transilluminator. Analytical sensitivity was evaluated as Limit of Detection (LOD). To calculate the LOD two independent series of certificate C. burnetii DNA (AMPLIRUN® Vircell, Microbiologists) were prepared in order to obtain 100, 50, 20, 10, 5, 2, 1, 0.5, 0.25, 0.1 and 0.05 copies of the genome per reaction. For each series, two dilutions before and two after of the lower dilution resulted positive were tested. In particular, for each dilution four replicates were prepared and tested. LOD was considered as the lower dilution where the all eight reactions resulted positive. Specificity was evaluated testing 30 microorganisms such as bacteria, moulds and yeasts.

RESULTS:
The real-time PCR LOD is set at 1 copy/reaction, while the end-point results to be 10 copies/reaction. The whole no-target microorganisms resulted negative with both PCR methods, according to the producer and the authors respectively.

DISCUSSION AND CONCLUSIONS:
Using DNA as a target for sample screening overcomes problems with culture conditions, allowing the rapid identification of positive samples, with the identification of a possible bacterial source within 48 hours. For this reason, PCR methods are helpful to identify C. burnetii in milk and dairy products where no official methods are present. Moreover for EU is important to understand the distribution of the zoonotic agents through Member States. Nowadays the oral-human-infectious dose is unknown, so it is important the use of more rapid and high sensitive methods able to detect this pathogen. Evaluation of performances of these two protocols in experimentally infected milk and dairy products is mandatory. Further tests with correlated species should be performed in order to evaluate the sensitivity of the above mentioned protocols.

REFERENCES:
INTRODUCTION:
Genetic typing based on sequence analysis distinguished BVDV-1 and BVDV-2 species and, recently, a new putative BVDV-3 species was detected. Up to date, on the basis of phylogenetic analysis of 5'UTR and Npro regions, at least 17 distinct subtypes were recognized for BVDV-1. The aim of this study is to further investigate the genetic heterogeneity of BVDV-1 in Italy.

MATERIALS AND METHODS:
A total of 225 isolates collected from 13 Italian regions over a 18-year period (1997-2014) was analyzed. Viral RNA was extracted from original biological samples identified as BVDV-1 positive. Reverse transcription and PCR assays targeting a 288 bp region of 5'UTR and a 428 bp region encoding autoprotease Npro were amplified as previously described (Vilcek S. et al., 1994, 2001) and sequenced. All the sequences were aligned with BVDV-1 reference strains using Clustal X2. Manual editing was performed with Bioedit software version 7.0. Phylogeny was estimated by the neighbor-joining algorithm (NJ) and the maximum likelihood (ML) method.

RESULTS:
The analysis of 5'UTR and Npro sequences confirmed the circulation of 11 subtypes as previous reported (Giammarioli M. et al., 2008; Luzzago C. et al., 2014). Interestingly, 4 isolates differed significantly from all the bovine Pestiviruses described so far. One isolate, collected in Sicily, showing sequence similarity from 71.50% to 81.00%, is very close related to the isolate Deer-GB1 (BVDV-1j). Two isolates, collected in the North and in the South of peninsula, clustered together with a range of similarity 71.97%-85.20%. The fourth isolate, collected in Central Italy, showed the highest identities with a BVDV strain 2561 from England and with a BVDV isolated from a mouse deer in Copenhagen Zoo (Grondahl C. et al., 2003) allocated within the 1f subtype (Tajima M. et al. 2001). The percentage of pairwise sequence similarity ranged from 71.40% to 86.30%.

DISCUSSION AND CONCLUSIONS:
According to the criterion adopted for the segregation of Pestivirus into defined genetic groups reported by Becher P. et al. (1999), our results provided evidence of 3 additional new subtypes, which have never been described before, thus revealing a very high BVDV-1 genetic heterogeneity. In Italy, this high genetic diversity is the result of the absence of any BVDV systematic control measures and of the management practices such as cattle trade and animal movement. The growing number of reports on BVDV-1 heterogeneity, including the recent findings reported herein, raises the main concern related to the emergence and spread of new BVDV variants with possible implications for animal health and disease control. Such issue has to be seriously considered at a global perspective.

REFERENCES:
INTRODUCTION:
C. perfringens is an anaerobic Gram-positive sporulating bacteria. It is widespread in the environment and although it is common in the intestinal flora of animals and humans, it can be pathogenic in certain circumstances, causing enterotoxemic disease and hemorrhagic enteritis.

C. perfringens is able to produce up to 30 potential toxins but four are considered major. The strains are then classified into five categories: A, B, C, D and E, according to the combinations of these toxins. Different other toxins can be included in the classification as minor. The β2 toxin, a 28 kDa protein, is one of the most important minor toxin; the pathogenic role of β2 in the clostridial disease is not fully understood. The gene cpb2 has been shown to be plasmid-borne. β2 toxin can be encoded from two different alleles: allele gene “consensus” expressed in porcine and allele gene “atypical” of non-porcine origin.

The aim of the study was to evaluate the expression of the entire and N-terminal deleted forms of typical and atypical β2 toxins in a baculovirus system.

MATERIALS AND METHODS:
Generation of construct: Porcine and bovine derived C. perfringens strains were obtained from our strain collection. The 800 bp cDNA encoding the atypical and typical entire β2 proteins and their corresponding N-terminal deleted forms (amino acids 28-265 for the Typical and 26-265 for the atypical), were amplified by PCR using specific primers designed according to sequences present in the database. The amplified fragments were digested by EcoRI-AgeI restriction enzymes and cloned, in frame with a 6XHis-tag, into POET2C_6xHis baculovirus transfer vector.

Transfection: Recombinant baculovirus were generated by transient transfection of SF21 insect cells according to the procedures reported in Flashbac expression system (Oxford Expression Technologies). Protein production: SF21 cells were infected with a recombinant baculovirus; three days post infection the supernatant fraction (for the secreted entire typical and atypical antigens) and the infected SF21 (for the deleted and intracellular typical and atypical protein) were harvested.

SDS-Page and Western blotting: the level of expression of the proteins was evaluated by Western blot using the anti-His C-Term HRP monoclonal antibody. The results showed the immunoreactive proteins bands with an expected molecular weight (Fig 1).

DISCUSSION AND CONCLUSIONS:
In the present study the procedure for the production of the recombinant β2 protein in baculovirus system we developed. All the proteins were expressed efficiently and in particular, the expression of intracellular forms was higher than the secreted forms. The high efficiency of recombinant proteins expression may pose the basis for a more extensive study, particularly focusing on the standardization of the purification methods. In fact the recombinant toxins obtained could be used for diagnostic purposes and vaccine production.

Work funded by the Italian Ministry of Health, research project code IZ5UM 12/11

REFERENCES:
1. The occurrence of cpb2-toxigenic Clostridium perfringens and the possible role of the β2-toxin in enteric disease of domestic animals, wild animals and humans. Alphons J.A.M. van Asten, Georgios N. Nikolau, Andrea Gröne. The Veterinary Journal 183 (2010)135-150;
INTRODUCTION:
Yersinia spp. are zoonotic bacteria able to infect humans, livestock, companion animals and wildlife. According to an EFSA report of 2009, Yersinia spp. represent the third cause of foodborne disease in Europe [1]. In Liguria region, the increase of wild boar (Sus scrofa) population in the last decades motivated extended hunting and a higher consumption of wild boar meat. Disembowelment procedures, when occurring in unhygienic conditions, may cause meat contamination by pathogenic bacteria (e.g. Yersinia enterocolitica) [2,3], thus posing a risk to human health. Few data on the safety of meat from wild boars hunted in Liguria are currently available [4]; the consume of uncooked meat accounts for the main human health related risk [5]. Focusing on wild boars hunted in Eastern Liguria (Genoa and La Spezia provinces), aim of this study was to evaluate Yersinia spp. presence in the liver, an organ often subject to cross-contamination at evisceration.

MATERIALS AND METHODS:
In the last hunting season (1/10/2013-31/01/2014), 514 livers of wild boars hunted in eastern Liguria were sampled. All specimens were analyzed according to ISO 10273-2003, and Y. enterocolitica suspected colonies were confirmed by biochemical tests. Positive strains were subsequently bio-typed and characterized for their serotype using specific antibodies: anti-O8, -O9, -O3, -O5 and -O1,2.

RESULTS:
Thirty four liver samples (6.6%) were detected positive for Yersinia spp.; particularly, on 61.8% of them Y. enterocolitica biotype 1A was isolated, and further characterized as O:5 (14.3%) and O:8 (14.3%) serotypes; 71.4% of Y. enterocolitica biotype 1A isolates were negative to serum-agglutination. Other Yersinia species (e.g. Y. bercovieri, Y. kristensenii and Y. frederikseni) were detected on 32.8% of the livers examined. In Genoa province (Fig. 1) 7 areas (Polcevera, Scofetta, Paradiso, Petronio, Fontana Buona, Sturla and Aveto) showed a Yersinia spp. prevalence lower than 10%; 4 areas (Genoa, Golfo, Scrivia and Stura) showed values in the interval 10-20%; in 3 areas (Trebbia, Graveglia and Riviera a Ponente) no positive cases were found. Eventually, the Entella zone presented a higher prevalence (>20%; Fig. 2). The different prevalence between sexes was not significant (Fisher’s Exact Test, P >0.05).

DISCUSSION AND CONCLUSIONS:
As reported by EFSA [1], Y. enterocolitica 1A strain, considered as a non-pathogenic biotype, is widely spread in the environment and frequently isolated from food, animal and human stools. Our data indicate that, despite the ubiquitous presence of Y. enterocolitica, it is reasonable to assume a low food-related risk for yersiniosis. Nevertheless, results obtained are noteworthy, as they suggest the occurrence of carcass contamination at disembowelment if good hygienic practices are dissuaded [2,3].

REFERENCES:
INTRODUCTION:
Shiga toxin-producing Escherichia coli (STEC) are responsible of human diseases and their pathogenicity is due to their ability to express verocytotoxin (VT) and to form attaching and effacing lesions in the intestine. The largest “reservoir” of STEC is represented by ruminants, not only cattle but also sheep and goats. Given the importance of ovine and caprine livestock in North Apulia (Capitanata), and the fact that many milk products from these species aren’t treated with any previous bacteriological sterilization procedures, the aim of the present study was to investigate the prevalence of STEC in ovine and caprine raw milk in order to determine the potential risk that the consumption of these products can have from the standpoint of public health.

MATERIALS AND METHODS:
In the period between November 2011 and December 2012 were analyzed 84 raw milk samples (67 from sheep and 17 from goat) from 66 farms distributed in Capitanata (Foggia-Italy). For the analysis of the samples was used the method ISO/TS 13136:2012 (1). The DNA extracts were screened by the Real time PCR detection of stx1, stx2 and eae, and the serogroups O157, O111, O26, O145, O103. As required by the protocol used, screening is followed by a microbiological isolation and a subsequent culture confirmation of isolates by Real time PCR. Furthermore, to determine the stx gene subtypes in isolated STEC strains, the procedures available at the EU-RL VTEC web site were followed, using primers designed on the basis of analyses of existing stx sequences (2).

RESULTS:
The results of screening showed a prevalence of 37% (31/ 84) of sheep and goat’s raw milk samples contaminated with STEC and so they were positive to at least one of virulence genes (vtx1, vtx2, eae). Furthermore, 26% (21/84) of the samples belonged to the one of five searched serotypes (9/21 O157, 1/21 O26, 1/21 O145, 1/21 O103). However, only 5% of analyzed samples (4/84) were isolated by microbiological confirmation. The four STEC isolated were milk ovine. All STEC strains carried both stx2 and eae genes and they belonged to O157 serogroup. Of 4 strains with genes encoding for Stx2 group, 50% (2/4) was positive for stx2 and 50% (2/4) for stx2c and stx2d.

DISCUSSION AND CONCLUSIONS:
In spite of the restricted number of samples, our findings are interesting because the epidemiological role of ewe’s milk remains to be established. In this study, the absence of isolation of E. coli non O157 from sheep and goat’s milk may be linked low concentrations of the pathogen in the presence of high levels contamination. Instead, as regards O157 STEC isolates, evaluation of associated virulence factors is required. The prevalence of serotype O157 in dairy products may be related to certain properties of this bacterium, not found in other STEC strains. The stx2d subtype that was predominantly detected among strains positive for genes of Stx2 group was also found to be the principal subtype in ovine isolates in other studies (3). Given the severity of infections caused by this organism, it is necessary to implement a task of surveillance and reporting of these infections.

REFERENCES:
1.ISO/TS 13136:2012 Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups
NICOLETella SEMOLINA IN A DONKEY: FIRST CASE REPORT FROM ITALY

Nizza S.[2], Vecchio D.[1], Martucciello A.[1], Alfano D.[1], Veneziano V.[1], Corrado F.[1], De Roma A.[1], Scognamiglio R.[1], Guarino A.[1], De Carlo E.[1]


Keywords: Nicoletella semolina, donkeys, Italy

INTRODUCTION:
Nicoletella semolina is gram-negative bacterium recently described as member of the Pasteurellaceae family[1]. N. semolina has been isolated from horses with respiratory disorders (2) however it is still unclear if this bacterium plays a role as potential pathogen, opportunistic or just as commensal of the normal bacteriological flora. To the best of authors’ knowledge there are no descriptions of the isolation of this bacterium in donkeys. The aim of the present report is to describe, for the first time, the isolation of N. semolina in a donkey foal from Italy.

MATERIALS AND METHODS:
Three 2-3-month-old crossbreed donkey carcass (A-B-C) were submitted to the necropsy. Anamnesis reported severe respiratory disorders before death. At necropsy in all foals was reported generalized pneumonia associated with purulent-catarrhal collection and emphysema diffuse; the foal A showed lesions in all lung lobes, pericardial effusion and subsequent cardiac tamponade. Samples were collected from lungs and were plated onto two Blood Agar Base and MacConkey agar SS plates, and incubated aerobically and in microaerophilia at 37°C±1°C for 72h. The plates were observed every 24h. On all isolates were performed identification using the Vitek 2 Compact system (bioMérieux). On the strain isolated from the foal A was performed the sequencing and analysis of 16S rRNA genes. PCR was performed with MicroSeq 500 rDNA PCR Kit (Life Thecnologies). Amplification products of the 16SrRNA genes were used for cycle sequencing with MicroSeq 500 rDNA Sequencing Kit. Sequence analysis and alignment were carried out using SeqScape v.2.5 (Applied Biosystem) and NCBI/BLAST/blasst suite.

RESULTS:
After 24h of incubation aerobic bacterial cultures of all 3 foals (A-B-C) in Blood Agar Base yielded heavy growth of Streptococcus equi subsp. zooepidemicus; at the same time point in MacConkey agar plates (foal C) was detected the co-presence of Salmonella kasenyi. After 48h of incubation in Blood Agar Base plates (foal B) was isolated Nocardia spp. Furthermore at 72h in in Blood Agar Base plates (foal A) was detected the appearance of colonies gray, rounded, waxy, nonhaemolytic, nonadherent, similar to a grain of semolina wheat (1). The organism was identified as a pleomorphic gram-negative coccobacillus, nonmotile, catalase and oxidase positive and negative for indole test. 16S rDNA sequencing revealed 99% homology with N. semolina.

DISCUSSION AND CONCLUSIONS:
The present report described in the donkey is fairly similar to that noted recently in young horses (3) where is described the isolation of N. semolina associated with Streptococcus equi subsp. zooepidemicus with respiratory disorders (2). In the donkey is still be determined whether N. semolina is a primary pathogen or a commensal which can occasionally cause disease. Further studies are required to clarify the potential pathogen of this bacterium in the donkeys.

REFERENCES:
INTRODUCTION:
Members of the genus Brachyspira include fastidious and strictly anaerobic spirochetes which colonize the large intestine of various animals and humans. Important diseases are swine dysentery caused by Brachyspira (B.) hyodysenteriae and porcine (PIS) and avian (AIS) intestinal spirochaetosis caused by B. pilosicoli, or in the latter case also by B. intermedia or alvinipulli (1). B. pilosicoli can also infect humans and thus very likely represents a zoonotic agent (2). Despite the economic importance of AIS and the zoonotic potential of B. pilosicoli, it has not received extensive study in poultry in Germany.

MATERIALS AND METHODS:
To investigate the occurrence of spirochetes in hens in Germany a total of 71 fecal swabs from laying hens were taken in 2009 to 2013 in eight flocks and sent to our laboratory using Amies transport media for culturing Brachyspira spp. Species determination was done by published PCR protocols or by nox-gene sequencing. Further studies focused on their antimicrobial susceptibility to macrolides, lincosamides and pleuromutilins by use of a microdilution assay and on their hemolytic activity.

RESULTS:
Brachyspira spp. were cultivated from 40 (56.3 %) swabs, distributed over all sampled flocks. Species determined were B. pilosicoli (n=16), B. intermedia (3), B. murdochii (1), B. pulli (9), B. alvinipulli (3), mixtures of B. innocens/murdochii (1), pilosicoli/intermedia (2), innocens/intermedia (1) and an unidentifiable species. Antimicrobial susceptibility testing has revealed minimal inhibitory concentrations (MIC) of >128 g/l (tylosin), 64 g/l (lincomycin), 8 g/l (tiamulin) and 4 g/l (valnemulin), respectively. All isolates were weakly hemolytic although three isolates contained the hlyA and tlyA haemolysin genes in combination.

DISCUSSION AND CONCLUSIONS:
Weakly haemolytic Brachyspira spp. could be successfully cultured from fecal swabs in Amies transport medium. Altogether, spirochetes, in particular the zoonotic B. pilosicoli, were widely present in laying hens in Germany as it was already reported for other European countries (1). Reduced antimicrobial susceptibilities of isolates to tylosin, lincomamine and pleuromutilins demonstrate that antibiotic resistance plays an important role in Brachyspira spp. from poultry. This could demand some variations on the control of AIS. Alternative strategies to antibiotic treatment, however, need a better knowledge on the virulence factors of the Brachyspira, e. g. their haemolysins. The simultaneous detection of hlyA and tlyA genes in weakly haemolytic Brachyspira spp. represented an interesting finding since this combination was otherwise found in the strongly haemolytic (pathogenic) B. hyodysenteriae only. This led to doubts on their role as haemolysin. Future work should unravel if specific gene combinations of haemolysins are associated with strong or weak hemolysis.

REFERENCES:
INTRODUCTION:
Clostridium perfringens is a Gram-positive, sporulated, anaerobic bacterium that is very commonly found in many environments, including soil, water, poorly preserved feeds, contaminated or improperly thawed colostrum or milk, calf housing environments (1). It normally inhabits the digestive tract without causing disease, but under right conditions could grow and proliferate, producing large amount of toxins. In cattle, this enterotoxaemia condition is characterized by a high fatality rate, sudden death, and hemorrhagic enteritis, particularly in small intestine (2). The acuteness of intoxication and death often occurs before treatment could be attempted.

Prevention of C. perfringens infection enterotoxaemia focuses on vaccinated young calves.

Our study investigates on a case of sudden death of five young buffaloes in Caserta district herd.

MATERIALS AND METHODS:
Five 3 to 4-month-old buffaloes suddenly died and they were submitted to the Istituto Zooprofilattico Sperimentale del Mezzogiorno in Caserta district, Department of Diagnostic for autopsy and further diagnostic investigation. They came from properly vaccinated herd. They were healthy few hours prior the death and then suddenly found dead without clinical signs. They are examined just few hours after death. During necropsy, it only was revealed a faint inflammation of the intestinal wall and characteristic gelatinous appearance of kidneys. Instead, it was not revealed hemorrhagic enteritis with intraluminal hemorrhage and/or ulceration. Intestinal contents were cultured on blood agar. The agar plates were incubated in an anaerobic condition at 37 °C for 24 h. In addition, samples from organs were cultured on blood agar plates, which were incubated under aerobic and anaerobic conditions at 37° for 24 h. Suspected colonies were identified by characteristic colony morphology, Gram staining and biochemical tests.

RESULTS:
Large numbers of C. perfringens were found in intestinal contents and bone marrow. Furthermore, during necropsy, it was observed a moderate inflammation of intestinal wall and a gelatinous appearance of kidneys surface.

DISCUSSION AND CONCLUSIONS:
Young buffaloes vaccination does not yield adequate protective immunity. So many vaccination plans target pregnant buffalo to impart maximum immunity to the young buffalo in colostrum. Our study describes an atypical case of clostridium infection. Died buffaloes consumed an adequate amount of high-quality colostrums, containing antibodies against C. perfringens within the first 18-24 hours but they didn’t gain adequate protection. Usually, post mortem examination of infected buffaloes revealed internal lesions such as inflammation of the intestinal wall, diarrhea with or without blood in the intestine, small areas of hemorrhage on intestinal and heart surface, fluid around the heart or rapid degeneration of the kidneys. Our autopsy, instead didn’t reveal these signs, intestinal wall only revealed a faint inflammation and kidneys surface appeared gelatinous. Other organs (liver, lungs, heart, spleen) were in normal condition. Diagnostic investigations showed a large numbers of C. perfringens in intestinal contents and bone marrow. Nowadays other herds of Caserta district are showing some atypical cases. Therefore, further investigation, should be performed to identify the type of C. perfringens and what kind of toxins give this unexpected clinic development.

REFERENCES:
EVALUATION OF LIQUID CULTURE SYSTEM FOR DIRECT IDENTIFICATION OF MAP FROM POSITIVE FECES SAMPLES

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Keywords: VersaTREK, M. avium subsp. paratuberculosis, Liquid culture

INTRODUCTION:
The isolation of M. paratuberculosis (MAP) provides the definitive diagnosis of infection. Since MAP is shed through feces, the fecal culture is the gold test, but it is difficult and time-consuming to carry out; primary colonies on solid media may be appeared on Herrold’s egg yolk medium (HEYM) from 5 weeks to 6 months after inoculation, in the other hand, it is the only method that doesn’t produce false positive results. VersaTREK/ESP Culture System II (VT) combines a liquid culture medium, two growth supplements and antibiotic supplement. The technology of VT is based upon the monitoring gas production/consumption due to microbial growth for the detection of headspace pressure changes within a sealed bottle. The purpose of this study was to evaluate the possibility to reduce requested time for detection of MAP using VT, applying different methods of feces decontamination.

MATERIALS AND METHODS:
Fifteen samples, that had already been tested for MAP and classified as Very High Shedding (>100 CFU, n=4 samples), High Shedding (50-100 CFU, n=3 samples) and Moderate Shedding (10-49 CFU, n=8 samples) and stored at -20°C were used. For each sample were used two different methods of decontamination: the procedure according to the technical VT system sheet (V) and the procedure (D) using for conventional culture by the Istituto Zooprofilattico of Piemonte, Liguria and Valle d’Aosta. Each decontaminated sample was inoculated on VT. Samples were retested on HEYM in order to assess its sensibility on frozen samples.

RESULTS:
As shows Table 1, 11/15 samples were positive using VT with D method and 13/15 with V method, so sensibility values were respectively 73,3% (IC 95%= 61,87%-84,72%) and 86,6% (IC95%: 77,81%-95,4%). Concordance between liquid culture and first solid culture was 86% when used decontamination V and 73% when used decontamination D. Mean time to detection of MAP by VT was six weeks. To confirm, positive samples were submitted to ZN staining and Simplex PCR. Table 2 summarises detection times of MAP by VT.

DISCUSSION AND CONCLUSIONS:
Despite specificity of 100%, Gold Test has a sensibility that is related with the progress of infection and symptoms, as well as the number of bacteria; in fact, the samples storage at -20°C can significantly reduce the viability and the initial charge. Nevertheless, VT method showed high sensibility (see table 1) unlike the conventional method that lacks sensibility after storage (2/15 positive samples). The effects of storage require further investigations, but VersaTREK performances can be interesting for a potential use of VT to detection infected animals at earlier stage of disease in which bacterial load of feces is still low. It can be noted a considerable reduction of time detection of MAP using VT (see Table 2); a rapid detection of MAP has become important in surveillance tools to confirm clinical diagnosis or to remove quickly infected animals during control or eradication programs.

REFERENCES:

Table 1: Results of VersaTREK Culture

<table>
<thead>
<tr>
<th>Sheding level</th>
<th>Positive V method</th>
<th>Positive D method</th>
<th>HEYM (retested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very High (&gt;100)</td>
<td>3/4</td>
<td>4/4</td>
<td>1/4</td>
</tr>
<tr>
<td>High (50-100)</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Moderate (&lt;50)</td>
<td>7/8</td>
<td>4/8</td>
<td>1/8</td>
</tr>
</tbody>
</table>

Table 2: Time detection of MAP by VersaTREK

<table>
<thead>
<tr>
<th>Sheding level</th>
<th>Mean day of Method (range)</th>
<th>SD</th>
<th>Mean day of Method (range)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very High (&gt;100)</td>
<td>46.5 (30.44-51.6)</td>
<td>3</td>
<td>40 (36-57.4)</td>
<td>5.44</td>
</tr>
<tr>
<td>High (50-100)</td>
<td>45 (31.0-50.6)</td>
<td>7</td>
<td>40 (39.29-51.6)</td>
<td>3.4</td>
</tr>
<tr>
<td>Moderate (&lt;50)</td>
<td>46.5 (34.4-51.6)</td>
<td>14</td>
<td>30 (24-39.3)</td>
<td>13.15</td>
</tr>
</tbody>
</table>
INTRODUCTION:
Contaminated poultry meat by C. jejuni is considered one of the main sources of campylobacteriosis in humans. Reducing Campylobacter in chicken could result in a reduction of human illness. A feeding trial was conducted to investigate the effectiveness on the Campylobacter reduction in chicken gut of a synbiotic product added to feed in experimentally infected poultry.

MATERIALS AND METHODS:
One-hundred-sixty 1-day-old chicks, were infected with 0.1 ml by oral gavage containing a challenge dose of 10^6 cfu/ml of C. jejuni M1 strain and were divided into four groups (Grp). Animals were fed with conventional feed differently supplemented with a probiotic strain (microencapsulated Bifidobacterium longum subsp. longum PCB133, 1 g > 10^9 cfu) and xylo-oligosaccharides (XOS) as prebiotic, added to the feed at 1% and 0.2% respectively. Control group A received only conventional feed without synbiotic; Grp B and C received conventional feed added with the synbiotic product from the 14th day and from 1-day of life respectively; Grp D received conventional feed added only probiotics from the 14th day of life. Nine chickens of each group were slaughtered at 10, 20, 30, 39 days of life and level of Campylobacter contamination in caeca faeces was tested. The analyses of the caecal microbiota were performed by real-time PCR. Data were elaborated using the analysis of variance with the GLM procedure by using SAS software and statistical significances were confirmed by using Tukey’s test. The variability between groups of Campylobacter presence in caeca was calculated using the analysis of variance (ANOVA).

RESULTS:
Average charge of Campylobacter contamination (log10 cfu/g) of caecal content in different groups, at different slaughtering, showed a reduction of Campylobacter in Grp B (3.8 cfu/g) and C (2.8 cfu/g), compared with GrpA (5.6 cfu/g). Moreover, only the GrpC revealed a statistically significant difference with the A (p-value 0.004), fig.1. In real time-PCR analysis, concerning Bifidobacterium genus, the GrpA had significantly higher bifidobacterial counts with respect to Grp B, C and D (p<0.01). B. longum quantification evidenced the presence of the administered probiotic bacterium in caecal samples. Campylobacter spp. quantification results showed no significant differences in GrpB and D compared to the control group. Concerning GrpC, ANOVA analysis evidenced a significant decrease (p<0.05) of Campylobacter spp. in animals treated with the synbiotic product. Animal of Grp C supplemented with the synbiotic product for 39 days evidenced on the contrary a significant reduction (p<0.01) of the pathogen in caecal samples.

DISCUSSION AND CONCLUSIONS:
Probiotics and prebiotics may be included in feed to reduce contamination of zoonotic bacteria. The use of synbiotics is an alternative and effective approach to antibiotic administration for livestock to reduce bacterial contamination. Our data are showing the effectiveness of these schemes at experimental level. The data obtained underline the effectiveness of the synbiotic product in reducing C. jejuni in infected chickens (2 log10 of reduction). Considering the high infective dose in 1-day-old chickens the product showed the effect only with a lifelong supplementation. The challenge data, will serve to define the best schema to be adopted in the chicken farm in the next step of our study.

REFERENCES:
POSTER PRESENTATIONS

ANIMAL DISEASES, VIRAL
AN UNUSUAL CASE OF LONG-TERM EXCRETION OF SCHMALLENBERG VIRUS GENOME IN BULL SEMEN

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Keywords: Schmallenberg virus, Semen, RT-qPCR, Austria

INTRODUCTION:
Schmallenberg virus (SBV), an orthobunyavirus of the Simbu serogroup, first emerged in 2011 in Germany and The Netherlands and has since spread to most of Europe, including Austria, where SBV was first detected in samples collected in late summer to autumn 2012 (1). SBV is transmitted by arthropod vectors, but trans-placental transmission also occurs, leading to abortions, stillbirths and birth of malformed and/or weak offspring. Recently, detection of SBV genome by reverse transcription quantitative real-time PCR (RT-qPCR) in semen of naturally and experimentally infected bulls was reported (2-4). Importantly, SBV viraemia and seroconversion were observed in cattle experimentally infected with SBV genome positive semen, raising the question if SBV could also be transmitted by means of artificial insemination or during mating (5). Here, we report about an exceptional case of long-term SBV genome excretion in a SBV seropositive breeding bull: so far, constantly SBV RT-qPCR positive semen has been collected from this bull for almost 47 weeks.

MATERIALS AND METHODS:
Individual semen samples were collected from a Tyrolean Grey bull at ten different time-points from June 2013 to May 2014. Blood was also collected from this animal at four different occasions (April 2013 to May 2014). Nucleic acid was prepared from blood by commercial silica-based extraction kits and from semen by Trizol LS/chloroform pre-treatment, followed by purification with commercial extraction kits. SBV genome detection was performed by duplex RT-qPCR, targeting both the SBV S-segment and beta-actin mRNA. The hyper-variable region of the SBV M-segment was amplified and sequenced from SBV RT-qPCR positive semen samples collected at three different time-points. Serum was analyzed by commercial ELISA and partly by serum neutralization test (SNT).

RESULTS:
Semen from the Tyrolean Grey bull was SBV RT-qPCR positive at all ten time-points, indicating continuous SBV genome excretion in semen for a period of almost 47 weeks (more than 10 months). semen fractionation performed on semen collected in Nov. 2013 and May 2014 showed that SBV genome was mostly associated with the cellular fraction. SBV M-segment sequencing revealed little variation in the hyper-variable region over a time-period of more than eight months. In blood, SBV antibodies were detected at all four time-points. No SBV genome was detectable in blood at two time-points tested.

DISCUSSION AND CONCLUSIONS:
Here, we present an unusual case of long-term SBV genome excretion in semen from a SBV seropositive bull. At present, observation of this bull is ongoing. Previous results from our laboratory indicated that in most cases SBV genome excretion in bovine semen is transient and limited to a single or a few consecutive ejaculates. However, continuous or intermittent SBV genome excretion in semen for longer time periods (weeks to months) may as well occur, as was also reported by others (2, 4). It is presently unknown by what mechanism SBV establishes persistence in the genital tract despite the presence of neutralizing antibodies.

REFERENCES:
038

ENHANCED SENSITIVITY OF AN ANTIBODY ENZYME-LINKED IMMUNOSORBENT ASSAY USING EQUINE ARTERITIS VIRUS PURIFIED BY ANION EXCHANGE MEMBRANE CHROMATOGRAPHY

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Keywords: Anion exchange chromatography, Equine arteritis virus, competitive blocking enzyme-linked immunosorbent assay

INTRODUCTION:
An improved competitive blocking ELISA (cELISA) based on a non-neutralizing epitope on Equine arteritis virus (EAV) GP5 was previously reported with an initial diagnostic sensitivity and specificity of 99.8% and 95.5% when compared to virus neutralization (VN) test results, respectively. In the field trial performed by AAVLD-accredited state laboratories and OIE reference laboratory, the diagnostic specificity of the same cELISA was 99.5% and the diagnostic sensitivity was 98.2%. The cELISA is not adversely affected by previous exposure of horses to non-EAV biologicals, which cause problems in indirect ELISA and the VN test due to the antibodies against the cell line used in vaccine production. The current study describes further improvement of the cELISA performance using a novel EAV purification method with an ion exchange chromatography.

MATERIALS AND METHODS:
Purification of EAV was performed by a differential centrifugation method and a method using ion exchange membrane capsule. The EAV fractions purified by two methods were analyzed in Western blot and used for preparing cELISA kits.
Four borderline sensitivity check sets were prepared by diluting EAV-positive sera in negative serum. Another sensitivity check set was prepared with sequentially collected time point sera from horse H681. Horse SR10387 was an EAV carrier, and the remaining four horses (HS37, H631, H632, and H681) were vaccinated with EAV MLV (Arvac® b). Four borderline sensitivity check sets determined in SN titer were used to evaluate the analytical sensitivity and specificity, intra-lab repeatability and inter-lab reproducibility of the cELISAs compared in this study. A borderline sample panel including 42 EAV VN-positive sera and 1 EAV VN-negative sera was prepared to test the proficiency of the cELISA made with EAV stocks by two different purification methods.

RESULTS:
AEC-purified EAV antigen contained ~86.3% GP5 monomer while differential centrifugation-purified EAV contained less than 29.4% GP5 monomer. Improvement of cELISA analytical sensitivity without sacrifice of analytical specificity was clearly evident when cELISAs based on the two purification methods were evaluated using sensitivity check sets composed of borderline positive/negative sera from three horses vaccinated with a commercial modified live attenuated vaccine (MLV), and a time point serum set sequentially collected from an MLV-vaccinated horse. Furthermore, the AEC-purified antigen cELISA had 44.2% to 46.4% higher agreement with the VN test than the cELISA derived from differential centrifugation-purified EAV when tested with 43 borderline EAV-seropositive samples as defined by the VN test. In addition, the AEC-purified antigen cELISA had highly significant (p = 0.001) robustness indicated by intra-laboratory repeatability and inter-laboratory reproducibility when evaluated with the sensitivity check sets.

DISCUSSION AND CONCLUSIONS:
The method of EAV purification was successfully developed based on an easily scalable protocol using the AEC. Currently, an extensive validation of the improved cELISA to confirm the diagnostic performance, particularly in diagnostic specificity and sensitivity, is ongoing using various sera from horses with MLV vaccination, experimental and natural infections of several EAV strains. The improvement of cELISA using anion exchange membrane chromatography may contribute to further harmonize the EAV antibody cELISA with the OIE-prescribed VN test. Furthermore, the results in this and previous manuscripts strongly support the use of this cELISA as a suitable alternative to the OIE-prescribed VN test for serodiagnosis of EAV.

REFERENCES:
INTRODUCTION:
Serology is one of the most important methods for PRRS diagnosis. During the last years, oral fluid samples have become an easier and cheaper way for monitoring both viremia (PCR) and sero-conversion (ELISA) after PRRSv infection and/or vaccination. Different ELISA kits for oral fluids have been launched into the market and it is needed to know their sensitivity and their specificity with this new kind of sample under field conditions.

The ultimate goal of this study was to evaluate the sensitivity and the specificity of CIVTEST SUIS PRRS E/S PLUS, comparing oral fluids with serum in field farms.

MATERIALS AND METHODS:
8 farms from Catalonia were selected, all of them with different PRRS vaccine protocols and different epidemiological situations. In all cases, it was done a seroprofile with weaners, growers and finishers, using serum (quantity of samples depending on field conditions, approximately between 40% and 60% of the animals) and oral fluid samples. Some sows of different parities were also tested, but only with serum. Serum samples were obtained by jugular puncture and oral fluid samples with cotton ropes located in the pens (1 rope every 15-20 animals). The ELISA used was CIVTEST SUIS PRRS E/S PLUS, HIPRA. When more than one rope was needed in the same pen, they were analyzed separately. The statistical analysis was performed with StatsDirect v.2,7,9 and Win Episcope 2.0. Correlation (R2) and kappa value were calculated. The sensitivity and the specificity of oral fluids kit, relating to serum, were also calculated.

RESULTS:
On the one hand, taking into account all samples, the correlation coefficient (R2) between serum and oral fluid samples was 0,48. In this situation, the sensitivity and the specificity for oral fluids were 89,8% and 81,8%, respectively. The kappa value was 0,648 (0,402 , 0,893)
But it was observed significant discrepancies in recently weaned pigs (between 4 and 5 weeks of age, when they still have maternal immunity), especially in 3 of the 8 farms. When these values were eliminated, the sensitivity (89,33%) and the kappa value (0,686 (0,448 , 0,924)) didn’t change a lot, but the correlation coefficient value increased until 0,76 and the specificity until 90%.

On the other hand, the correlation value (R2) was also calculated when there was more than one rope in the same pen. In this case the result obtained was 0,92.

DISCUSSION AND CONCLUSIONS:
There were different results when the samples from pigs 4-5 weeks old were included or not. In the one hand, it can be expected that some false positive results are due to animals with maternal immunity. On the other hand, neither sensitivity nor kappa value changed which means that kit is able to detect post-infection and/or post-vaccination seroconversion, including or not the animals within these ages. In some farms (3 of the 8) a peak of positivity was observed between 14 and 20 weeks of age, which was higher with oral fluids than with serum; more studies are needed to know if this higher sensitivity is because of the oral fluids or there is anything else that increases the positivity.

Another important point to note is the excellent correlation between different ropes located in the same pen, which means that, when is necessary to use more than one rope, they give a very similar results.

So, CIVTEST SUIS PRRS E/S PLUS detects the post-infection and/or post-vaccination seroconversion with a sensitivity and specificity of 89,3% and 90%, respectively, in animals which are more than 5 weeks of age. In this situation the correlation with serum is 0,76.

REFERENCES:
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THE OPTIMIZED HERD CONCEPT (OHC) - A COMPREHENSIVE SOLUTION STRATEGY TO CONTROL PRRSV

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Keywords: PRRSV, Optimised Herd Concept, PrioCHECK PRRSV AB VIA

INTRODUCTION:
PRRSV outbreaks are feared in the swine industry because of the devastating economical impact. Even though various diagnostic tests for PRRSV including ELISA and PCR systems exist, correct result interpretation with respect to the actual PRRSV status pose a major challenge. Noteworthy are the genetic diversity of PRRSV isolates, the prolonged PRRSV persistence and the complex immunological behavior of the virus[1]. Vaccination against PRRS has shown to be an effective tool to reduce clinical disease, however, PRRSV infection is not prevented. MLV vaccine virus may be shed and transmitted to non-vaccinated contact pigs[2] and vaccine virus can persist in boars and be disseminated through semen[3]. The complex nature of the PRRSV disease indicates that single diagnostic tests may not be enough to successfully subdue PRRS virus but rather comprehensive solution strategies are needed for the effective control of the disease. In particular to discriminate type I from type II on herd level with an ELISA approach, to detect single or mixed infections of different PRRSV types, assess the chronological order of different infections, estimate the time of infection and the virus load and finally support decisions to choose the optimal time for PRRSV vaccination and the respective schedule.

MATERIALS AND METHODS:
The following diagnostic tools have been applied to establish the optimized herd concept (OHC) PrioCHECK®-PRRSV RT-PCR, PrioCHECK®-PRRSV Ab porcine and PrioCHECK Ab VIA. Additionally, herd management data were introduced to complete the IHC.

RESULTS:
Here we show results that demonstrate the reliability and robustness of the PRRSV detection tools PrioCHECK®-PRRSV RT-PCR, PrioCHECK®-PRRSV Ab porcine and PrioCHECK Ab VIA. External validation using PrioCHECK®-PRRSV RT-PCR showed a highly specific and sensitive detection of the PRRS virus in different tissues. Furthermore a comprehensive concept for PRRSV disease control is discussed combining herd / site information, results of molecular tools (RT PCR) as well as immunological detection systems (ELISA) translating to a reliable statement about the immunological fitness of a pig herd and hence lead to optimized measures for a sustainable disease control. Analysis of over 1000 pig serum samples derived from different herds and countries with the PrioCHECK®-PRRSV Ab porcine indicated a high agreement with competitor ELISA systems. The PrioCHECK Ab VIA could be shown to be an effective tool to not only discriminate type 1 from type 2 PRRSV but also to give an assessment about single or mixed infections of different PRRSV types on herd level, assessment of the chronological order of different infections, estimation on the time of infection and the infection status based on the serological titre level.

DISCUSSION AND CONCLUSIONS:
A comprehensive concept for PRRSV disease control is discussed combining herd and site information, results of molecular tools (RT PCR) as well as serological detection systems (ELISA). Finally, the IHC may support the farmer to improve the herd management in terms of identifying the best vaccination time point and to establish a herd risk assessment e.g. identifying suitable pig populations for change of housing during the fattening period within the breeding units.

REFERENCES:
INTRODUCTION:
The Schmallenberg virus (SBV) is an arthropod-borne virus of the genus Orthobunyavirus of the Bunyaviridae family and is the only bunyavirus of the Simbu serogroup detected in Europe so far. After its first isolation in summer 2011 (2), SBV has been reported in more than 25 European countries (5).

The higher genetic divergence of the envelope glycoprotein (Gc) compared to the capsid protein within the Simbu serogroup (1) makes the SBV Gc the antigen of choice to develop a test for the detection of SBV-specific antibodies.

In this study, the serological reaction of infected animals against the SBV full-length Gc and two truncated forms was evaluated in an indirect ELISA.

MATERIALS AND METHODS:
The SBV Gc protein and two truncated forms of it comprising either the amino terminal third or the carboxyl terminal two thirds were affinity purified. The protein preparations were then used as antigen in an indirect ELISA.

A total of 23 bovine and 8 murine sera (4) of known status were analyzed. From the 23 bovine sera, 21 were field sera that included 10 negative and 11 positive. From the positive sera, 5 were obtained from calves before colostrum intake. One bovine hyperimmune serum and one negative serum were used as controls. From the 8 mouse sera, 6 were positive and 2 were negative.

Each serum was evaluated with the three antigens in an indirect ELISA. The ELISA was performed using the ELISA starter accessory kit II (Bethyl).

RESULTS:
The SBV Gc protein was successfully purified in high amounts from transiently transfected eukaryotic cells. The N terminal domain could be readily isolated from the supernatant as a monomer, whereas the full-length Gc and the Gc with an N terminal truncation were purified from detergent-treated cell lysates. Interestingly, the N terminally truncated Gc was predominantly found as a disulfide-linked oligomer.

The reactivity of each antigen was tested with bovine and murine sera in an indirect ELISA and all positive sera reacted against the three antigens tested. These results indicate, that the recombinant proteins are similarly folded as the glycoprotein present in the viral particle, since the sera were obtained from infected animals. Hence, two subdomains of the Gc were isolated that remained reactive to antibodies present in sera from two different species of SBV-infected animals.

DISCUSSION AND CONCLUSIONS:
The results of this study indicate that the SBV virion-associated Gc is strongly immunogenic, generating an immune response against both tested subdomains in two animal species. Similar observations have been reported when analyzing the specificity of antibodies against other orthobunyaviruses (3). The reactivity of the sera tested with the three recombinant proteins suggest that epitopes for antibodies that developed in the course of an infection are present in these antigens. Therefore, the Gc and especially its truncated forms presented here, are functional promising tools to detect SBV-specific antibodies. Besides that, these data are relevant for the development of a DIVA (Differentiating Infected from Vaccinated Animals) vaccine against SBV. Furthermore, it can be hypothesized that the immuno-dominant region og SBV-Gc is mainly represented by the construct expressing the N-terminal part of Gc.

REFERENCES:
INTRODUCTION:
Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) appears in two genotypes (EU and US); for both genotypes attenuated live-vaccines are available. Vaccinated herds are currently monitored by PCR and ELISA; however, by these methods immunity is not assessed. Thus, serum neutralization test (SNT) might be an attractive alternative to estimate the level of immunity.

MATERIALS AND METHODS:
A cross-sectional study in 38 Bavarian sow herds was performed to assess the level of neutralizing antibodies (nabs). Per herd 38 blood samples were collected from 10 weaned piglets at the age of 8 weeks, 10 gilts and 6 sows of 1./2., 3./4. and 5./6. parity, respectively. Sera were tested by ELISA (Idexx, Ludwigsburg, Germany), SNT against EU- (Porcilis® PRRS) and US-vaccine virus (Ingelvac® PRRS MLV), and pooled sera (n=5 or n=6) were tested by real-time RT-PCR.

Herds were classified by the last vaccination of sows as ‘Vacc EU’, ‘Vacc US’ and ‘nv’ (non-vaccinated) and by the detection of PRRSV-EU in the group of weaned piglets (EU+ and EU-). The detection of PRRSV-US and the vaccination of piglets were not included as variables. Six groups were analyzed: (1) Vacc EU/EU+ (n=7), (2) Vacc EU/EU- (n=9), (3) nv/EU+ (n=1), (4) nv/EU- (n=7), (5) Vacc US/EU+ (n=4) and (6) Vacc US/EU- (n=10).

RESULTS:
Sows of the group (2) Vacc EU/EU- showed the highest EU-SNT-titers followed by (5) Vacc US/EU+ and (1) Vacc EU/EU+ in descending order (Kruskal-Wallis, p<0.05). Very low EU-SNT-titers were observed in the three remaining groups (3) nv/EU+, (4) nv/EU- and (6) Vacc US/EU-.

All parity groups of sows in (2) Vacc/EU- showed a homogenous and high level of SNT-titers which corresponded to the absence of EU-virus in weaned piglets. In contrast, in addition to a generally lower level of SNT-titers in the group (1) Vacc EU/EU+ sows of 1./2. parity showed a lack of SNT-titers (Kruskal-Wallis, p<0.05). In contrast, in US-vaccinated herds (5) Vacc US/EU+ this parity group had higher SNT-titers than sows of higher parity.

A weak and strong ELISA-reactivity was observed in (6) Vacc US/EU- and (5) Vacc US/EU+, respectively. Thus detection of virus corresponded to a strong ELISA-reactivity in all animal groups. In contrast, EU-vaccinated sows showed a strong ELISA-reactivity irrespective of detection of virus in weaned piglets.

DISCUSSION AND CONCLUSIONS:
PRRS-vaccination should induce SNT-titers at least against the vaccine virus; moreover, a homogeneous level of titers is expected. Indeed, in EU-vaccinated herds the absence of EU-virus in weaned piglets corresponded to a high and homogeneous level of EU-SNT-titers in sows as compared to EU-vaccinated herds with concurrent virus circulation. In the latter herds a lack of nabs was observed in sows of 1./2. parity. Thus by means of EU-SNT a gap in herd immunity was identified. Such an inhomogeneous distribution of SNT-titers among sows increases the time period of virus circulation in piglets as those without and with maternally derived nabs get infected early and late, respectively.

A completely different picture was observed in US-vaccinated herds: In absence of EU-virus EU-SNT-titers were rarely observed. The presence of EU-virus corresponded to elevated levels of EU-SNT-titers; and this time sows of 1./2. parity showed higher EU-SNT-titers than older sows. Probably, EU-virus spreads more efficiently in the presence of heterologous antibodies across the herd (antibody-dependent enhancement of virus infection). This view is further supported by a strong ELISA-response in all animal groups of US-vaccinated herds if EU-virus was detected. Additionally, it was confirmed that low ELISA-reactivity in US-vaccinated sows corresponds to the absence of EU-virus. In contrast, in EU-vaccinated herds sows showed high-level ELISA reactivity irrespective of EU-virus detection. The value of the ELISA is restricted to the certification of PRRSV-free herds. The EU-SNT reflects the level of herd immunity at least against vaccine virus; it indicates gaps in herd immunity.
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SUBTYPING AVIAN INFLUENZA ANTIBODY POSITIVE SERA FROM THE DUTCH POULTRY MONITORING PROGRAMME USING A PROTEIN MICROARRAY

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Keywords: Avian Influenza, protein microarray, subtyping

INTRODUCTION:
Avian influenza (AI) outbreaks in poultry cause huge economic losses and are a major threat to public health worldwide. Migratory water birds form a natural reservoir of AI and pose a risk for introduction into poultry. When introduced in poultry, AI normally causes mild or no disease (low pathogenicity avian influenza (LPAI)). LPAI of the H5 and H7 subtypes, however, can evolve to high pathogenicity (HPAI) viruses and are therefore notifiable. Measures to control these subtypes have been included in European Union (EU) legislation and, consequently, surveillance programmes have been implemented by EU member states. In the Netherlands a part of the surveillance is based on monitoring, in which each poultry farm is tested with an AI ELISA at least once a year. Although for control purposes detection of only H5 and H7 subtypes is required, it is important from a public health point of view to determine all subtypes. The aim of the study was to get insight in the different Avian Influenza (AI) subtypes detected in commercial poultry flocks in 2012 and 2013.

MATERIALS AND METHODS:
The subtypes of the AI virus are characterised by two envelope proteins haemagglutinin (H1-16) and neuraminidase (N1-9). Gene constructs representing all H and N subtypes were produced using an in vitro expression system. Proteins were used to develop a microarray. A similar microarray was described before (Koopmans et al., 2012). Antibody positive sera from the Dutch poultry monitoring programme (2012–2013) were tested using our protein array.

RESULTS:
Results show that the HA subtypes 2, 5, 6, 7, 8, 9, 11 and all NA subtypes were detected although in different frequency.

DISCUSSION AND CONCLUSIONS:
Not all known AI subtypes were detected in commercial poultry. Some subtypes were detected more frequently than others which might suggest that transmission from wild birds to terrestrial poultry is not random.

REFERENCES:
A FIELD EVALUATION OF GE INDIRECT ELISA FOR SURVEILLANCE OF IBR MARKER VACCINATED AND IBR FREE MILK HERDS

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INTRODUCTION:
Bulk milk samples have been proven to be a practical and cost-effective biological material for surveillance of many animal diseases. The application of bulk milk testing in IBR vaccinated herds has been limited for the intrinsic low sensitivity of blocking gE ELISA (1), when applied to a matrix with low IgG content. We recently developed a recombinant based gE indirect ELISA which overcome these limitations. Objective of this study was to evaluate the applicability of the method under field condition.

MATERIALS AND METHODS:
Bulk milk samples have been proven to be a practical and cost-effective biological material for surveillance of many animal diseases. The application of bulk milk testing in IBR vaccinated herds has been limited for the intrinsic low sensitivity of blocking gE ELISA (1), when applied to a matrix with low IgG content. We recently developed a recombinant based gE indirect ELISA which overcome these limitations. Objective of this study was to evaluate the applicability of the method under field condition.

RESULTS:
Laboratory simulation suggested a detection limit of one positive sample out of 40 samples (theoretical prevalence of infection: 2.5 %), which seemed adequate to reveal minor seroconversion, as it could be expected in marker vaccinated herds according to EU regulation (2). Bulk milk samples were collected in three different Italian provinces (Cuneo, Torino and Trento) from positive (n=51), vaccinated (n = 114) and IBR-free (n = 55) herds. Among positive farms, 37 had a seroprevalence greater than 2.5% in lactating animals. Each samples was subjected to IgG purification/concentration and assayed in indirect ELISA. Results were compared with IBR status as detected by officially method according to the last available individual blood testing (using blocking gE ELISA), estimating the correct seroprevalence in lactating animals. When this estimation was available, ELISA test returned positive results if the seroprevalence greater than 2.96%, but tested negative if the prevalence was lower than 2.38%. This result confirmed the 2.5% of seroprevalence as theoretical limit of detection and allows to asses the sensitivity of the diagnostic process to 100% (95%CI 90.5% – 100%) in farms with a seroprevalence greater than 2.5%. On the other hand, results on gE negative farms showed a nearly perfect specificity, with no differences in term of reactivity between vaccinated and IBR-free farm samples. Indeed, in the first test session, only 1 IBR-free and 1 vaccinated farm showed positive results in ELISA. In order to better investigate the two discordant situations, we collect individual blood and milk samples and re-tested all the animals. In the IBR-free farm, the second test detected the presence of a single positive animal out of 67. The positivity was proved using both individual milk and blood sample. The positive cow had been vaccinated in 2003 with an attenuated conventional vaccine: its antibody titers decreased until 2013, testing negative at the previous gE blocking ELISA. This animal, erroneously not removed by the farmer, probably reactivated the vaccine strain with no viral excretion: this scenario was sufficient to obtain positive outcomes in ELISA test using bulk milk and can explain our results. The second discordant farm is still under investigation. To date, given these results on gE-negative samples, we can fix the specificity of the diagnostic protocol to 99.4% (95%CI 96.7%-100%), reaching the 100% in IBR-free farms (95%CI 93.4%- 100%).

DISCUSSION AND CONCLUSIONS:
Performances of the proposed method seems to overcome the limitations of the blocking ELISA, in term of both sensitivity and specificity. As far as sensitivity in bulk milk is concerned, it has been significantly improved in comparison with conventional gE-Elisa reactions, and now it is close to the sensitivity of the most sensitive Elisa kits for total antibodies which are commercially available.

REFERENCES:
1) OIE Terrestrial Manual 2010 – Chapter 2.4.13 Infectious Bovine Rhinotracheitis / Infectious pustular vulvovaginitis
2) 64/432/EEC on animal health problems affecting intra-Community trade in bovine animals and swine
POTENTIAL APPLICATION OF A DIVA QUANTITATIVE REAL-TIME RT-PCR ASSAY FOR PESTE DES PETITS RUMINANTS VIRUS

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Keywords: Peste des petits ruminants virus, DIVA test, RRT-PCR

INTRODUCTION:
Peste des petits ruminants virus (PPRV) is a Morbillivirus belonging to Paramyxoviridae family which induces a contagious disease affecting small ruminants. The clinical outcome in the infected animals is diverse, but showing high morbidity and mortality in the acute forms. It is endemic in sub-Saharan Africa, Middle East and Asia and emerged in Northern Africa in 2008. Currently, the attenuated PPRV Nigeria75/1 strain is the only live vaccine recommended for control strategies (1). In a previous study, a real-time RT-PCR (RRT-PCR) assay for detection of the PPRV Nig75/1 vaccine strain was developed (2). The aim of the present study was to adapt this assay into a duplex test, incorporating the simultaneous detection of an internal control, and to evaluate this method for its potential application as a DIVA test in clinical material.

MATERIALS AND METHODS:
Nucleic acids were extracted from viral suspensions and clinical samples using QIAamp®cador Pathogen Kit (Qiagen). An exogenous internal positive control (IPC) (Intype IC-RNA®, Qiagen) was spiked into the lysis buffer and was co-purified with the sample RNA as a positive control.

Probe and primers described previously (3) were selected to amplify the IPC and were incorporated to the developed RRT-PCR for PPRV vaccine strain detection for optimization of duplex RRT-PCR assay. An in vitro transcribed RNA standard was obtained and quantified including the PPRV Nig75/1 target fragment. PPRV field strains of the four genetic lineages, and other ruminant viruses were used for specificity tests. Diagnostic specificity and sensitivity were evaluated with target samples from healthy animals and experimentally vaccinated/inoculated sheeps, including blood, faeces and swabs. Generic PPRV RRT-PCR technique described by Kwiatek (4) was used as reference test, being adapted as a duplex method for incorporating the detection of the IPC.

RESULTS:
Comparative analytical sensitivity assays performed between the uniplex RRT-PCR previously developed for PPRV vaccine detection and the duplex format including the IPC showed minimum Ct differences. Low Ct differences were obtained as well when comparing both duplex RRT-PCR tests for PPRV generic or vaccine-specific detection established in this study. Triplicates of serial dilutions of the quantified RNA standard were assayed and the detection limit of the PPRV-vaccine RRT-PCR was estimated to be fewer than 60 viral RNA copies, producing a standard curve with a linear dynamic range over 7 log10.

Analytical specificity assays proved the competence of the developed duplex system to detect only the vaccine strain and the incorporated IPC. A panel of blood samples collected from sheep (n=150), red deer (n=7) and roe deer (n=1) at different locations in Spain, and different clinical material obtained from healthy donor sheep remained as negative when analysed by the duplex method, assuring its diagnostic specificity. Finally, the application of the developed RRT-PCR as a DIVA test was evaluated testing clinical material obtained throughout an experimental ovine vaccination/challenge study, using the PPRV Nig75/1 vaccine and further challenge with PPRV Morocco 2008 field strain. Samples were tested in parallel by both vaccine-specific and generic PPRV RRT-PCRs. During the vaccination stage, positive results correlated between both tests when Ct values were ≤37 but were inconsistent for higher Ct values, while negative samples remained undetected. The vaccine RRT-PCR maintained its specificity all along the experiment.

DISCUSSION AND CONCLUSIONS:
The proposed duplex RRT-PCR assay resulted specific and sensitive for PPRV vaccine strain detection, enabling the DIVA principle application if a vaccination campaign is implemented. Additionally, the presented test can be applied for obtaining an estimate of genome copy number per sample. Also, the incorporation of an IPC in the samples allows monitoring the extraction and amplification procedures.

Support
Work funded by Spanish INIA project RTA2011-00072-00-00 and INIA-MAGRAMA agreement EG13-020.

REFERENCES:
1. OIE Manual, 2012, chapter 2.7.11.
**A SIMPLE AND RAPID DUPLEX LFDCOMB ASSAY TO DETECT ANTIBODIES TO CSFV AND ASFV**

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**Keywords:** Lateral flow test, African swine fever virus, Classical swine fever virus, Rapid diagnostics, Field test

**INTRODUCTION:**
Classical Swine Fever (CSF) and African Swine Fever (ASF) are both highly contagious diseases of domestic pigs. Due to the clinical and pathologic similarity, the differentiation of CSF and ASF is impossible either by clinical syndrome or by post-mortem examination. The two diseases have to be distinguished by laboratory diagnosis, which is currently performed by ELISA to detect antibodies or by PCR to confirm the virus in blood, lymph nodes, spleen or serum samples of infected pigs. Both methods have to be carried out in well equipped laboratories; they are time consuming and need highly trained personnel. A simple diagnostic method to rapidly differentiate the two diseases may be important for front line disease control of CSFV and ASFV.

**MATERIALS AND METHODS:**
The Duplex LFD-Comb test is a lateral flow device (LFD) technology carried out in a microtiter plate format. In the Duplex LFD-Comb CSFV/ASFV, the recombinant E2 derived from CSFV and the affinity purified VP72 are sprayed as test lines onto the nitrocellulose strip, a monoclonal antibody against CSFV was applied in the control line. The E2 and VP72 proteins conjugated with latex microsphere particles were used for detection and are supplied in dried form in the microtiter well and reconstituted with assay buffer and serum sample. The LFD-Comb is then placed into the microtiter wells and the reaction mixture flows through the nitrocellulose membrane by capillary forces to pass the test and control lines and is absorbed in the waste pad. The presence (or absence) of antibodies against CSFV and ASFV in a serum sample is visualized in the test line(s). The control line serves as validity test and has to be visible in all the tests.

**RESULTS:**
Evaluation of the LFD-Comb CSFV/ASFV test was performed on a total of 134 sera: 75 CSFV serum samples (40 positives, 14 from BVDV/BDV infection, 21 negatives) from the Prionics sample archive and the Friedrich-Loeffler-Institute (FLI) in Germany. In addition, 59 ASFV serum samples from 12 pigs immunized with inactivated ASF virus and 3 control pigs from the same study (FLI) were used. The serum samples were taken at post immunization day (PID) 21, 28, 36 and 41. 26 sera were confirmed as positives in ELISA from the 12 immunized pigs, 6 sera were doubtful and 27 sera were negative. The Duplex LFD-Comb CSFV/ASFV correctly identified all the 40 CSFV positive samples suggesting a diagnostic sensitivity of 100%. Furthermore, the test correctly identified 89 out of 94 negative samples suggesting a diagnostic specificity 94.7%. Similar to the reference ELISA for CSFV, the Duplex LFD-Comb CSFV/ASFV showed cross reactivity with some of the samples positive for BVDV/BDV. The results of Duplex LFD-Comb CSFV/ASFV to detect antibodies against ASFV are comparable to a commercial ELISA. The Duplex LFD-Comb assay showed earliest detection of antibodies from animals at PID21. It detected 24 positives from 26 ELISA positive samples suggesting a relative sensitivity of ASFV antibody detection of 92.3%. The diagnostic specificity for ASFV was calculated using all the 75 CSFV samples as true negatives for ASF, from which 2 false positives were observed.

**DISCUSSION AND CONCLUSIONS:**
The diagnostic sensitivity of the Duplex LFD-Comb CSFV/ASFV is comparable to commercial ELISA tests. In the case of CSFV antibody detection, it showed slightly reduced cross reaction with BDV/BVDV samples. The Duplex LFD-Comb CSFV/ASFV may be applied to rapid differentiation of CSFV and ASFV infection in pigs, particularly as a front line screening test. The simple workflow of the Duplex LFD-Comb CSFV/ASFV test requires only very limited training of involved personnel and is therefore, suitable for small field laboratories requiring a high throughput to test samples for the presence and discrimination of CSF and ASF.

**REFERENCES:**
Acknowledgement
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DEVELOPMENT OF A COMPETITIVE ELISA FOR AHS DIAGNOSIS USING THE RECOMBINANT AHSV-VP7

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Keywords: AHSV, recombinant AHSV-VP7, ELISA

INTRODUCTION:
The African Horse Sickness (AHS) is a non-contagious disease of solipeds transmitted by hematophagous insects of the genus Culicoides and caused by a double-strand RNA virus belonging to the genus Orbivirus (Reoviridae). Up to date, 9 serotypes have been serologically identified. The disease has a variable course depending on the species and age of the infected animals. The mortality rate in horses can be higher than 90%. The World Organization for the Animal Health (OIE) recommends ELISA, complement fixation and virus neutralization as prescribed tests for the serological screening of the susceptible population (1). Among these assays, ELISA has proved to be a good method for the detection of anti-AHSV group-reactive antibodies, targeting the Viral Protein 7 (VP7). The aim of this work was to produce the recombinant AHSV-VP7 (rVP7) for use in the serological diagnosis of AHS by competitive ELISA (c-ELISA).

MATERIALS AND METHODS:
Recombinant VP7 production
The rVP7 was expressed in Baculovirus System: the entire sequence of the segment S7 serotype 1 was cloned into a plasmid vector transfer and expressed as a fusion protein, where at the N-terminus was inserted a V5 epitope, for detection in Western blotting. At 72 h p.i., the baculovirus-infected Sf9 cells were harvested, lysed, purified on sucrose gradient according to Maree et al. (2) and solubilized with sarcosyl as described by Rutkowska et al (3). rVP7 was analysed by SDS-PAGE and detected in Western blotting with anti-V5-HRP antibody (Invitrogen) and MAb 7F11E14-HRP vs VP7-AHSV (IZSAM).

c-ELISA
Polysorp Nunc microplates, coated with rVP7 and blocked with 3% skim milk, were incubated for 1 h at 37°C with horse positive and negative sera diluted 1:5 in blocking buffer. Then MAB 7F11E14-HRP was added for 1 h at RT. After incubation, plates were washed with TMB substrate was added. After 30 minutes, reaction was terminated by adding H2SO4 0.5N. Optical densities (OD) were measured at 450 nm and converted in B/B0 % values, where B is sample OD and B0 is MAB control OD. Fifty AHSV negative horse sera and 10 AHSV positive sera (8 hyperimmune guinea-pig antisera against serotypes 1, 2, 3, 4, 5, 6, 7, 8; 1 horse serum positive for serotype 9 and 1 hyperimmune sheep antiserum positive for serotype 7) were tested.

RESULTS:
The production of the rVP7 was confirmed in Western blotting with anti-V5-HRP antibody and MAB 7F11E14-HRP: a protein band with an approximate molecular mass of 40 KDa was observed (Fig. A). c-ELISA results of AHSV negative and positive sera are shown in Fig. B.

DISCUSSION AND CONCLUSIONS:
The production and purification of the rVP7 described above is a simple method to manufacture large quantities of quality immunodiagnostic reagents. The rVP7 reacted with MAB 7F11E14, produced using AHSV-9 whole virus as immunogen, and with antibodies from AHSV positive sera. It may mean that our rVP7 is biochemically and antigenically similar to its native counterpart. In fact Baculovirus System guarantees a correct folding and all post-translational modifications that allow to obtain a recombinant protein biologically active and similar to the native one. The c-ELISA was able to discriminate AHSV positive sera from negative sera. In conclusion, the preliminary results indicate that the rVP7 produced can be used in the development of diagnostic tools for the diagnosis of AHS.

REFERENCES:

Fig. A. Comassie stain of Sf9 raw lysate (lane 1) and purified rVP7 (lane 2). Western blotting of purified rVP7 with anti-V5-HRP antibody (lane 3) and MAB 7F11E14-HRP (IZSAM).

Fig. B. c-ELISA: B/B0 % values of AHSV negative and positive sera
INTRODUCTION:
European brown hare syndrome (EBHS) is an acute and severe, contagious, necrotising viral hepatitis that occurs in Lepus europaeus and Lepus timidus resulting in high mortality amongst wild and farmed animals. The EBHSV virus (EBHSV) is a small non-enveloped, single stranded RNA (~7.5-8 kb) virus belonging to the genus Lagovirus, family Caliciviridae. Virions have a diameter of about 35 to 39 nm and a single major capsid protein (CP) with a molecular mass of approximately 60 kDa (1).

EBHSV CP self-assembles into virus-like particles (VLPs) that are morphologically and antigenically identical to the infectious particles (2). Generally speaking, VLPs are appealing as vaccine candidates since they lack the viral genomic material essential for replication and infection (3). They have been shown to successfully prime the immune system due to the similarities in structure and antigenicity to the viruses which are derived from (4).

Here we present the expression of the C-terminally 6xHis-tagged CP in baculovirus system and its purification by Ni++ affinity chromatography.

MATERIALS AND METHODS:
Cloning of the CP gene: Total RNA was isolated from 40 mg of homogenized liver of infected farmed hares using RNeasy Micro Kit (Qiagen), and cDNA synthesized with random primers using Superscript II Reverse Transcriptase (Life tech.). The full sequence of CP gene was amplified with Accuprime Taq-DNA polymerase (Life tech.) using CP-F/CP-R primers pair. The PCR product was cloned in frame with 6xHis-tag at the C-terminal end into pOET-2C and confirmed by sequencing.

Generation of recombinant baculovirus: Plasmid DNA containing the CP encoding sequence, was co-transfected with Flashbac baculovirus genome (Oxford Expression Technologies) into sf21 cells as reported in the manual’s instructions.

Expression and purification of recombinant CP: Sf21 cells were infected with 1 Plaque Forming Unit for cell and incubated at 27 °C for 3 days. Cells were harvested and lysated in ice with 50 mM sodium phosphate (pH 8) buffer plus 1% triton X100. Protein was purified from the clarified lysate by His-Select Ni++ affinity chromatography (Sigma-Aldrich) in native conditions according to the manual’s instruction. Fractions were tested by ELISA (RHD/EBHS kit, IZS Lombardia and Emilia Romagna, Italy) and SDS-PAGE/Coomassie blue staining. VLPs integrity was examined by electron microscopy after negative staining with 2% phosphotungstic acid, pH 6.9.

RESULTS:
The sequence amplified from cDNA was of the expected size (1731 nucleotides).

DISCUSSION AND CONCLUSIONS:
The presence of the recombinant CP was confirmed by ELISA both in the supernatant and cell extract. However, most of the recombinant protein was evidenced on the cell fraction. Self-assembly of CP subunit into VLPs was confirmed in the growth medium by electron microscopy (data not shown). SDS-PAGE/Coomassie of the eluted fractions revealed a protein band of ~55-60 kDa with high degree of purity (Fig. 1 lanes 6, 7).

REFERENCES:
REOCCURRENCE OF HAEMORRHAGIC SEPTICAEMIA IN GERMANY

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Keywords: Haemorrhagic Septicaemia, PCR, cross reaction

INTRODUCTION:
Haemorrhagic septicaemia (HS) is an acute to peracute, OIE listed disease with high morbidity and mortality mainly in cattle and buffaloes, however, deer and pigs can be affected, too. HS is caused by certain serotypes of Pasteurella (P.) multocida, i.e. Asian serotype B:2 and African serotype E:2 according to the Carter and Heddleston system. In Germany, HS occurred for the last time in 1986 and was withdrawn from the list of notifiable diseases in 1999. In summer 2010, acute disease with high mortality rates was observed in fallow deer, cattle and pigs and diagnosed as HS by pathological and bacteriological investigations (Soike et al. 2012).

MATERIALS AND METHODS:
One fallow deer, six pigs and four cattle were submitted for diagnostic investigation to the Berlin-Brandenburg State Laboratory during the summer of 2010. Necropsy was performed and relevant tissue samples were histologically and bacteriologically examined using standard procedures. Furthermore a PCR protocol for differentiation of isolated P. multocida was established according to the OIE manual (Townsend et al. 1998, Townsend et al. 2001).

RESULTS:
Pathological lesions in cattle consisted of severe fibrinous pleuropneumonia associated with pulposus swelling of the spleen and septic haemorrhages on the serosal surfaces of the thoracic and abdominal cavity. The fallow deer died from a septic shock with massive pulposus swelling of the spleen and haemorrhagic diathesis. In the pigs, the main lesion consisted of a severe phlegmonous inflammation of the whole neck resulting in death by suffocation due to the massive swelling of the soft tissues surrounding larynx and trachea. P. multocida was isolated from all the tissue specimens examined, mainly in heavy growth and pure culture. All isolates were identified as capsular type B using the P. multocida multiplex capsular PCR typing system. Additionally, they were positive in the so called HS-causing type-B-specific PCR assay. During validation of the OIE PCR protocol, which was formerly assumed to be specific for HS-causing type B P. multocida isolates, specificity testing resulted in a cross reaction with some, but not all, P. multocida capsular type D strains.

DISCUSSION AND CONCLUSIONS:
As a re-emerging disease, HS was detected in fallow deer, cattle and pigs for the first time in Germany since 1986. During validation of the OIE recommended PCR assays (Townsend 1998, Townsend et al. 2001) it was found that the PCR described as specific for HS-causing capsular type B P. multocida also detected some but not all strains of capsular type D. This cross reaction led to an adaptation of the diagnostic strategy. Multiplex PCR for differentiation of the P. multocida capsular type and PCR for detection of HS-causing type B P. multocida are always run in parallel to overcome the specificity problem and to assure a correct diagnosis of HS-causing type B P. multocida isolates.

REFERENCES:
050
SPECIAL PLAN FOR SWINE VESICULAR DISEASE (SVD) CONTROL OF SLAUGHTERED PIGS FROM OWN-CONSUME FARMS IN CAMPANIA REGION

Caligiuri V.[1], Ottaiano M.[1], Desio G.[1], Fusco G.[1], Carbone C.[2], Bove D.[1], Palermo P.[1], Vitale V.[1], Baldi L.[1], Guarino A.[1]

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Keywords: Swine, Virus, Campania

INTRODUCTION:
The European Commission has approved, by Ministry of Health recommendation, an Special Plan for Swine Vesicular Disease (SDV) control in Campania Region in own-consume farms, to eradicate the virus circulation in herds excluded by O.M. 04.12.2008. Those farms hold a maximum of two growing pigs/year, for non-commercial use but exclusively for production of private consume sausages: own-consume farms represent 95% of the pig population.

MATERIALS AND METHODS:
The Plan, carried out between 12.16.2013 and 05.26.2014, provided blood sampling of a representative population, performed by the AASSLL among farms during own-slaughtering, localized on the basic risk areas within cells 10*10(Ath.1).
The farms sampled were 4.495, compared to 6.600 estimated, for a total of 5.595 samples (intracardiac blood clots): blood samples were analyzed with cELISA for IgM and IgG by the IZSM Portici labs and for the Serum Neutralization (SN) by the IZSLER (CERVES). “National Data Bank” (BDN) and SIGLAWEB were used for data sources. “Downgraded” holdings, excluded from the commercial circuit, due to major failures in respecting basic bio-security and management requirements (Decree n.93/2011), have been included in the Plan. Those “downgraded” holdings must detain up to two swine and slaughter all others within 7 days from the order.

RESULTS:
Only 20 (on 4.495) holdings were found positive (0.0045%: 0.0025-0.0064 C.I. 95%) in the Salerno and Napoli provinces. Furthermore, epidemiological studies on serum-positives samples, highlighted connections between positive farms and transportation used.

DISCUSSION AND CONCLUSIONS:
About positive farms, the prevalence of 0.0045% can be considered real and acceptable, considering the own-consume farms peculiarities: up to 2 pigs; allowed to buy only from companies that are already checked for National Plan for SDV (O.M. 04.12.2008); those pigs are breed only for few months in order to be slaughtered for own-consume sausages production.
Campania Region aims to define clearly the update of these holdings in the BDN, in order to have a proper real-time display of those farms. At the same time positive holdings and transportation will be checked during 2014 to verify disinfection and transport practices, identified as critical points.

REFERENCES:
BDN
SIGLAWEB
O.M.12/04/2008
Decree n.93/2011
DEVELOPMENT AND VALIDATION OF A NEW REAL TIME TAQMAN REVERSE TRANSCRIPTION-PCR ASSAY FOR EQUINE ARTERITIS VIRUS DETECTION

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Keywords: Equine Arteritis Virus, Horse, PCR

INTRODUCTION:
Equine Arteritis Virus (EAV) belongs to the family Arteriviridae, genus Arterivirus, which also includes porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus, and lactate dehydrogenase elevating virus of mice. EAV is the etiologic agent of the disease Equine Viral Arteritis (EVA) which is a reproductive and respiratory disease of horses, donkeys and mules. EAV infection of horses is endemic in much of the world and results in occasional outbreaks of respiratory disease in adult horses, abortion in pregnant mares, and interstitial pneumonia in young foals. Clinical signs of EAV infection may vary between outbreaks but most of them are subclinical. Many stallions become persistently infected carriers following EAV infection and continue to shed the virus in their semen for variable periods of time and act as a natural reservoir. In this study, we have developed a new qRT-PCR to detect the virus in semen of infected stallions. Indeed, a qRT-PCR targeting the Open Reading Frame 7 (ORF7) of the virus has been developed in 2002 by Balasuriya et al. at that time full genome sequences of EVA were not available. Since the last 10 years, evolution in knowledge regarding EVA genome sequences enables us to develop a qRT-PCR targeting specifically the most conserved region of the genome, ORF1

MATERIALS AND METHODS:
We have designed seven primers set and probes targeting the most conserved regions, among 10 field isolates sequenced in the laboratory and 29 sequences on GenBank database, along the ORF1 sequence using Primer3 software (http://frodo.wi.mit.edu/). Primer sets #1, #2, #3, #4 target non structural protein 1 (nsp1), Primer set #5 targets nsp 8 and sets #6 and #6bis on nsp10. Validation of PCR protocol has been performed following the AFNOR XP U47-600 standard in order to define the PCR limit of detection (LOD) and the method LOD for each of the 7 primers set and probes designed in comparison of the reference method published by Balasuriya et al. in 2002

RESULTS:
Bucyrus RNA, in-vitro transcribed, range from 10-2 to 108 copies. PCR LOD is determined after 3 independent sessions with 8 replicates of each dilution. PCR LOD is the number of copy of genome where 95% (23 out of 24) of repetitions gave a positive result. In our hands, PCR LOD of the PCR targeting ORF7 developed by Balasuriya et al. in 2002 reached 102 copies of EAV genome, the exact same result was obtained for primer set #6bis targeting ORF1. Serial dilution, range from 100 to 0 TCID50, of Bucyrus virus strain in horse semen has been used. Method LOD is determined after 2 independent sessions with 4 replicates of each dilution. Method LOD is the smallest amount of doses where 100% (8 out of 8) of repetitions gave a positive result. In our hands, method LOD of the PCR targeting ORF7 developed by Balasuriya et al. in 2002 reached 20 doses (TCID50), the exact same result was obtained for primer set #6bis targeting ORF1

DISCUSSION AND CONCLUSIONS:
In conclusion, we were able to developed and validate a new qRT-PCR, targeting ORF1, for EVA diagnosis that can be used in addition or/and in place of the reference one

REFERENCES:
-Norme AFNOR XP U47-600 Juin 2011
DEVELOPMENT OF STABILIZED RT-QPCR ASSAYS FOR IDENTIFICATION OF RUMINANT PESTIVIRUS

Benito Zuñiga A.A., Arnal Bernal J.L., Serrano Muro J.D., García Manrique B., Pradas Osuna L.
EXOPOL ~ Zaragoza ~ Spain

Keywords: Stabilized, RT-qPCR, Pestivirus

INTRODUCTION:
Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) are considered relevant pathogens for livestock industries worldwide. These ruminant pestiviruses can cause significant economic losses, mainly by reproductive diseases but also by respiratory and enteric processes (1,2). RT-qPCR is currently accepted as a reliable diagnostic tool for several animal diseases (3), including pestivirus infections; however, its complexity and high relative prices have limited its use as a routine diagnostic test. In this study, we propose a novel technology to develop stabilized RT-qPCR assays for a rapid, simple and reliable diagnosis of ruminant pestivirus.

MATERIALS AND METHODS:
Stabilized RT-PCR: Three assays for specific detection of BVDV, BDV and pan-pestivirus were designed targeting 5’UTR region. An assay for β-actin gene was also included as endogenous control (EC) in each reaction. A patented technology (ES 2180416) was used to stabilize all reagents of the RT-qPCR, including RT enzyme, polymerase, primers and probes for pathogen (FAM) and EC (VIC), into a 96-well PCR plate. After that, plates were stored at 4°C for 6 months before use. A synthetic positive control (PC) was designed for each assay.

Validation of assays: 3 BVDV vaccine strains, 10 isolated field strains (8 BVDV, 2 BDV) and a specificity panel of 42 related pathogens were included. For BVDV assay, a concordance study was performed with 94 ruminant cases using a commercial kit (VetMAX BVDV; A.B.). Serial 10-fold dilutions of each PC were tested by triplicate to obtain the linear range, limits of detection (LOD) and coefficient of variation (CV).

Evaluation of clinical cases: 315 bovine, 86 sheep and 25 goats; with reproductive (63%), respiratory (12%) or digestive (26%) process. Samples included placenta, fetal tissues, lung, reproductive and digestive swabs, intestine and feces. RNA extraction and setup: RNA was extracted using an automatic device and a commercial kit (Taigen Co.). Setup for stabilized assays only needs addition of 15µl of DEPC water and 5µl of RNA sample into each well. PCR conditions were: 45°C 10min, 95°C 2min, and 40 cycles (95°C 5sec, 60°C 50sec).

RESULTS:
Analytical Specificity: The BVDV and BDV assays showed specific detection of respective strains; all of these strains were also positive for the pan-pestivirus RT-qPCR. By the other hand, none of 42 pathogens in the specificity panel resulted positive.

Linear range and analytical sensitivity: Linearity experiments showed a dynamic range from 102 to 109 copies/rxn, R2 values ranged from 0.94 to 0.99 and slopes were from -3.12 to -3.49. LOD resulted 1.5x102, 1.9x102 and 1.2x102 copies/reaction for BVDV, BDV and pan-pestivirus assays respectively.

Precision: In intra-assays tests, CV ranged from 1.3% to 20.6%, obtaining higher CV in the lowest copy number dilution (102 copies/reaction).

Concordance study: A high concordance level (κ= 0.956) was obtained between BVDV assay and the commercial kit. One goat resulted BVDV positive for the commercial kit but negative in our BVDV assay; moreover, this case was positive for pan-pestivirus and BDV assay.

Evaluation of clinical cases: BVDV was not detected in goats (0/25) or sheep (0/36). In cattle BVDV was identified in 7% (22/315) of samples, mainly in respiratory process (8/47). Furthermore, BDV was not found in cattle (0/25) but it was detected in 1,2% (1/86) of sheep and 12,0% (3/25) of goats. All negatives samples were positive for EC.

DISCUSSION AND CONCLUSIONS:
An accurate detection and differentiation of ruminant pestiviruses, is a key factor to implement effective control measures in infected herds (4). RT-qPCR is a powerful tool for health animal diagnosis, however, its complexity and costs have limited its use in many laboratories. This study describes a novel stabilized RT-qPCR format that solved these inconveniences. In addition, these assays probed to be a specific tool for identification of BVDV, BDV and pan-pestivirus in different samples including several tissues, swabs and feces.

REFERENCES:
2. S. Vilcek; PF Nettleton. 2006. Veterinary Microbiology 116:1–12
VALIDATION OF A REAL-TIME RT-PCR FOR THE DETECTION OF EQUINE VIRAL ARTERITIS (EVA) VIRUS IN HORSE SPERM

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Keywords: equine viral arteritis, real-time PCR, semen

INTRODUCTION:
EAV is a contagious viral disease of equids caused by equine arteritis virus, an RNA virus. Although infrequently reported in the past, number of EVA outbreaks seem to have increased over the last years. For international trade virus isolation (VI) from semen is the prescribed test. However, real time RT-PCR assays on semen are at least equally sensitive, less expensive and more rapid to perform. The aim of this project was to develop a sensitive and reliable real-time PCR test for the detection of EAV nucleic acid in semen.

MATERIALS AND METHODS:
Sperm samples are diluted 1:10 in FCS containing 1% penicillin/ streptomycin and 1% fungizone prior to isolation of RNA using the MagNaPure isolation robot (Roche). The EAVorf7 region was detected using the primer/probe set depicted in table 1. Briefly reactions were done in 20µl total volume consisting of the appropriate buffers using the following cycling conditions: RT at 61°C for 30 min followed by an initial denaturation step at 95°C for 3 min followed by 45 cycles at 94°C, 1 min; 45°C, 1 min; 72°C, 1 min and a final extension phase at 72°C for 4 min.

Table 1. EAVorf7 primers and probe

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>EAVorf7f (forward primer)</td>
<td>5’ – CAACCCACTCAGGCTATYAT – 3’</td>
</tr>
<tr>
<td>EAVorf7r (reverse primer)</td>
<td>5’ – TGRATTTGTTTGGTAGGAAC – 3’</td>
</tr>
<tr>
<td>EAVorf7taq2 (probe)</td>
<td>5’ – 6FAM-TCATGCCCTTAGGCTCCAGGCT–TMR</td>
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RESULTS:
Limit of detection (analytical sensitivity):
To test for the detection limit of the PCR test dilutions of ring trail samples were compared. Results show that the PCR test is almost 10 times more sensitive than the virus isolation on RK13 cells.

In silico selectivity (analytical specificity):
The selectivity of the PCR test was determined in silico (BLAST search) as well as in vivo. The phylogenetic most related PRRS virus was not detected with the PCR test.

Repeatability:
To determine the repeatability of the PCR test Ct values of the weak positive control (WPC) which is present in each individual test were analysed. Over a period of two years 46 PCR tests were performed. The mean Ct value of the WPC was, 35.1 with an SD of 1.13 and a variance of 1.24.

Diagnostic sensitivity and specificity:
255 defined negative EAV samples and 9 samples that tested positive in the virus isolation were tested with the PCR test. All 9 positive samples were confirmed whereas in 5 negative samples EAV RNA was detected. Taking into account that the PCR test has a lower detection limit than the virus isolation, the diagnostic specificity and sensitivity of the PCR test are 100%.

DISCUSSION AND CONCLUSIONS:
A highly robust, sensitive and specific real-time RT-PCR for the detection of EAV in horse semen was developed. The PCR test is almost ten times more sensitive in comparison to the OIE prescribed virus isolation test and takes only three hours to perform. In ring trails organised by the EU reference laboratory the PCR test scores 100%.

REFERENCES:
EVALUATION OF DIAGNOSTIC PERFORMANCE OF INFLUENZA A AND SUBTYPE-SPECIFIC REAL-TIME RT-PCRS FOR DETECTION OF SWINE INFLUENZA VIRUSES IN FINISHING HERDS WITH ACUTE RESPIRATORY CLINICAL SIGNS

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Keywords: SIV, PCR, Diagnosis, Subtyping

INTRODUCTION:
Swine influenza is an acute respiratory disease of swine caused by type A influenza viruses. In Europe, swine influenza is considered one of the most important primary pathogens of swine respiratory disease and infection is primarily with H1N1, H1N2 and H3N2 influenza A viruses. For diagnosis of swine influenza infections serological and virological tests can be used. In this study we evaluated the relative diagnostic performance of three different real-time influenza A RT-PCRs and two subtype-specific RT-PCRs for oral fluids, pooled nasal swabs and individual nasal swabs as compared with paired serology in finishing herds with acute respiratory signs suspected from SIV infection.

MATERIALS AND METHODS:
Eight herds were included in this study, three or four pens per herd were sampled. Nasal swabs of all pigs were collected with plain cotton swabs. Also blood samples were collected and five acutely diseased pigs per pen were earmarked for collection of a second blood sample 3–4 weeks later. During the first and second visit in each pen a chewing rope was placed according to standard procedures. All samples were transported cooled overnight by courier service to the laboratory.

Pooled swab extracts, oral fluids and a selection of individual swab extracts were tested using the same RNA extraction procedure. RNA extracts were tested for the presence of influenza A viral RNA using two commercially available and one in-house influenza A PCR, and were also tested with SIV Subtype Specific RT-PCRs consisting of H1H3 duplex primer-probe-mixes and N1N2 duplex primer-probe-mixes.

RESULTS:
The results show a good qualitative and quantitative agreement for pooled and for individual nasal swabs between the three influenza A RT-PCR tests evaluated in this study. Internal controls of all tests were within limits for pooled and individual nasal swab extracts, indicating no issues with inhibition. For the five PCR positive herds the percentage of positive pooled swabs and individual swabs varied between 25% and 100%, and between 20% and 100%, respectively. Also oral fluids collected at the first sampling showed good diagnostic performance on a pen level with for the five PCR positive herds 50 - 100% positive results. Two completely PCR negative herds clearly showed serological proof of H1N1 infection. The diagnostic performance of the subtype-specific PCRs was overall good. SIV infections in three herds were clearly subtyped as H1N1, as confirmed by serology. SIV infection in one herd was clearly subtyped as H3N2, whereas in another herd SIV infection was clearly subtyped as H3 with a question mark for the neuraminidase. The serology and phylogenetic analysis clearly confirmed that this was also a SIV H3N2 infection.

Six out of seven SIV positive herds showed serological proof of infection with at least 40% of the pigs seroconverting or showing a significant increase in titre. One PCR positive herd failed to seroconvert.

DISCUSSION AND CONCLUSIONS:
In this study the usefulness of molecular diagnosis of SIV infections is clearly demonstrated. All influenza A PCR tests and the subtyping real-time PCR test showed good diagnostic performance and can be used for testing pooled or individual nasal swabs and oral fluids. The results of this study underline that for molecular and virological diagnosis sampling in the acute phase of disease, i.e. in the first few days after the first clinical signs, is of utmost importance. When sampling is done relatively late, also acute phase serum samples should be collected. When all samples are negative in the influenza A PCR it should be considered to collect convalescent phase serum samples and test paired serum samples to confirm or exclude SIV infection in the herd.

REFERENCES:
VALIDATION OF THE ID SCREEN FMD TYPE O COMPETITIVE ELISA

Pourquier P., Comtet L., Malzac M., Roche M., Robles-Félicité L., Lecoq A.

IDvet ~ Montpellier ~ France

Keywords: FMD TYPE O, SEROLOGY, ELISA

INTRODUCTION:
The ID Screen® FMD Type O Competition ELISA specifically detects antibodies against FMDV serotype O. It can be used with serum or plasma from cattle, swine or other susceptible species. This study summarizes validation data obtained for this ELISA.

MATERIALS AND METHODS:
The ID Screen FMD Type O cELISA was used as per manufacturer’s instructions. The test makes use of inactivated FMDV type O coated antigen and an anti-serotype O-HRP concentrated conjugate.

RESULTS:
ANALYTICAL SENSITIVITY: 8 bovine sera from Argentina, vaccinated with a trivalent FMD vaccine (serotype O, A and Asia 1), were serially-diluted and tested in parallel using the ID Screen® ELISA and commercial Kit A. The ID Screen® ELISA showed equivalent or superior analytical sensitivity to Kit A.

EXCLUSIVITY: 6 FAO / IAEA positive bovine sera from cattle infected with different serotypes and 11 reference sera for different serotypes / strains from the Pirbright Institute were tested in parallel using the ID Screen® ELISA and Kit A. The ID Screen® ELISA correctly identified the serotype O sera as expected. No cross-reactions with other serotypes were observed for these panels, except for the SAT1 serum, which was found positive by both kits.

SPECIFICITY: The following sera from disease-free, certified herds were tested:
- 248 naïve cattle sera (France); measured specificity: 100% (CI 95%: 98.47 - 100%);
- 263 naïve goat sera (France); measured specificity: 99.62% (CI 95%: 97.88 - 99.93%);
- 210 naïve sheep sera (France); measured specificity: 99.05 % (CI 95%: 96.6 - 99.74 %);
- 248 pig sera (France) and 152 Iberian pig sera (Spain); measured specificity = 100% (CI 95%: 99.05-100%).

COMPARATIVE SPECIFICITY: 32 swine sera and 32 bovine sera from disease-free, certified French herds were tested in parallel using the ID Screen® ELISA and Kit A.
- Measured specificity for the ID Screen® ELISA= 100% (CI 95%: 94.34-100%).
- Measured specificity for Kit A= 98.44% (CI 95%: 91.67-99.72%).

DISCUSSION AND CONCLUSIONS:
The ID Screen® FMD Type O cELISA shows excellent test performance. The test is particularly rapid and easy-to-use. All reagents are supplied ready-to-use (not freeze-dried), and results are obtained in 90 minutes. External validation work has been performed on samples from infected regions (data available upon request). IDvet welcomes propositions for collaborative validation work on this disease.

REFERENCES:
INTRODUCTION:
A new variant of Rabbit Hemorrhagic Disease Virus (RHDVb) has been widely detected in Spain since 2011. Vaccination against classic RHDV strain might be ineffective in terms of protection and fatal consequences are often presented in animals as young as 11 days. Hence, the need of a rapid, specific and sensitive diagnostic technique is evident. RT-qPCR is an updated tool which detects specifically new variant RHDV strain. We proposed to design, develop and validate a RT-qPCR for detecting and quantifying RHDVb.

MATERIALS AND METHODS:
Sixty six VP60 gene sequences of RHDVb facilitated by UniOvi were aligned to select primers and probe which detect specifically this virus. Due to the duplex format of the assay, RHDVb (FAM) and an endogenous control (EC) (HEX) were simultaneously detected in every well. A synthetic ssDNA which contains the target sequence was designed as positive control (uRHDvar).

RNA isolation from liver was carried out in an automatic extraction device following its respective instructions manual. RT-qPCR was set up in 20 µL with 5x102 nM of each primer, 2.5 x102 nM of probe as well as Precision OneStep qRT-PCR MasterMix (2X) from PrimerDesign (United Kingdom). Thermal profile consisted in a reverse transcription step followed by an enzyme activation step and 42 cycles of amplification. Results were analyzed considering as positive samples those whose Cq value was lower than 38.

UniOvi provided a panel of samples determined as RHDVb by sequencing. This validation panel included 103 samples and consisted of 23 samples of classic strain RHDV, 69 samples of RHDVb strains, 2 samples of type A variant RHDV strain and 9 RHDV negative samples. Specificity test was performed with a group of 22 pathogens including main bacteria, virus and parasites which affect rabbits or are supposed to be genetically related to RHDVb.

Moreover, 61 clinical samples suspected of RHD and submitted from 14 different Spanish provinces and Portugal were analyzed. Liver samples were firstly analyzed by antigen capture double antibody sandwich ELISA INgezim RHDV, which detects either RHDV classic strains or RHDV variant strains. After that, RNA from livers was isolated and analyzed by RHDVb RT-qPCR.

Repeatability intra assay test was performed using ten-fold dilutions of uRHDvar. For that purpose, a standard curve of quantification was set up with 7 dilutions with their respective 3 replicates. Linear range, coefficient of variation range (CV) and lower limit of detection (LOD) were determined.

RESULTS:
Analyzing the validation panel, every primary classified as positive sample resulted RHDVb RT-qPCR positive. 33 samples resulted negative. EC amplification was observed in 100% of samples. RHDV classic strains and type A variant RHDV strains resulted negative by RHDVb RT-qPCR. Kappa value was 0.978. None of the pathogens tested in specificity panel resulted positive. Statistical parameters were: slope=-3.44, efficiency=95.18% and R2=0.99. Linear range resulted from 1.2x1010 copies/rxn to 1.2x102 copies/rxn. CV values ranged from 0.23% to 12.77%. LOD observed was 1.2x102 copies/rxn.

38 clinical samples resulted positive by ELISA whereas 23 resulted negative. On the contrary, RT-qPCR resulted positive in 42 samples and negative in 19 samples. Samples ranged from 1.12x109 copies/
**INTRODUCTION:**

Feline Coronavirus (FCoV) is a common viral infection in cats. FCoV is a member of the family Coronaviridae, a group of enveloped, positive-stranded RNA viruses. FCoV has been divided into two biotypes, known as feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) [1]. FECV is often being asymptomatic or causing mild enteric infectious. This biotype remains confined to the digestive tract and not to spread beyond the intestinal epithelium and regional lymph nodes. The second biotype FIPV infects blood monocytes and tissue macrophages and causes Feline infectious Peritonitis (FIP) - a highly fatal immune-mediated condition in cats. The pathogenesis of FIP is still poorly understood. FIPV is considered to be a result of genetic mutation of FECV, which is believed to occur in about 1-5% of FCoV infected cats. Antibodies against FCoVs are found in 50–90% of the animals. At present there is no serological test available to distinguish FECV and FIPV and a definite diagnosis of FIP can be established only by histopathologic examination of biopsy or postmortem material. Compared with serology reverse transcriptase PCR (RT-PCR) provides the advantage of direct detection of FCoV RNA in blood, tissues and feces of infected cats and differentiation of FIPV infected cats and asymptomatic FCoV shedding based on virus location.

**MATERIALS AND METHODS:**

Material submitted for the PCR detection of FCoV included blood, ascitic fluid samples and feces from naturally infected diseased and healthy cats. FIPV strain Bagira was obtained from infected Crandell feline kidney (CrFK) cells. Fecal samples were suspended 1:3 (vol/vol) in PBS and homogenized by vortexing. Insoluble components were pelleted for 3 min at 3000 rpm, and 100 ml of the supernatant fraction used directly in the RNA extraction. Plasma was collected from EDTA-blood. Viral RNA from 50-100 ml of plasma or ascitic fluid was extracted using Ribo-prep kit. Reverse transcriptase (RT) reactions were made using Reverta-L kit (AmpliSens, Russia). The oligonucleotide primers and TaqMan probe were chosen from the 7b gene sequence of FCoV. A BLAST search at NCBI site (http://www.ncbi.nlm.nih.gov) was performed using blastn algorithm for calculating sequence similarity. Optimization of the critical parameters of the PCR for the FCoV detection was performed, including concentration of reagents and PCR cycling parameters. The 25 ml reaction mixture contained 10 ml cDNA, 0.5mM each primer, 0.3mM probe, RT-PCR-mix and 2.5 units of TaqDNA polymerase (AmpliSens, Russia). Real-time PCR was performed using a Rotor Gene Q cycler (Corbett Research). The sensitivity and specificity of assays were analysed.

**RESULTS:**

To determine the sensitivity of PCR serial dilutions of known concentrations of strain Bagira with a titer of 5.0 lgTCID50/mL were assayed. The analytical sensitivity of the assay was observed to be 0.1 lgTCID50/mL. The 7b Real-time PCR was more sensitive than conventional N-gene PCR used previously. The viral genomes of a related coronaviruses and other feline infectious agents as follow: FCV, FHV, FeLV, FIV, FPV, Mycoplasma haemofelis were assayed giving amplification signal only for FCoV and Canine COV. A total number of 7411 cat samples were analyzed in 2011-2013, 3387 were RT-PCR positive for FCoV. FECV biotype was found in 40% samples. Viral RNA was detected in about 5.7% of blood and ascitic fluid samples from naturally infected cats in Moscow region (Russia). We found that total amount of FIPV positive samples was decreased from 17.8% in 2006 to 5.3% in 2013.

**DISCUSSION AND CONCLUSIONS:**

We have developed an RT-PCR assay for the detection of coronavirus RNA in plasma, ascitic fluid samples and feces of infected cats. The detection of FCoV in the feces of healthy cats provides direct evidence for virus shedding by asymptomatic carriers, detection of viral RNA in ascitic fluid samples can be used to establish a definite diagnosis of FIP.

**REFERENCES:**

INTRODUCTION:
Pigs are susceptible not only to swine influenza but also to human and avian influenza viruses [1]. In Sweden the classical H1N1 swine influenza was first reported in the spring of 1983, causing severe clinical signs in pigs of all ages. Serological survey in 2003, 2006 and 2010, indicating the circulation of swine influenza based on detection of antibodies against classical swine H1N1, avian like H1N2 and H3N2 swine influenza viruses in pig populations in Sweden [2]. Since the first report of the detection of pandemic influenza A (pH1N1)pdm09 in early May 2009 in pigs in Canada [3], pH1N1pdm09 has been isolated from pigs throughout the world including several European countries such as Germany, Italy, Denmark, Norway, Island and Finland [4,5]. The A(H1)pdm09 virus was detected in Swedish pigs for the first time in May 2013.

MATERIALS AND METHODS:
The surveillance program will target the following swine populations: Active surveillance- In collaboration with farmer’s association, ten field veterinarians who agreed to participate in the study were asked to select ten pig farms that were representative of the pig production systems in Sweden and that were owned by producers interested in participating in the study. From first week of February 2014, participating farms were visited every second week for 6 consecutive visits by the field veterinarian. A total of 15 nasal swab samples were collected at each visit. During the visit, the age of the pigs and any respiratory clinical signs (absence or presence of sneezing, coughing and nasal secretion) among the sampled individuals were recorded. The nasal swabs and submission sheets were shipped overnight to the laboratory for testing. Nasal swab samples were initially being screened for influenza A virus by real-time reverse transcription PCR(rRT-PCR) selective for the matrix gene. Samples that shown to be positive by rRT-PCR were further analyzed for determination of subtype, including the influenza A(H1N1)pdm09 virus using specific rRT-PCR specific for heamagglutinin gene of influenza A(H1N1)pdm09 virus. The heamagglutinin and neuraminidase fragments from all positive swine isolates were sequenced by the Sanger sequencing method.

RESULTS:
Active surveillance- From first week of February to third week of April 2014, participating farms were visited six times by the field veterinarian. No clinical signs of disease were observed during these visits. From the total number(800) of samples collected, 74 (9%) were positive for influenza A viruses with rRT-PCR. Sixty nine (93%) of influenza A positive samples were also positive for pH1. Out of ten participating farms five farms had at least one positive result during this period and four farms were tested positive in at least two occasions. The molecular characterization and phylogenetic analysis of the newly isolated viruses revealed that they possess pandemic H1N1 lineage HA and internal genes. However the NA gene was closely related to H1N2 SIV strains, previously isolated in Swedish pig population. So far a total of 90 human samples have been submitted for analysis with no positive result.

DISCUSSION AND CONCLUSIONS:
In last five years two new influenza A viruses were detected in the Swedish swine population. Both of these viruses were the result of multiple reassessments between avian or/and human and swine influenza A viruses. In the present study the absence of any clinical signs in examined pig farms with pigs harboring influenza A virus is highly notable.

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INTRODUCTION:
Infectious bursal disease (IBD) is a highly contagious disease, caused by an Avibirnavirus (family Birnaviridae). It is a non-enveloped double stranded bi-segmented RNA virus, called the infectious bursal disease virus (IBDV). Chicken and turkeys are its natural hosts. The virus targets immature B lymphocytes, in which it has a cytolysis action. Although morbidity reaches up to 100%, the mortality varies widely, from 1-2% to almost 50% for hypervirulent strains, and peaking and receding within 5 to 7 days. Beside the virulence of the strains, clinic depends also on different parameters, e.g., the age, the breed, and the level of passive immunity of the birds. Below 3 weeks of age, the virus leads to immunodepression and subclinical disease. In older chicken, animals may display exhaustion, watery diarrhoea, and prostration. After its first description in 1962 in the USA (1), IBDV was also observed in Europe and was reported in Switzerland in 1967 for the first time (2). In 1996, the last Swiss cases were announced to the OIE. Since then no IBDV routine vaccination was done until its re-emergence at the end of 2013.

MATERIALS AND METHODS:
In November 2013, mortality with unspecific clinical signs were observed in a broiler flock (flock size: 16000 animals, breed JA 987, 35 days old, vaccinated parental line). The 12th November, 3 birds (2 sacrificed and 1 dead, lot 1) were presented for necropsy at the Institute Galli-Valerio (IGV; Lausanne, Switzerland). The day after, 200 animals died and 10 were collected for further analysis (4 dead and 6 sacrificed, lot 2). One of the neighbour farms, about 400 m distant, announced an increased mortality in 41 days old broiler. Eight animals out of 3200 died on the 17th, followed the day after by 150. Ten of them (lot 3) were collected and send for further analysis. Both farms received chicks (same breed) and feed from the same provider. They also shared transport boxes. Necropsy, bacteriology, parasitology, and histology were performed at the IGV. Cloacal and trachea swabs were collected from lot 2 and analysed by real-time PCR for avian influenza and Newcastle disease at the Institute of Veterinary Bacteriology (Zurich, Switzerland).

RESULTS:
Gross pathology of the lot 1 did not reveal particular sign. Despite a lack of lesions, cultures indicated the marked presence of Clostridium perfringens in the small intestines. For this lot, no material was collected for histology. Real-time PCR for avian influenza and Newcastle disease was negative for the lot 2. Although necropsy of lot 2 and 3 was as inconclusive as the one for the first lot, histology of the cloacal bursa was highly suggestive of infectious bursal disease: a marked oedema and important necrosis herds in lymphonodules, combined with infiltration of heterophile granulocytes were present in each broiler.

DISCUSSION AND CONCLUSIONS:
In those two cases, several factors made the diagnosis challenging; the disease was considered as absent in Switzerland, the clinical signs are unspecific and the gross pathology inconclusive. As other frequent possibilities were ruled out (bacterial or parasitic diseases, management problems), the hypothesis of an easily contagious viral disease was closely examined by using the least expensive method (wish of our customer), histology.
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SURVEILLANCE OF TICK BORNE ENCEPHALITIS AND OTHER TICK BORNE DISEASES IN UMBRIA REGION

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Keywords: TBDs (Tick Borne Diseases), TBE (Tick Borne Encephalitis), surveillance

INTRODUCTION:
Ticks can transmit viruses, bacteria and protozoa playing an important role in the epidemiology of tick borne diseases (TBDs). Because the ticks are reliable indicators for the existence of pathogens in a specific area, it is recommended to periodically screen animals and vectors for pathogen carriage. Surveillance of TBDs occurrence is required to map local risk, to know the prevalence of pathogens and the emergence of new infectious agents.

The aim of the present study was:
1) to get data on TBE infection in wildlife and domestic animals (sheep) in Umbria
2) to verify the TBE flavivirus presence in Ixodes spp. ticks
3) to detect the presence of other emerging pathogens in all the collected ticks

MATERIALS AND METHODS:
The study was conducted in the year 2012. Ticks were collected directly from the skin of the animals during necropsy or into the farms. Environmental sampling was done by dragging. Once collected, ticks have been identified by species and life stage. DNA and RNA were extracted with “AllPrep DNA/RNA mini kit” (Qiagen®). Ticks were examined individually or pooled (5-6 ticks belonging at the same species and from the same host/area). Several and specific PCR methods were applied in order to detect the following infectious agents: TBE Flavivirus (1), Rickettsia spp. (2), Anaplasma phagocytophilum (3), Babesia spp. (4), Borrelia spp. (5), Francisella spp. (6), Coxiella burnetii (7).

Serum samples from 390 sheep, randomly collected from 130 farms, were analysed to detect antibodies against TBE virus, by a c-ELISA (EIA TBEV Ig, TEST-LINE Clinical Diagnostics, Czech Republic). In addition, 211 sera collected from 141 wild boars, 66 roe deers and 4 fallow deers, were also analysed.

RESULTS:
A total of 505 ticks was collected. Six different species were identified: 207 Rhipicephalus sanguineus (40,9%), 105 Hyalomma marginatum (20,8%), 78 Ixodes ricinus (15,4%), 68 Dermacentor marginatus (13,5%) 46 Rhipicephalus bursa (9,1%), 1 Ixodes hexagonus (0,2%).

All PCR tests resulted negative for TBE virus in Ixodes spp. The seroprevalence for TBE virus was 0,8% (I.C.95%: 0,2% - 2,4%) in sheep population whereas the wild animals resulted negative.

PCR tests and DNA sequences performed in ticks detected several pathogens as listed in the table 1 and 2.

DISCUSSION AND CONCLUSIONS:
The low seroprevalence for TBE in sheep and the absence of antibodies in wildlife sera, confirmed the non endemic status of the Umbria Region for TBE. The limited number of ticks/animals submitted to laboratory tests, could affect any speculation on the true epidemiological situation in this central part of Italy. However we believe that these results represent a first step towards TBDs surveillance in our region. Up to few years ago it was thought that the only tick-borne rickettsiosis in Europe was the Mediterranean spotted fever (MSF) caused by Rickettsia conorii. In the last decade new rickettsial agents have been identified and the current opinion is that human rickettsial infections are supported by several species of Rickettsia and by different ticks genera, as also confirmed by the results of our study. Borrelia and Anaplasma are also circulating in ticks of our region however the clinicians fail to report cases of disease; this encourages us to enhance our surveillance activities.

REFERENCES:
DIARRHEA IN AUSTRIAN PIGLETS: VIRAL AND HISTOLOGICAL FINDINGS


Keywords: porcine diarrhea, virus, diagnosis, pathohistology

INTRODUCTION:
Diarrhea in pigs is a complex problem resulting from interaction between infective agents, host immunity and management procedures. It causes considerable economic loss to the pig production, especially in suckling and weaner pigs. Bacteria are often assumed to be the primary causative agents of diarrhea; tests for viral infections are in general less initiated or, like in many recent publications from American and Asian investigators, concentrate on the occurrence of porcine epidemic diarrhoe virus (PEDV) or Swine Deltacononavirus (SDCV). In Europe, the most prevalent agents of diarrhea in young pigs are enterotoxigenic E. coli, Cl. perfringens, Coccidia but also rotavirus. Since 2006 infections with PEDV became less frequent in Europe (report EU Commission 13/05/2014). In Austria, several recent investigations aimed to determine the occurrence of single pathogen outbreaks, but studies on mixed infections are rare or either reported many years ago. This part of the study concentrates on viral pathogens. Identification of the prevalent viruses in combination with other pathogens will help us to improve our understanding of the epidemiology of diarrhea, as well as providing information about the most appropriate therapies or vaccination.

MATERIALS AND METHODS:
In this study different parts of the gastrointestinal tract (stomach (pars glandularis), duodenum, jejunum, ileum, colon and rectum) of 55 diarrheic pigs of different age from 19 commercial farms in Styria/Austria were submitted for diagnosis. Virological diagnosis was performed by electron microscopy (TEM, negative staining) because of its “open view”; samples were: different digestive tract contents and tissue suspensions. Digestive tract samples were also investigated by means of routine histology (H&E) and immunohistochemistry (ABC-technique) for rotavirus. A monoclonal antibody raised against recombinant rotavirus capsid 2B4 (Fa. Santa Cruz Biotechnology Inc./ USA) was used. PCR was performed to exclude Hepatitis E virus and to determine PCV1 and PCV2.

RESULTS:
The occurrence of viruses was proven in 80% of the diarrheic animals. 63.6% of the virus positive animals were infected with one virus type, 36.4% with more than one virus type. Corona- and rotavirus predominated. Presence of coronavirus was dominant in 45.5% of the sampled virus positive animals followed by rotavirus (in 40.9%). In two diarrheic suckling pigs a high amount of circovirus could be detected and confirmed by PCR as PCV1-type and PCV2-type. Caliciviruses were detected in 16.6% of the infected animals. Caliciviruses belonged to the noro- or sapovirus group because Hepatitis E virus was excluded via PCR. No other virus type (e.g. entero-, toro-, adeno- and parvovirus) was detected in our samples. In the animals with coinfection the combination rota-/coronavirus was dominant, followed by rota-/ circovirus, corona-/calicivirus and rota-/calicivirus. Virus concentration was high in colon, moderate in rectum and low in small intestine. Histology gave information on the relevance of detected bacteria (haemolytic and non-haemolytic E. coli, Clostridium perfringens A and Cl. difficile) and viruses for the acute disease process. Intestinal lesions from diarrheic pigs could be grouped in four different types (A, B, C, D) by reason of histological examination. Mixed types were also present.

DISCUSSION AND CONCLUSIONS:
Rotavirus was found more often in suckling pigs whereas coronavirus was detected both in suckling, weaner and grower pigs. Pathogenic bacteria were detected in all cases of diarrhea with virus incidence. Therefore histological lesions induced by viruses or pathogenic bacteria were often detected simultaneously in the same sample. Tissue lesions typical for virus infections were shortening and fusion of intestinal villi. Viral lesions dominated in infected weaner and grower pigs whereas bacterial lesions were more often found in suckling pigs. Pig holders must be aware that in porcine health management diarrhea in piglets is a complex infection process which demands appropriate management techniques.

REFERENCES:
STABILITY OF ANTIBIOTIC RESIDUES IN INCURRED MEAT SAMPLES DURING FROZEN STORAGE

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Keywords: drug residues, stability, incurred samples

INTRODUCTION:

Penicillins (PEs), Sulfonamides (SAs) and Tetracyclines (TETs) are antibiotics commonly used in veterinary medicine for treatment of bacterial infections in various food-producing animals. Their use is allowed by the EU legislation but this may give rise to residues in meat products. European regulation (EC) N.37/2010 established the maximum residue limits (MRLs) at different values in various animal origin matrices. Decision 98/179/EC lays down detailed rules on official sampling for the monitoring of certain substances and residues thereof in live animals and animal products by an Official Residue Control Program. When samples analysed within the program contain concentrations of antibiotics that exceed their MRLs, an official legal proceeding starts and this may require that samples are retested even after months or years, therefore it is necessary to store them under frozen conditions (-24 ± 6°C) for long time. Consequently, in order to guarantee the reliability of the results, it is of fundamental importance to assess the stability of the residues over time. Here, we investigated the stability of Penicillins, Sulfonamides and Tetracyclines in incurred bovine and swine muscle samples stored at frozen temperature (-24 ± 6°C) within a period of 6 months to 1 year.

MATERIALS AND METHODS:

The analytical procedure involved the following steps: a) homogenization and weighing of samples (10 g for SAs and 5 g for TETs and PEs); b) extraction with solvent (ethyl acetate for SAs, succinic acid 0.1M/Methanol for TETs and water for PEs); c) purification by Solid Phase Extraction (SPE) cartridges (SCX 150mg/3mL for SAs, Chelating Sepharose tandem OASIS HLB 3 mL/60 mg for TETs and ENV+ 50 mg/3 mL for PEs); d) derivation for PEs; e) liquid chromatography (LC) analysis. The LC system consisted of a Hewlett-Packard (HP) 1100 Serier Quaternary Pump, a HP 1100 Serier DAD detector, a HP1100 Serier autosampler all controlled by a HP computer using HP chemstation software. We selected six meat samples that, within the national Official Residue Control Program 2013, were found to contain concentrations above MRLs for one out of six different antibiotics, namely Ampicillin, Benzylpenicillin, Sulfadimethoxine, Sulfapyridine, Oxytetracycline or Doxycyline. The material was homogenized at room-temperature, stored frozen at -24 ± 6°C and analysed in replicate by liquid chromatography on 6 sampling dates over a period of about 6 month for Benzylpenicillin and Doxycyline or about one year for the remaining analytes.

RESULTS:

We show that Benzylpenicillin and Ampicillin are not stable in meat samples stored at frozen temperature: a decrease greater than 50% for Benzylpenicillin and Ampicillin was detected after one month and 6 months respectively. On the contrary, concentration of Sulfadimethoxine decreases very slowly within one year with a reduction of 10%, while the concentration of Sulfapyridine decreases about of 40% within one year. Oxytetracycline is stable within one year while Doxyxycline concentration decreased about of 20% after 6 months.

DISCUSSION AND CONCLUSIONS:

Our results show that Penicillins are unstable in incurred meat samples, and are in agreement with previous reports1,2. Therefore, very low freezing temperatures (-75°C) should be tested as a possible optimal storage for penicillin containing samples. On the contrary, Tetracyclines are more resistant to frozen storage. Stability of Sulfonamides depends on the specific analyte. It will be interesting to establish if any of the sample preparation steps play a role in the subsequent stability of the analytes, i.e. the grinding step, which as previously discussed1 seems to protect analytes desactivating degrading actions of endogeneous compounds in the bulk matrix.

REFERENCES:

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DETERMINATION AND PREVALENCE OF CANINE ROTAVIRUS INFECTION IN DOGS

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Keywords: Canine rotavirus, ELISA, Diarrhea

INTRODUCTION:
Canine Rotavirus (CRV) is the essential cause of neonatal puppy diarrhea. The aim of the present study was to determine serological prevalence and CRV antigen in diarrheic dogs of Konya district.

MATERIALS AND METHODS:
A total of 104 dog sera and feces samples were collected in order to investigate the presence of antibodies against CRV and CRV antigens from Konya in Central Anatolia of Turkey between September 2013 to March 2014. All sampled animals selected by using randomly methods. Sampled dogs were unvaccinated against CRV. The sera samples were controlled for the antibodies against CRV by a commercially available indirect enzyme linked immunosorbent assay (ELISA). Feces samples were examined for CRV antigens by presence of CRV antigens commercially rapid test kit.

RESULTS:
Specific antibodies were detected in 91(77.11\%) sera samples of 104 dogs while there were two animals positive for CRV antigens.

DISCUSSION AND CONCLUSIONS:
It was obtained 77.11\% seropositivity and concludes that this high rate was due to sampling dogs carrying a high risk of CRV infection. This is the first serological survey for CRV antibodies performed on dogs in Konya. The results of this study suggest that CRV infections may play a role in causing digestive discharging. In addition the etiology of diarrheic viral disease of dogs needs further attention.

REFERENCES:
DETECTION OF EQUINE HERPES VIRUS 1, EQUINE HERPES VIRUS 4, AND EQUINE ARTERITIS VIRUS ANTIbODIES IN KYRGYZSTAN BY ELISA

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Keywords: Equine herpes virus 1, Equine herpes virus 4, Kyrgyzstan

INTRODUCTION:
Equine Herpes virus 1 (EHV-1) and Equine Herpes virus 4 (EHV-4) are the member of the subfamily alphaherpesvirinae in the family Herpesviridae. These pathogens are the important viral pathogens of horses worldwide. EHV-1 and EHV-4 can be diagnosed by using serological tests such as type specific enzyme linked immunosorbent assay (ELISA), serum neutralization and complement fixation. The aim of the present study was to estimate the existence of Equine herpes virus 1 (EHV-1), Equine herpes virus 4 (EHV-4), and Equine Arteritis Virus (EAV) antibodies in domestic horses in Kyrgyzstan.

MATERIALS AND METHODS:
In this study, 116 blood sera samples of unvaccinated domestic horses were tested for equine herpes virus (EHV-1, EHV-4) and EAV specific antibodies by commercially available indirect Enzyme Linked Immunosorbent Assay (ELISA). A total of 116 blood serum samples were collected between November 2012 to February 2013. Antibodies to EHV-1 and EHV-4 in sera were detected using a commercially available ELISA test kits. The test was performed as per the manufacturer’s instructions.

RESULTS:
EHV-1, EHV-4, and EVA specific antibodies were detected as 81.89% (95/116), 96.55% (112/116), and 20.68% (24/116), respectively. 95 horses (81.89%) were detected positive for EHV-1 plus EHV-4. 20 horses (17.24%) were detected seropositive both EHV-1 and EVA while 22 horses (18.96%) were seropositive both EHV-4 and EVA. Only 2 (1.72%) horses were determined as seronegative and 22 (18.96%) horses were found to be seropositive all mentioned infections.

DISCUSSION AND CONCLUSIONS:
Different serological methods can be used for the determination of specific antibodies against EHV-1 and EHV-4 but it is not enough for distinguish two serotypes because of a strong antigenic cross-reactivity. ELISA is commonly used in routine diagnosis preferred other tests for its high sensitivity and its practical advantages. There is no information about horse viral infections in Kyrgyzstan. This study reveals that the horse population in the Kyrgyzstan consisted of 81.89% EHV-1, 96.55% EHV-4, and 20.68% EVA. Vaccination for these viruses has not been applied in Kyrgyzstan, so these seropositive results indicated that natural infections. The reason of high seroprevalence rates of this region can be explained that climatic extremes, suspected and clinically respiratory symptoms animals were sampled so investigated animals were potential risk for these infections. In conclusion, horses have a potential risk to other species. To our best knowledge, this is the first seroprevalence survey of horses infected by EHV-1, EHV-4 and EVA in Kyrgyzstan. These preliminary observations should be followed by a further large-scale survey to establish the extent of EHV-1, EHV-4 and EVA infections in Kyrgyzstan. Also, effective control and preventive measures must be taken for prevent the exposure and spread of mentioned infections.

REFERENCES:
DETECTION AND PRELIMINARY CHARACTERIZATION OF HEPATITIS E VIRUS (HEV) IN DOMESTIC PIGS FROM SLOVAKIA

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Keywords: hepatitis E virus, domestic pigs, phylogenetic analysis

INTRODUCTION:
Hepatitis E virus (HEV) is the causative agent of hepatitis E - an emerging zoonotic disease distributed worldwide. HEV is non-enveloped virus with single-stranded positive sense RNA genome approximately 7.2 kb composed of a short 5’ untranslated region (UTR), three partially overlapping open reading frames (ORFs) and a short 3’ end terminated by a poly(A) tail (Tam et al., 1991). ORF1 encodes non-structural polyprotein, ORF2 encodes the capsid protein and ORF3 encodes the cytoskeleton-associated phosphoprotein (Meng et al., 2002). The virus belonging to the family Hepeviridae, genus Hepevirus, is subdivided into four genotypes (King et al., 2012). Two of genotypes (HEV-1 and HEV-2) contain strains infecting only human population. The other two genotypes HEV-3 and HEV-4 can be isolated from humans, pigs and other animal's species (Schlauder and Mushahwar, 2001). The aim of this preliminary study was to genetically characterize HEV isolates first time detected in domestic pig farms from Slovakia.

MATERIALS AND METHODS:
Clinical samples (rectal swabs, n = 162) were obtained from five domestic pig farms in categories suckling, weaning and finishing. Some of them showed enteric problem associated with diarrhea, but majority was asymptomatic. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s manual. The cDNA was prepared using gene specific primer and SuperScript reverse transcriptase (Invitrogen). Nested RT-PCR according to Erker et al. (1999) was used to amplify a partial ORF1 region. Purified PCR products were sequenced using automatic sequencer ABI PRISM. The sequences from both strands of the PCR products were determined with the same primers as used for the nested PCR amplification. Partial ORF1 sequences (242 nt) were assembled using MEGA 4.0 software.

RESULTS:
The fragment of expected size was detected in 42 clinical samples (26 %). Positive samples were detected predominantly in finishing pigs. The nucleotide sequences of ten HEV isolates were aligned and compared to each other and to selected strains deposited in GenBank. The comparison of nucleotide sequences Slovak isolates to each other showed 81.0-100 % identity. By comparison of nucleotide sequences with neighbouring countries, Czech Republic and Hungary, was observed identity 81.8-86.8% and 80.6-86.8%, respectively. Phylogenetic analysis of partial ORF1 revealed that Slovak isolates clustered into two genetic groups. All Slovak HEV isolates were typed as genotype 3.

DISCUSSION AND CONCLUSIONS:
The occurrence of HEV was observed in four pig farms. In only one pig farm HEV was not detected. Relatively high number of positive samples was detected in category finishing pigs, lower in weaning category but none in suckling. Many studies of other enteric viruses have presented the high occurrence just in suckling category. Our study revealed that all Slovak HEV isolates belong to genotype 3 which is common genotype of animal and human isolates. This is considered as important character of the possible source of human infection. In the near future we would like to intend analysis for another pig farms in Slovakia and more HEV isolates.

ACKNOWLEDGEMENTS:
This work was supported by Slovak scientific projects APVV-0379-10, VEGA 1/0342/14 and INFETZOOON, ITMS: 26220120002 supported by the EU.

REFERENCES:
INTRODUCTION:
African swine fever (ASF) is a highly contagious, viral disease of pigs. Endemic in more than 20 sub-saharan Africa countries, ASF has spread throughout Russia and outbreaks have occurred in a number Eastern European countries. To prevent introduction and spread of the disease, strict control and eradication programs are implemented which require accurate and reliable diagnostic tests. The ID Screen® African Swine Fever Indirect ELISA detects anti-AFSV antibodies in both domestic and wild pigs.
Unique features of the ID Screen African Swine Fever Indirect ELISA include the coating of three recombinant ASFV antigens (P32, P62, and P72), and the ability to use the test with blood filter paper and meat juice as well as serum and plasma. Thanks to a new protocol and lowered cut-off, IDvet has improved the test performance. This study presents validation data obtained for this new cut-off (30-40%).

MATERIALS AND METHODS:
The ID Screen ASF Indirect ELISA was used as per manufacturer’s instructions. The coated antigen is comprised of 3 recombinant ASFV antigens (P32, P62, and P72); the test is revealed with an anti-multi-species IgG-HRP conjugate.

RESULTS:
SPECIFICITY
Serum: 763 disease-free sera from domestic pigs (France and Norway), wild boars (France), and Iberian pigs (Spain) were tested. Measured specificity = 99.61% (CI95%: 98.96% - 99.90%), n=763.
Meat juice: 100 negative samples were tested using the ID Screen® ELISA. These samples were obtained from disease-free animals in France. Measured specificity = 100% (CI95%: 96.30% - 100%), n=100.
Filter paper: 90 negative animals were tested by both the serum and filter paper protocols. All sera were found negative by both protocols. (Collecting blood using filter paper facilitates sample collection in field. With the ID Screen protocol, filter paper samples may be tested in a 96-well deep-well plate, making sample processing faster and less prone to mix-ups.)
SENSITIVITY
Serum:
- 3 sera were tested from pigs vaccinated on day 0 and day 24 with the ASF strain Ourt88/3, challenged on day 42 with a mild ASF strain (ANSES, Ploufragan, France), and bled on day 62 or 63. After challenge, all three animals gave strong positive results with the ID Screen® ASF Indirect ELISA.
- 8 reference sera provided by the Community Reference Laboratory (CRL), CISA-INIA, Spain were analysed. The ID Screen® ASF Indirect ELISA accurately identified all sera.
Filter paper: 3 sera were titrated and tested by both the serum and filter paper protocols. The measured analytical sensitivity was similar regardless of the sample type tested (serum, Whatman 1 or Whatman 3 filter paper).
Meat juice: IDvet has not been able to test pig carcasses from infected animals. Given that the concentration of antibodies in meat juice samples is generally 15 to 20 times less than in serum samples, test sensitivity was evaluated through the analysis of spiked samples: 5 positive sera were diluted 1:30 in negative meat juice in order to obtain spiked samples. All spiked samples were correctly identified as positive.

DISCUSSION AND CONCLUSIONS:
The ID Screen® African Swine Fever Indirect ELISA is the only commercial ELISA based on 3 recombinant antigens. The serum application has been validated by the ASF European Reference Laboratory. It shows excellent performance in terms of sensitivity and specificity, including for sera from wild boars. It is an efficient and reliable tool for the diagnosis of ASF in wild and domestic species. This test can also be applied with filter paper and meat juice samples.

REFERENCES:
EFFECT OF DIFFERENT STORAGE TEMPERATURES ON THE STABILITY OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) RNA IN BLOOD SAMPLES

Avci O. [1], Bulut O. [1], Yapici O. [2], Simsek A. [1], Yavru S. [1], Dik I. [1], Atli K. [1]

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Keywords: Area Under the Curve, BVDV, Stability

INTRODUCTION:
Bovine viral diarrhea virus (BVDV) is one of the viral agents that cause economical losses all over the world. There are studies in which different tests (IP, reverse transcription PCR) were used to determine the stability of BVDV RNA by storing tissues (kidney, thymus, spleen) and blood samples at different temperatures and on different days. The present study was conducted to determine stability of Bovine viral diarrhea virus (BVDV) RNA stored at different temperatures.

MATERIALS AND METHODS:
A total of 6 blood samples obtained from a private cattle farm, which were found to be antigen positive (Ag +) by direct ELISA method, were used in this study. BVDV Ag+ samples were stored separately at +4, 21 and 37°C for 1 month. The samples were analyzed on the 0, 1st, 2nd, 3rd and 4th weeks by ELISA for the presence of BVDV antigen and by RT-PCR for the presence of BVDV RNA. Stability of BVDV RNA was calculated using maximum concentration (Cmax) and area under the curve (AUC) as kinetic parameters of each sample.

RESULTS:
All of the samples were found positive both by ELISA and RT-PCR on each week. Cmax values of BVDV RNA for the storage temperatures of +4, 21 and 37°C were 356, 346 and 338 ng/µL respectively, and AUC0→4 values for the same temperatures were 1151, 1106 and 1077 week.ng/µL respectively.

DISCUSSION AND CONCLUSIONS:
It was determined that storage at different temperatures for one month does not statistically influence the kinetic parameters of BVDV RNA (P>0.05). Based on the Cmax values obtained, it was determined that the amount of BVDV RNA in the positive samples stored at specified temperatures remained detectable until the end of the 4th week and that the lowest RNA loss was in the samples stored at +4 °C. In conclusion, it can be expressed that storage of BVDV RNA at +4, 21 and 37°C for one month has no effect on the stability of BVDV RNA.

REFERENCES:
SURVEY ON THE DIFFUSION OF HEPATITIS E VIRUS IN WILD BOARS IN THE LIGURIAN REGION (ITALY)

Battistini R. [1], Ercolini R. [2], Rossini I. [1], Tomei L. [1], Mignone W. [3], Arossa C. [4], Dellepiane M. [4], Ferrari A. [5], Razzuoli E. [6], Peletto S. [6], Serracca L. [1]


Keywords: HEV, wild boar, zoonosis

INTRODUCTION:
Hepatitis E virus (HEV) is a non-enveloped single stranded RNA virus belonging to the Hepeviridae family. Until now, four genotypes and several subtypes have been defined. HEV strains closely related to human HEV have been detected in pigs, deer and wild boar indicating the possibility of a zoonotic transmission (1). The meat products from HEV-infected reservoir animal species are capable of transmitting HEV to humans and are a public health concern. Cases of HEV were linked to the consumption of contaminated wild boar meat (2, 3). Direct exposure to wild boar or environmental contamination might also represent a source of HEV transmission especially for hunters or butcher (1). A limited amount of data is available on HEV prevalence in wild boars in Italy (4, 5) and no data are available from Ligurian Region so, in this study, was evaluated the circulation of HEV in wild boars from this area.

MATERIALS AND METHODS:
Liver samples were collected from 570 wild boars (Sus scrofa) during the 2012-2013 and 2013-2014 hunting seasons in the 4 provinces of Ligurian region in Italy (Table 1). The samples were homogenised in 10% (w/v) sterile phosphate-buffered saline and clarified by centrifugation at 10,000 g for 5 min. Total RNA was extracted from 140 µl supernatant using E.Z.N.A. viral RNA kit (Omega bio-tek). HEV RNA was detected by real Time RT-PCR (6) and confirmed by sequencing and phylogenetic analysis.

RESULTS:
HEV RNA was detected in the livers of 26/570 (4.6%) wild boars tested. Three provinces of Ligurian region resulted positive for HEV, with a prevalence of 4.5% in Imperia, 6.1% in Savona and 6.7% in Genova while no samples resulted positive in La Spezia. Phylogenetic analysis showed that all wild boar HEV sequences belonged to genotype 3 and in particular 12 sequences belonged to subtype 3, 3 to subtype 3a, one to subtype 3c and one to subtype 3f (Table 1).

Table 1: HEV RNA detection in wild boar liver samples related to the place of hunting

<table>
<thead>
<tr>
<th>Location of sampling</th>
<th>Number of liver samples</th>
<th>Number of positive samples (%</th>
<th>Number of positive sequences</th>
<th>Genotype (Subtype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imperia</td>
<td>370</td>
<td>14 (4.5%)</td>
<td>5</td>
<td>3c(3)</td>
</tr>
<tr>
<td>Savona</td>
<td>171</td>
<td>8 (4.7%)</td>
<td>7</td>
<td>3c(2)</td>
</tr>
<tr>
<td>Genova</td>
<td>80</td>
<td>4 (5.0%)</td>
<td>2</td>
<td>3c(2)</td>
</tr>
<tr>
<td>La Spezia</td>
<td>59</td>
<td>0 (0.0%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>570</td>
<td>26</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES:

DISCUSSION AND CONCLUSIONS:
The data obtained in this study showed an active circulation of the virus in wild boar populations along Ligurian territory with the exception of the most southern part of the Region. In addition the presence of HEV in liver samples indicates a potential zoontic risk in handling and/or consumption of raw or undercooked meat and products made from liver from this animal, since the genotype 3 found in the samples is most isolated in industrialized countries and include viral strains affecting both man and animals.
SEROPREVALENCE OF AUJESZKY'S DISEASE IN PIG HERDS IN SOUTH SARDINIA

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Keywords: Aujeszky’s Disease, seroprevalence, Sardinia, Italy

INTRODUCTION:
Aujeszky’s Disease (AD) is a notifiable disease caused by Suid Herpesvirus 1 (SuHV1; syn. AD Virus, ADV), showing a worldwide distribution, particularly in regions with dense populations of domestic pigs. Pig is the primary host of ADV, and the only species able to survive a productive infection, so serving as reservoir host (Mettenleiter et al., 2012). AD causes serious losses to swine producers, both as a result of the disease and because of movement restrictions. For these reasons, in several parts of the world, national eradication programmes has been implemented in order to eliminate the AD from pig herds. Aim of this survey was to investigate and update the ADV seroprevalence in pig herds in south Sardinia.

MATERIALS AND METHODS:
In 2013, serum samples coming from pig herds located in south Sardinia were tested to detect ADV antibodies, using a commercial competitive ELISA (c-ELISA) kit (PRV/ADV gI Ab Test, IDEXX Lab., Westbrook, USA). The assay was performed according to the manufacturer’s instructions. Pig herds and serum samples were grouped in 4 herd categories according to the number of pigs present in the herd: very small (1-10 pigs), small (11-37), medium (38-100), and large (>100). Data were summarized in terms of number and percentage both of pig herds and serum samples with positivity to ADV. Chi-square test was employed to verify the presence of any significant difference in seropositivity among herd categories, and the Odds Ratio (O.R.) with 95% Confidence Interval (95% C.I.) was calculated for each herd category.

RESULTS:
A total of 18032 serum samples coming from 2080 pig herds were analysed with a c-ELISA. Number and percentage of herds and serum samples and O.R. with 95% C.I. for each category are summarized in Table 1. Overall, positivity was detected in 15.0% of herds, and 12.6% of serum samples. Chi-square test showed a significant difference among herd categories both in herds (prevalences ranged 10.3 to 38.5%) and in serum samples (prevalences ranged 5.8 to 19.2%) (p<0.05).

DISCUSSION AND CONCLUSIONS:
Our results showed that in Sardinia the ADV seroprevalence is currently relatively high, although recent data about AD prevalence in pig to which compare our results are scarce in literature. Foti et al. (2008) in Sicily found seropositivity in 42.2% of herds and 14.6% of serum samples, and a CRMA report about surveys on AD in 2013 showed its presence in the entire Italian territory (CRMA, 2013). However, AD prevalence has dramatically decreased in Italy during the two last decades, as compared with previous reports (Zanardi, 2000). In our survey, small and medium herds showed to have higher risk to be positive to AD than very small and large herds. This is in partial agreement with Tamba et al. (2002) that found a positive association of ADV infection of the herd with increased herd size. The low prevalence and risk of positivity in large herds can be related both to higher levels of biosecurity adopted and to regular vaccination programmes. In conclusion, a wider compliance and major effort must be paid by pig farmer in order to achieve the eradication of AD in Sardinia.

REFERENCES:
INTRODUCTION:
Aujeszky’s disease (AD) is one of the most economically important diseases of swine. The disease is caused by Suid herpesvirus 1 (SuHV1) for which domestic pigs (DP) as well as wild boar (WB) are the only natural hosts (1). Although successful eradication of AD in DP populations in many parts of the world was achieved (1), the role of WB as a potential source and reservoir of AD virus (ADV) is still very important. Poland is close to finish its AD eradication programme and therefore it was very interesting to investigate the distribution of SuHV1 in WB population.

MATERIALS AND METHODS:
In the study a total of 10541 blood samples taken from WB shot in all 16 voivodships of Poland was used. The samples were collected during 2012 and 2013. A commercially available ELISA was used for screening of antibodies to ADV according to manufacturer’s instruction (HerdChek Anti-PRV gpI, IDEXX, Inc., USA).

RESULTS:
From 10541 blood samples that have been received for testing purposes 10472 were used. Bad quality of 69 (0,65%) of them was the reason to exclude them from the investigations. Antibodies to ADV were found in 3108 (29,7%) of 10472 samples and the prevalence differed among voivodships as shown in Table 1.

DISCUSSION AND CONCLUSIONS:
Presented data indicate that SuHV-1 infection still occurs in the WB population in Poland. In western part of Poland the percentage of seropositive WB was higher than calculated for the whole country and ranged from 30,8% to 47,7%. What is interesting is that similar studies conducted in Poland in the years 1997-2000 revealed an average 14,52% seropositive WB (2) and now the average ADV seroprevalence amounted 29,7%. It means an important increase of SuHV1 infected WB in Poland. Similar observations were made among other also in Germany (3, 4) and Croatia (5). Taking into account that the overall seroprevalence of ADV in WB in neighboring countries is on similar level as was detected in Poland it can be concluded that together with increasing WB population observed across Europe (1), the number of infected with SuHV1 WB arose also. What is also important from practical point of view is that in countries free of AD or in those that are close to finish the AD eradication programme WB should be taken into consideration as a potential source and reservoir of SuHV1 for DP. Therefore, a continuous monitoring of these disease in WB population would be advisable (6).

REFERENCES:

Table 1. Results of ADV antibody detection in wild boar in Poland in 2012-2013

<table>
<thead>
<tr>
<th>Voivodship</th>
<th>received</th>
<th>used</th>
<th>positive (%)</th>
<th>doubtful (%)</th>
<th>negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP 4. v. śląskie</td>
<td>1024</td>
<td>1016</td>
<td>412 (40,7%)</td>
<td>32 (3,2%)</td>
<td>566 (56,1%)</td>
</tr>
<tr>
<td>KFP</td>
<td>772</td>
<td>776</td>
<td>237 (30,8%)</td>
<td>20 (2,6%)</td>
<td>512 (66,6%)</td>
</tr>
<tr>
<td>LP Łódzkie</td>
<td>1219</td>
<td>1219</td>
<td>334 (27,4%)</td>
<td>30 (2,5%)</td>
<td>555 (70,0%)</td>
</tr>
<tr>
<td>ELU Lubuskie</td>
<td>475</td>
<td>474</td>
<td>215 (45,9%)</td>
<td>14 (2,9%)</td>
<td>446 (64,0%)</td>
</tr>
<tr>
<td>LO Łódzkie</td>
<td>394</td>
<td>393</td>
<td>117 (29,7%)</td>
<td>7 (1,8%)</td>
<td>269 (68,5%)</td>
</tr>
<tr>
<td>MB Małopolskie</td>
<td>352</td>
<td>351</td>
<td>65 (18,5%)</td>
<td>6 (1,7%)</td>
<td>280 (79,8%)</td>
</tr>
<tr>
<td>WWM Mazowieckie</td>
<td>588</td>
<td>588</td>
<td>169 (28,7%)</td>
<td>17 (2,9%)</td>
<td>402 (68,4%)</td>
</tr>
<tr>
<td>OŚ oświęcimie</td>
<td>428</td>
<td>421</td>
<td>139 (44,9%)</td>
<td>9 (2,1%)</td>
<td>223 (35,0%)</td>
</tr>
<tr>
<td>KPK Kujawsko-Pomorskie</td>
<td>639</td>
<td>637</td>
<td>169 (26,3%)</td>
<td>15 (2,3%)</td>
<td>452 (71,4%)</td>
</tr>
<tr>
<td>PO podkarpackie</td>
<td>794</td>
<td>792</td>
<td>224 (28,2%)</td>
<td>23 (2,9%)</td>
<td>546 (69,9%)</td>
</tr>
<tr>
<td>PO podlaskie</td>
<td>1315</td>
<td>1299</td>
<td>325 (25,3%)</td>
<td>17 (1,3%)</td>
<td>956 (73,4%)</td>
</tr>
<tr>
<td>SL śląskie</td>
<td>463</td>
<td>461</td>
<td>111 (24,0%)</td>
<td>17 (3,7%)</td>
<td>335 (72,3%)</td>
</tr>
<tr>
<td>SW świętokrzyskie</td>
<td>166</td>
<td>164</td>
<td>13 (8,0%)</td>
<td>4 (2,4%)</td>
<td>149 (89,6%)</td>
</tr>
<tr>
<td>SM małopolskie</td>
<td>1024</td>
<td>1024</td>
<td>256 (25,0%)</td>
<td>25 (2,4%)</td>
<td>742 (72,6%)</td>
</tr>
<tr>
<td>WO województwo</td>
<td>195</td>
<td>196</td>
<td>65 (33,2%)</td>
<td>3 (1,5%)</td>
<td>128 (65,3%)</td>
</tr>
<tr>
<td>ZPB zielonogórskie</td>
<td>446</td>
<td>446</td>
<td>119 (26,3%)</td>
<td>15 (3,4%)</td>
<td>212 (49,6%)</td>
</tr>
<tr>
<td>Total</td>
<td>10641</td>
<td>10472</td>
<td>3108 (29,7%)</td>
<td>284 (2,7%)</td>
<td>7111 (67,9%)</td>
</tr>
</tbody>
</table>
EPIDEMIOLOGY OF EQUINE VIRAL ARTERITIS (EVA) IN ARABIAN HORSES IN POLAND - PRELIMINARY STUDY

Witkowski L.[1], Paschalis Trela K.[1], Cywinska A.[1], Czopowicz M.[1], Kita J.[1], Mickiewicz M.[1], Trela J.[2], Aymeric H.[3], Lecouturier F.[3], Gaudaire D.[3]

[1]Faculty of Veterinary Medicine, Warsaw University of Life Sciences - SGGW ~ Warsaw ~ Poland, [2]Medical Teaching Hospital, School of Veterinary Medicine, University of California ~ Davis ~ United States, [3]ANSES-Dozulé Laboratory for Equine Diseases ~ Goustranville ~ France

INTRODUCTION:
EAV infection has been shown to occur in equine population in Poland, however, little is known about its epidemiology in Arabian horses. Semen of breeding stallions is routinely monitored but seroprevalence remains unknown. Total Arabian population in Poland counts approximately 4000 animals. Vaccination against EAV is not allowed in Poland.

MATERIALS AND METHODS:
Investigation of seroprevalence of EAV in Arabian horses in Poland. Serum samples were collected in 2010 and 2011 in Poland. Data for epidemiological investigation were collected. The sera were tested using virus neutralization test according to the OIE recommendations.

RESULTS:
Total number of 612 horses (422 mares and 190 stallions) was tested (approximately 15% of the population). Antibodies against EAV were detected in 34.8% horses (CI 95%: 31.1%, 38.7%). Seroprevalence among mares was 43.1% (CI 95%: 38.5%, 47.9%) while in stallions 16.3% (CI 95%: 11.7%, 22.2%).

The increase of seroprevalence with age was observed. Among the horses less than 4 years of age seroprevalence was 10.5% (CI 95%: 7.7%, 14.1%), in the group of 5-12 year-old – 62.8% (CI 95%: 56.0%, 69.1%) and in the horses older than 12 years – 86.8% (CI 95%: 75.2%, 93.5%). However, seroprevalence rose faster in mares than stallions. In mares younger than 4 years it reached 12.9% (CI 95%: 9.0%, 18.1%), between 5-12 years – 68.4% (CI 95%: 61.2%, 74.8%) and in older than 12 years – 94.4% (CI 95%: 81.9%, 98.5%). In stallions 7.0% (CI 95%: 3.8%, 12.4%), 30.0% (CI 95%: 16.7%, 47.9%) and 70.6% (CI 95%: 46.9%, 86.7%) animals were seropositive, respectively.

DISCUSSION AND CONCLUSIONS:
Results suggest that EAV is prevalent in the Arabian population but its prevalence differs among sex and age. According to the collected information all seropositive breeding stallions on main breeding farms have been tested for shedding EAV with their semen, and all of them were negative. Stallions which spread the virus with semen are a main reservoir of EAV in the equine population but horizontal transmission via respiratory route is also possible. The Polish Arabian population is relatively small and homogenous but most of horses travel a lot and have close contact with horses from Poland and foreign countries (e.g. during events).

The research was supported by a grant from the National Science Centre on the basis of the decision No. DEC-2011/03/B/NZ6/04682.

REFERENCES:
INTRODUCTION:
Immunohistochemical methods can be used to confirm the diagnosis of a Schmallenberg virus (SBV) infection during postmortem investigations and to study the pathogenesis of this viral infection. Using specific antibodies the presence of viral antigens can be demonstrated in sections of paraffin embedded tissues. Since SBV was at the time a new emerging virus no specific antibodies were available, but the Australian Animal Health Laboratory (AAHL) in Geelong, Australia had ample experience with other Simbu serogroup viruses and generously supplied a monoclonal antibody (MAb) raised against Tinaroo virus with a broad reactivity within the Simbu serogroup.

MATERIALS AND METHODS:
After deparaffinization, heat-induced epitope retrieval was carried out on 4 µm tissue sections at 100 °C during 20 minutes at pH 6, thereafter sections were incubated with Mab 4H9/B11/F1 in a 1:500 dilution during 15 minutes at 37 °C, and incubated with a polymer-based immunoperoxidase (DAKO EnVision™). 3,3’-Diaminobenzidine was used as chromogen and the slides were counterstained with haematoxyline. As a negative control Mouse IgG1 was used as isotype control in duplo-sections, replacing the monoclonal antibody against Tinaroo virus. The cerebellum of a SBV infected lamb that was strongly positive by PCR and immunohistochemistry was used as a positive control on the lower part of each slide.

Immunohistochemistry was performed on samples from brain tissue and spinal cord, and on tissue samples from lung, liver, spleen, muscle, thymus, pancreas and, if available, placenta, belonging to eighteen lambs with positive RT-qPCR Ct-values in brain tissue, ranging from 18.6 to 33.5.

RESULTS:
Optimisation of the SBV IHC method was straight forward since, using the above described antigen retrieval method, a specific staining of neurons and axons was obtained in SBV infected tissues with a low background staining (optimal dilution of Mab 4H9/B11/F1 was 1:500) as shown in figures 1 and 2.

Sixteen out of 18 tested lambs showed immunoreactive neurons and neuronal processes in the brain and/or spinal cord, indicating the presence of viral antigen. Eight out of these 16 IHC-positive cases showed a nonsuppurative meningoencephalitis and/or poliomylitis, the other eight cases showed immunoreactivity of neurons in hypoplastic brain tissue, while the spinal cord remained negative due to severe hypoplasia. Immunoreactive neurons were located in the cerebral and cerebellar cortex, including Purkinje cells, in the midbrain and brainstem, and immunoreactivity was also found in neuroblasts, located in the ventricular zone. Muscular tissue, organs and placenta did not show immunoreactivity.

DISCUSSION AND CONCLUSIONS:
International collaboration allowed for the development of an immunohistochemical method for detection of SBV in a short period of time. This method is a useful tool for studying the pathogenesis of SBV infections, but the diagnostic performance is relatively low compared to real-time RT-PCR. For example the majority of SBV infected malformed calves scored a negative IHC result (results not shown). The limited amount of tissue in a section, lysis of target cells and hypoplasia, the long interval between fetal infection and stillbirth with clearance of viral antigens may explain these results.

REFERENCES:
INTRODUCTION:
Two types of commercial enzyme-linked immunosorbent assays (ELISA) for the detection of antibodies to Bovine viral diarrhoea virus (BVDV) are currently available in the market. One detects antibodies to the p80 (NS2/NS3) protein, typically using a blocking or competitive ELISA format, while the other detects antibodies to a wide range of viral proteins using an indirect ELISA format (ELISA plates coated with whole virus particles). Differences in ELISA kit performance according to format and sample type have been reported before (Kramps et al., 2004). The objective of this study was to compare the performance of a panel of ELISA kits when testing serum pools of samples that had been individually tested with the LSI Vet Ruminant BVD/BD p80 kit (Life Technologies).

MATERIALS AND METHODS:
A panel of 300 pools representing a range of individual seroprevalences from 0-100% was assembled and used for further testing with a range of antibody ELISA kits and protocols including p80 and whole virus kits. Results obtained were compared to the proportion of seropositive animals in each pool as tested with the LSI p80 kit using the individual sample protocol. The tests characteristics were evaluated using WinEpiscope 2.0. The sensitivity (Se), specificity (Sp) and the Youden index (J) for each test were calculated. The level of agreement (expressed as Kappa value) including 95% confidence intervals (95% CI) between the result obtained when the individual samples in the pool were tested with the LSI p80 ELISA using the individual sample protocol with short incubation and each ELISA result was established. Pools with one or more seropositive samples were considered positive.

RESULTS:
All kits except the LSI E protocol obtained a relative sensitivity (Se)>94%. All p80 kits returned a relative specificity (Sp)>87% and the whole virus ELISAs a Sp>73%. Kappa values were over 0.7 for all kits with the exception of the LSI E. Three out of the five p80 protocols had a higher kappa value (>0.8) than whole virus kits (>0.69). The other two p80 kits obtained kappa values of 0.7 and 0.35. Seven out of the 30 pools classified as ‘all individual animals seronegative’ gave positive or inconclusive results when tested with other kits/protocols. These seven pools were constituted by 12 and 24 months old animals from vaccinated herds which were therefore considered too old to have maternally derived antibodies. The analytical sensitivity of a selection of whole virus and p80 kits was compared.

DISCUSSION AND CONCLUSIONS:
Overall, the kits were able to detect antibodies in pooled samples containing one or more individual positive samples in almost all cases, highlighting the potential to use pooled samples as a cost-effective screening tool. A number of possible explanations for the discrepant results were explored including differences in ELISA format (competitive/indirect), and differences in analytical sensitivities between kits and/or protocols which were sufficient to detect low levels of antibody present as a response to vaccination. It is recognised that serological responses to inactivated vaccines are more readily detected by indirect ELISAs containing whole virus antigen than is the case when using ELISAs that target the p80 response specifically (González et al., 2014). The results obtained in this study also support this finding. The type of kit used (p80 or whole virus), whether the sampled group had been vaccinated for BVDV or not and the cut off applied were found to have an impact on the agreement obtained between the results of individual and pooled testing.

REFERENCES:
INTRODUCTION:
The plaque reduction neutralization test (PRNT) is the most specific serological test for serological identification of flaviviruses (1,2). The most stringent PRNT test which is represented by the 90% endpoint PRNT (PRNT90) is considered as the gold standard protocol for the sero-diagnosis of flavivirus infections. In differential PRNT, a neutralizing titer of fourfold or higher for one virus compared to those of the others is considered virus specific (1). Possible occurrence of co-infections could not be discarded in case of similar titers against two or more flaviviruses. PRNT presents some drawbacks. Firstly, it takes around 1 week to be completed. Secondly, it is expensive and requires highly trained staff. Thirdly, PRNT using wild type flaviviruses can be technically difficult, as many flaviviruses grow slowly and have pinpoint-sized plaque phenotypes. Last but not least it requires a quantity of serum difficult to obtain when dealing with wild animals and particularly with some species of wild birds.

MATERIALS AND METHODS:
In order to test the consistency of the serum-neutralization (SN) in quantitating specific WNV antibodies, we tested by SN (3) and PRNT90 (OIE, 2012) 1348 serum samples from equids (horses and donkeys) and 38 sera of human patients. Furthermore, 25 serum samples from horses previously showed to be positive for Usuv, were tested at the same time by SN and PRNT90 using Usuv and WNV as antigens. Sera with a titre of at least 1:10 were considered positive. In order to compare SN and PRNT90 assays, two different comparisons were performed for resulting titres and positive/negative result values: titres were compared by Wilcoxon non parametric test for dependent samples; positive/negative results were compared using McNemar 2 test for dependent samples and the level of agreement was evaluated by the k-Cohen coefficient (4).

RESULTS:
As for serum samples from equids, the two diagnostic tests were not statistically different in terms of titres and positive/negative results. Contingency table (Tab 1) shows the level of agreement between the two tests. The McNemar 2 test gave a value of 0.83 (p>0.05) showing a not significant difference between the two methods and the k-Cohen, equal to 0.78 (p<0.05), gave a good level of agreement beyond the chance. Also titres were not significantly different (Wilcoxon test = -1.69, p>0.05). Similar results were also obtained when human sera. Contingency table (Tab 2) shows the level of agreement between the two tests for human samples. The McNemar2 test gave a value of 1.33 (p>0.05) showing a not significant difference between the two methods and the k-Cohen, equal to 0.81 (p<0.05), gave a good level of agreement beyond the chance. Also titres were not significantly different (Wilcoxon test = -1.67, p>0.05). All 25 samples from horses were shown to be positive for WNV and Usuv in both tests with a broad range of titres (Tab 3). Results from serum samples from horses 6, 8, 18, 19, 21 and 25 suggest the presence of antibodies for both viruses as results of a double infection whereas serum from horse number 10 shows specific antibodies only for Usuv. The remaining serum samples seemed to be positive only for WNV. Horses 6-10, 12, 23 and 25 showed a recent infection by WNV as suggested by the IgM Elisa test.

DISCUSSION AND CONCLUSIONS:
We showed on a large number of serum samples, that SN can efficiently replace PRNT at quantitating WNV antibodies. The two diagnostic tests were not statistically different in terms of titres and positive/negative results thus supporting the use of SN during the daily activities of diagnostic laboratories dealing with WNV serological investigation. SN is faster and requires less labor when compared to PRNT. Cross-reactivity exists between WNV and Usuv when both serological methods are used. However, specific antibodies can be attributed to one or the other virus as there is a neutralizing titer of fourfold or higher for one virus compared to the other. Importantly, both viruses contemporarily circulate in the same areas thus leading to the presence of sero-reactors for both viruses as it seems to be the case for horses 6, 8, 18, 19, 21 and 25.

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075
EVALUATION OF THREE ALTERNATIVE METHODS FOR DIAGNOSIS OF EQUINE HERPESVIRUS ABORTION

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Keywords: Equine herpesvirus, abortion, diagnosis, PCR, aspiration biopsy

INTRODUCTION:
The gold standard for diagnosing an equine herpesvirus (EHV-1/EHV-4) abortion is a combination of necropsy, histopathology, virus isolation, PCR and/or immunostaining of the aborted foetus and placenta. The corresponding costs of these diagnostics, including transport logistics of foetuses to a qualified laboratory, are a serious hurdle for many owners in the Netherlands. Therefore three alternative diagnostic methods were hypothesised and investigated: EHV-1/EHV-4 real-time PCR on 1) an aspiration biopsy of the fetal lung; 2) a vaginal swab of the mare collected within 2 days after abortion; 3) detection of EHV-1 specific antibodies in a blood sample of the mare collected within 2 days after abortion.

MATERIALS AND METHODS:
Real-time PCR on aspiration biopsies (n=41) was first optimised by testing 5 different DNA extraction methods in comparison with the gold standard of full necropsy, histopathology and immunohistochemistry. For EHV-1 real-time PCR primer and probe sequences were generously provided by Dr. Mats Isaakson, SVA, Uppsala, Sweden. EHV-4 real-time PCR was performed as described [1]. EHV-1 and EHV-4 specific antibodies were determined with a commercially available gG-ELISA (Svanova). The three methods were evaluated in a population of mares that delivered normally (group A; n=86), and a population of mares that aborted their foals (group B; n=83) using both classical and Bayesian statistical analysis.

RESULTS:
Aspiration biopsies of foetal lungs (n=41) showed 100% agreement with the gold standard. All EHV-1 positive aspiration biopsies (n=12) were strongly positive with a mean Ct-value of 15.6. All samples scored negative for EHV-4. Relative sensitivity and specificity of real-time PCR on aspiration biopsies as compared with the gold standard was 100%. In the abortion group of the field study, using the PCR result of the aspiration biopsy as a gold standard, the relative sensitivity and specificity of EHV1-PCR on vaginal swabs was 100% and 96.8%, respectively, whereas the relative sensitivity and specificity of EHV1-ELISA on serum samples of the mares was 37.5% and 88.3%, respectively.

Posterior results of Bayesian analysis showed for EHV1 PCR on vaginal swabs and EHV1-ELISA on serum samples point estimates for sensitivity of 87% and 68%, and for specificity of 93% and 95%, respectively.

After implementation in routine diagnosis from January 2012 onwards, that year 52 submissions were received of which 43 were complete (both an aspiration biopsy and a vaginal swab). Fifteen out of 52 submissions (29%) were found positive for EHV1 by real-time PCR. Thirteen out of these 15 submissions were complete, and were found consistently positive for EHV1 by real-time PCR in both samples with mean Ct-values of 20 and 29 for aspiration biopsies and vaginal swabs, respectively.

DISCUSSION AND CONCLUSIONS:
Real-time PCR on an aspiration biopsy of the foetal lung, preferably combined with a vaginal swab from the mare within two days after abortion, provides a good alternative for diagnosis of equine herpes virus abortion as compared to full necropsy of the foetus. This diagnostic approach cannot replace pathology in general terms, but provides a cost-effective and practical tool for monitoring and surveillance of viral causes of abortion like EHV1 and EHV4. In contrast to virus isolation, diagnostic performance by PCR is much more robust in samples that are poorly preserved. In this study samples were simply submitted by post at environmental temperatures. Therefore this approach can also be valuable in remote areas with complicated logistics for transport of foetuses to an institute.

REFERENCES:
**MOLECULAR DETECTION OF FELINE CALICIVIRUS IN CLINICAL SAMPLES: A VALIDATION STUDY COMPARING DETECTION BY RT-qPCR DIRECTLY FROM SWABS AND AFTER VIRUS ISOLATION**

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**Keywords:** Feline Calicivirus, real-time RT-qPCR, Virus isolation

**INTRODUCTION:**
Feline Caliciviruses (FCV) are non-enveloped RNA viruses responsible for upper respiratory tract disease(1). They show a high genetic variation(2). Conventional, nested and real-time reverse-transcriptase PCR (RT-qPCR) assays have been developed to detect FCV in clinical specimens. In comparison to virus isolation (VI), molecular methods are faster and more specific; the latter, however, may result in a lower diagnostic sensitivity (not all strains are recognized). In contrast, VI may fail particularly due to virus inactivation during transport(3). This study was initiated to validate and compare two RT-qPCR assays, apply the assays with samples from a FCV field study and compare them to VI. Moreover, the influence of storage conditions on the detectability of FCV was evaluated.

**MATERIALS AND METHODS:**
Two previously published RT-qPCR assays (4,5)(designated S1 and S2) targeting different regions on the open reading frame 1 were evaluated. Analytical sensitivity and efficiency were optimized using ten-fold serial dilutions of synthetic RNA standards applying a primer-probe concentration matrix and different commercially available RT-qPCR mastermixes.

To optimize the sample collection and transport/storage conditions, the stability of FCV on dry swabs and using different transport media was assessed over time and at different temperatures.

The PCR assays were applied using 300 samples from a Swiss FCV field study and compared with VI. From each cat oropharyngeal cytobrushes and nasal and conjunctival swabs were collected; samples from the same cat were pooled. For VI, Crandell-Rees feline kidney cells (CRFK) were incubated at 80% confluency with sterile-filtered samples. Cultures were observed daily for cytopathic effect as a sign of virus replication. Cell culture supernatants were tested by FCV RT-qPCR. A sample was considered FCV positive if one of the tests was positive. The target regions of the samples resulting in incongruent results for RT-qPCRs S1 and S2 were sequenced.

**RESULTS:**
Both RT-qPCR systems reached an analytical sensitivity of 100 copies per reaction and a dynamic range over 6 logs with the same reaction composition but using different mastermixes and different thermal profiles. RT-PCRS1 and S2 showed “100% and 81% efficiency, respectively. FCV stability on swabs was similar at both 4°C and -20°C but viral burdens rapidly decreased to undetectable values within 4 days. FCV was more stable when using a viral transport medium in comparison to dry swabs. 97 (32%) of the 300 samples were rated FCV-positive. Of these, 81% were positive in the swabs by S1; 84% were positive in the VI supernatants by S1; 77% were positive in the swabs by S2 and 81% were positive in the VI supernatants by S2. S1 was more sensitive compared to S2 and VI combined with RT-qPCR was more sensitive compared to RT-qPCR directly from swabs. None of the methods detected all FCV-positive samples. When sequencing samples that gave discordant results in the two RT-qPCR assays, mismatches in the binding regions were detected.

**DISCUSSION AND CONCLUSIONS:**
To reach a high sensitivity of FCV detection, samples have to be processed within 3 days after collection in a proper transport medium and stored at ≤4°C. Moreover, amplification of the virus in cell culture followed by analysis of the cell culture supernatant by RT-qPCR increases the sensitivity of detection. However, no method was able to recognize all of the FCV-positive cats. The sensitivity of the RT-qPCR assays is mainly hampered by the high genetic variability of FCV. The design of improved primers and probes will be necessary. The combination of VI with RT-qPCR as readout may be recommended to receive a high sensitivity and specificity of detection, but for routine diagnostics purposes this approach is usually too time-consuming and expensive.

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INTRODUCTION:
Zoonotic viruses are thought to be the highest risk of epidemic diseases for next years. Among others, these also include rotavirus, agent of gastroenteritis in man and animals and hepatitis E virus (HEV), which causes acute hepatitis in humans and infect domestic and wild animals.
Group A rotaviruses (RVA) infection is preferentially species-specific, heterologous rotavirus infections may also occur, including animal-to-human virus transmission.
Recently, a human RVA of rabbit origin was described in a child with gastroenteritis, and a bovine-like RVA was isolated from a laboratory rabbit [1].
Hepatitis E is an emerging disease now recognized to be a zoonosis. Swine, wild boar, deer and rabbit are considered reservoirs of the zoonotic HEV genotypes 3 and 4. Recently HEV was detected in both farmed and wild rabbits in several areas of China, USA and France. The prevalence of serum antibodies against HEV ranges between 7% and 57% in young animals.
Several evidences support a risk of zoonotic transmission of HEV from rabbits to humans, including experimental infection of non-human primates and swine with rabbit HEV and detection of HEV closely related to rabbit HEV in a man [2].
HEV and RVA infections were studied in 21 farmed and 121 pet rabbits in Italy. Virus infection was investigated by serological analysis in sera and by detection of RNA in fecal samples.
MATERIALS AND METHODS:
During 2013, a total of 121 rabbits attending veterinary examination were enrolled in this study. Sera and feces were collected from the same animals (except 7 fecal samples). Seventy-seven pets were asymptomatic, most others were affected by different pathologies. Between October 2013 and February 2014, 21 samples were collected from stud-mare rabbits affected by reproductive failure.
Western blotting (WB). The capsid protein of an Italian gt3 swine HEV strain expressed in Sf9 cells from a recombinant baculovirus (BacΔ111ORF2HEV), the purified SA11 RV and crude extract of mock Sf9 (negative control) were subjected to WB. Sera were diluted 1:100.
Immunocytochemistry. Sf9 cells infected with BacΔ111ORF2HEV and mock cells were fixed and incubated with rabbit sera (1:500). Replication foci were visualized by reaction with amino-ethylcarbazole.
Reverse-transcription–polymerase chain reaction (RT–PCR) and DNA sequencing. RNA was extracted using Qiagen RNA-Easy-Mini kit. For both viruses, RT-PCRs were performed using the OneStep RT-PCR kit (Qiagen). For Rotavirus the NSP5 segment was amplified [1]. For HEV an RT-PCR and a nested-PCR were conducted, amplifying a 300 bp ORF2 fragment.
RESULTS:
None of the 135 fecal samples, (114 pets and 21 farmed rabbits), showed presence of HEV genome. IgG against HEV were detected in 4 of 121 pet rabbits tested. Two seropositive animals were asymptomatic, one was affected by rhinitis. Rabbits showed no clinical signs of RVA infection. Twelve out of 52 sera recognized specific RVA proteins (Fig.1). Twelve samples resulted positive in RT-PCR, one was confirmed through sequence analyses. One animal was positive for both RVA and HEV IgG.
DIAGNOSTIC PERFORMANCE AND EVALUATION OF RABIES SEROLOGICAL TESTS BY FAVNT, RFFIT AND ELISA FOR PET QUARANTINE


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Keywords: Rabies, Pet animal, FAVNT, RFFIT, ELISA

INTRODUCTION:
The rapid fluorescent focus inhibition test (RFFIT), the fluorescent antibody virus neutralization test (FAVNT) and enzyme-linked immunosorbent assay (ELISA) are diagnostic tests for determining levels of rabies neutralizing antibodies in vaccinated animal. The blood test result must indicate a rabies antibody titre equal to or greater than 0.5 IU/ml for interantional movement according to the OIE international standards and guidelines on animal health.

As pet travel continues to increase, the new animal quarantine regulations that rabies-neutralizing antibody test is mandatory on pet animals have implemented from Dec.1st 2012 in the Republic of Korea.

The rapidity with which the test can be carried out and the ease with which several samples may be tested at a time make it suitable for routine animal quarantine diagnosis. Each one has its own good properties, but the specificity and sensitivity should be discussed further.

This study was designed to assess the performance in 2013 to 2014 of Korean pet quarantine and compare of sensitivity and specificity between above serological methods in dogs and cats.

MATERIALS AND METHODS:
A total of 787 serum samples of dogs(n=635) and cats(n=152) were collected from December, 2012 to June, 2013. Serums samples were titrated out and compared to ANSES standards with a known antibody concentration to convert the titres into IU ml⁻¹ and analyzed individual information including species, vaccination history, sex, age.

For serological comparison against Rabies, 152 samples selected and were performed the indirect ELISA (Bio-Rad Platelia Rabies II Kit, France), FAVNT and RFFIT according to the OIE Manual of Diagnostic.

RESULTS:
First, 787 serum samples were done and analyzed by FAVN as the authorized lab. The most numerous countries is European Union (n=289, 29%) among those planning abroad. The other continents were Asia (n=224, 28%), US(n=156, 20%) and Oceania (n=56, 7%). Most tested animals is under the age of five and requested two to 8 months after vacciantion. The average titer of cat (66.63 IU/ml) was higher than dogs (39.14 IU/ml).

The comparison of titres obtained by three tests showed 92.3% agreement in vaccinated dogs and 100% in non-vaccinated ones.

The agreement, sensitivity and specificity of Bio-Rad Platelia Rabies II ELISA when compared to FAVN and RFFIT were 91.3 %, 92.1 % and 93.5 %, respectively. Two sera with titres were detected as positive by RFFIT and as negative by the FAVN test.

DISCUSSION AND CONCLUSIONS:
When comparing the serological methods used in diagnostics for quarantine, it is important to evaluate particularly their sensitivity, specificity, and reproducibility.

The comparison of neutralizing antibody tests for sensitivity, specificity and reproducibility of rabies antibodies showed no significant differences between ELISA, RFFIT and FAVN method, recommended by for international trade. However, there were differences in rapidity, simplicity and easiness of performance for them, costs of the rea-gents and equipment, skill of technician.

In accordance with the increasing of pet movement abroad, pet quarantine procedures are demanded to meet the need of owners and importing countries in which rabies does not occur or had been eradicated.

Therefore, it is inevitable to handle various diagnostic procedures to prevent errors in test results and cope with lab conditions.

REFERENCES:
INTRODUCTION:
The porcine reproductive and respiratory syndrome virus (PRRSV) is recognised worldwide as an important cause of reproductive failure and pneumonia in pigs and causes high economic losses (1, 2). Therefore, PRRSV eradication programs have been developed and PRRSV negative herds and farms have been established. For monitoring of unsuspected herds, tests with a high specificity are required. The tests should have a high sensitivity as well, since positive animals may not be overlooked. Different commercial ELISAs have been developed for detection of PRRSV antibodies (Ab) in serum. Aim of the study was to validate the Ingezim PRRS 2.0 (Ingenasa) in comparison to the until now as Gold standard used HerdCheck PRRS X3 (IDEXX) ELISA (3, 4) for detection of PRRSV Ab in serum of pigs.

MATERIALS AND METHODS:
A whole of 664 serum samples of pigs were included in the study. The pigs were divided into 4 groups: Group 1 consisted of three pigs from a PRRSV negative farm that were vaccinated with attenuated live vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim, Germany). Blood samples were taken from each pig at day five, nine, 12, 18, 21 and 26 post vaccination. Group 2 included 245 pigs from PRRSV positive farms of different origins in Europe and Asia. Group 3 included 309 pigs from six monitored PRRSV negative boar studs and one pig breeding farm from Germany and Austria. Group 4 included residual blood samples of 92 Austrian wild boars. All samples were tested with the HerdCheck PRRS X3 as well as with the Ingezim PRRS 2.0. Cut-off of both ELISAs is at the S/P value of 0.4. Agreement (kappa) between both ELISAs was tested. Specificity of the Ingezim PRRS 2.0 compared to the HerdCheck PRRS X3 was estimated.

RESULTS:
In all pigs of group 1 a seroconversion at day 14 post vaccination was estimated with both ELISAs. One pig was Ab positive already at day 9 tested with the HerdCheck PRRS X3, whereat the Ingezim PRRS 2.0 was near the cut-off (0.38). In group 2, 186 samples (77%) were tested positive for PRRSV Ab with the HerdCheck PRRS X3, 197 samples (80%) were positive with the Ingezim PRRS 2.0. In 220 samples (90%) the result was the same in both ELISAs. This means a kappa of 0.7 (good agreement). Not concordant results were mostly elevated but still beneath the cut-off in one ELISA, while the other ELISA was (weak) positive. All 309 samples of group 3 were tested negative with the HerdCheck PRRS X3. Three of these samples, however, were tested PRRSV Ab positive with the Ingezim PRRS 2.0. The specificity of the Ingezim PRRS 2.0 is therefore 99%. In the wild boars of group 4, only two positive results were found with the HerdCheck PRRS X3 and three positive results with the Ingezim PRRS 2.0. The positive results were not corresponding.

DISCUSSION AND CONCLUSIONS:
Antibodies developed by with PRRSV (NA type) vaccinated pigs were detected by both ELISAs similarly well. There were some slight differences in detection of antibodies in samples of PRRSV positive farms. All positive farms, however, could be reliably detected with both ELISAs. In PRRSV negative farms, a high specificity could be found in the Ingezim PRRS 2.0, although it is not as high as in the HerdCheck PRRS X3. Positive antibody results in unsuspected herds measured with the Ingezim PRRS 2.0 should be confirmed with an alternative test.

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AN OVERVIEW OF SURVEILLANCE OF EQUINE INFECTIOUS ANAEMIA (EIA) IN FRANCE IN 2012, WITH A FOCUS ON THE INVESTIGATION OF TWO CLINICAL EPISODES EPISODES

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Keywords: Equine Infectious Anemia, Horse, Epidemiology

INTRODUCTION:
The equine infectious anaemia virus (EIAV) is a member of the genus Lentivirus of the family Retroviridae. Only equines can be infected by EIAV. Once infected, the animal is infected for life and remains a source of contagion for other equines, even in the absence of any clinical signs. The virus is transmitted primarily via biting flies, but also iatrogenically if non-sterile needles or surgical equipment are used. Insects — mainly horseflies and stable flies — are mechanical vectors: although the virus does not replicate within the insect, the infectious virus can remain in its mouthparts for several hours after biting.

MATERIALS AND METHODS:
Surveillance of EIA in France was performed via the NRL and 12 accredited laboratories. Serological analysis was performed using Agar Gel Immuno-Diffusion test (AGID) as described by OIE chapter 2.5.6. For each positive case, an epidemiological survey was implemented. Genotyping was performed as described by Quinlivan et. al 2007.

RESULTS:
In 2012, French accredited laboratories carried out 15,691 serological analyses of which 27 tested positive. The 27 positive analyses involved eight equines in two distinct outbreaks in two counties: Vaucluse and Gard. The first primary outbreak was declared in January 2012, after confirmation of the infection in a 16 year-old gelding that showed suggestive clinical signs such as fever, nasal bleeding, swelling of the lower abdomen, hind legs and the penile sheath, and pale mucus membranes. This site held seven other horses, three of which were also diagnosed with an EIAV infection on 6 February 2012. The epidemiological surveys covered a six-month period from August 2011 to January 2012 and focused on identifying (1) all horses that had been in ‘contact’ and at risk for EIA infection and (2) all horses present within a 1 km radius of the outbreak site. These surveys revealed that some of the four infected horses had participated in an endurance race in September 2011 in Vaucluse. As a result, more than 180 horses were tested and no cases of infection were detected.

The secondary primary EIA outbreak declared in September 2012 in Gard involved six horses. The index case, which exhibited fevers (40°C) and low haematocrit level (16%), was originally thought to be infected with babesiosis (tested positive), and was treated in consequence. In the absence of improvement and, noting the poor state of its health (massive weight loss of about 100 kg), the veterinarian supposed an EIA infection.

The epidemiological survey was carried out on horses present within 2 km around the outbreak site as well as on all horses that had been in contact with the infected horses during the four months period preceding the confirmation of the outbreak. The surveyed sites and farms, belonging to 67 different owners and involving 378 horses, were placed under surveillance. Of the 378 horses, 2 belonging to the primary outbreak site tested positive for EIA. Isolated strains were genotyped by sequencing the Gag gene (1400 nucleotides long). Virus isolates were characterised from five of the eight EIA positive horses. Phylogenetic tree shows that the isolates from the two different outbreak sites were not only different from each other but also from those identified previously in France (Ponçon et al., 2011). Although epidemiological survey indicate that there was an epidemiological connection between the two outbreaks via the purchase, sale/trade of horses, this hypothesis was not confirmed by the phylogenetic analysis. In contrast, phylogenetic study shows that the Vaucluse and Gard outbreaks had two distinct origins.

DISCUSSION AND CONCLUSIONS:
Given the attenuated and discreet clinical signs of EIA, screening for EIAV should also be considered on a more regular basis when symptoms lead veterinarians to suspect babesiosis.

REFERENCES:
EVALUATION OF AN SCHMALLENBERG VIRUS (SBV) ANTIBODY ELISA FOR ANALYZING ANTIBODIES IN MILK

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Keywords: Schmallenberg virus, milk antibodies, indirect ELISA

INTRODUCTION:
In the late summer of 2011 a new disease syndrome appeared in several European countries characterized as an epizootic outbreak of abortions and stillbirth and, malformations in calves and lambs (1, 2, 3, 4). In November 2011, the causative agent was identified as an Orthobunyavirus of the Simbu serogroup (5). The new virus was named Schmallenberg virus after the location of the first case. A year later, in November 2012, the first case was confirmed in the south-west of Sweden when the SB virus was detected in a lamb (6). To monitor the introduction and spread of the SB virus in Sweden an indirect ELISA to detect antibodies in serum was developed. Since the monitoring programme also included analysis of bulk milk there was a need for an ELISA adapted for serological analysis of milk.

MATERIALS AND METHODS:
The indirect ELISA was based on whole virus antigen and a monoclonal anti-IgG1 labelled with horseradish peroxidise for the detection of serum antibodies to SBV in ruminants. Optimal working dilutions of antigen and conjugate were estimated with checkerboard titrations. An optimal cut-off value (sample value as percentage of positive control value (S/P value)) of 15 % was selected by comparative studies including ROC analysis with a serum dilution of 1:100. An end point titration by 5 fold serially dilution was performed on individual skim milk- and serum samples of cattle and sheep to compare the antibody titres in milk and serum.

RESULTS:
It was found that the ratio of SBV antibodies between serum and milk in analysed sheep sera varied between 10 and 67 and in cattle between 10 and 57. To compensate for the generally lower level of antibodies in milk a dilution of 1:2 was selected for milk compared to the 1:100 dilution of serum. The compliance between serum- and milk antibody titres on an individual basis was confirmed by the SBV ELISA with results expressed as percentage of the positive control serum (S/P %).

DISCUSSION AND CONCLUSIONS:
The indirect SBV ELISA is a useful tool to determine exposure to SBV infection and is very well suited for larger-scale serological bulk milk surveys. The equivalency between milk and serum antibody titres makes the SBV ELISA also useful for milk analysis in individual animals. The possibility of testing for SBV antibodies in milk allows for easier sampling, which also makes collecting samples possible for animal owners.

REFERENCES:
INTRODUCTION:
Ruminant pestiviruses are characterized by high antigenic and genetic variations which may impact not only on diagnostic outcome but also on the vaccine efficacy. Analysis of genetic diversity of BVDV in Polish dairy cattle population in years 2004-2011 indicated that only BVDV-1 species was present with predominance of subgenotypes 1b and 1d (Kuta et al., 2013). Here, the first identification of BVDV-2 in a dairy herd vaccinated with a killed vaccine is reported.

MATERIALS AND METHODS:
Serum samples were collected from all 68 animals in a dairy herd with a history of respiratory problems and deaths of young calves showing signs of mild bloody diarrhea. Vaccination with a killed vaccine was introduced as a prophylactic measure in 2012 with no prior laboratory testing. The ELISA testing for antibodies and viral antigen were used according to the manufacturer (IDEXX Laboratories). Virus isolation was done in bovine kidney cell line (MDBK). Total RNA was extracted using TRI reagent (Sigma-Aldrich). Pooled and individual serum samples were tested using real time RT-PCR with primers and probes specific for BVDV species 1, 2 and 3 within the 5’ untranslated region (5’-UTR) of viral genome (Baxi et al., 2006; Liu et al., 2008). The RT-PCR amplicons were gel-purified and sequenced. Nucleotide sequences were aligned using CLUSTALW software. Bootstrap analysis was carried on 1000 replicates. The sequences were compared with the reference strains available in the GenBank database using the identity matrix in BioEdit v.7.1.9 software. Phylogenetic trees were constructed using the neighbor-joining method of MEGA software (ver. 5.0). Additionally, palindromic nucleotide substitutions (PNS) method was used to confirm the genotypes of positive samples (Giangaspero et al., 2013). This method is based on the comparisons of secondary structure of nucleotide sequences at the variable loci, V1, V2 and V3 within the 5’-UTR.

RESULTS:
Four seemingly healthy animals (twin calves 4 months of age and two 1 year old heifers) were identified as BVDV positive. Calves were devoid of maternal antibodies, despite a history of 2 years of herd vaccination. Virus isolation was positive on the first passage of an ear-notch sample from one calf and from three serum samples of other positive individuals. Genotype analysis of 5’-UTR region of viral genome revealed the presence of BVDV-2a in all four animals with 99.6%-100% homology of nucleotide sequences among them indicating the presence of a single isolate in that herd. Identical results were obtained using PNS software (ver. 2.0) and the genotype was defined as 2a1.6.

DISCUSSION AND CONCLUSIONS:
This is the first report of the presence of BVDV-2 in Polish cattle. In 2013, both Germany and the Netherlands reported severe outbreaks of hemorrhagic syndrome in calves and cows caused by a virulent BVDV-2c. Similar outcome was observed in this herd although the losses were restricted only to young animals and BVDV-2a was identified. However, the economic impact of this infection was significant confirming that this species of BVDV can cause a severe sickness in a herd.

REFERENCES:
INTRODUCTION:
Bovine herpesvirus (BoHV1) and Bubaline herpesvirus (BuHV1) are closely related viruses, belonging to the extensive subfamily of alphaherpesvirinae characterized by a relatively large host range, a short replication cycle and the ability to induce latent infection (1). Both cattle and water buffalo are susceptible to heterologous infection. Water buffalo is the primary host and reservoir of BuHV1 and was recently isolated also in southern Italy (2). Limited information are available on its pathogenic potential (3); moreover, the lack of diagnostic test to distinguish among alphaherpesviruses infections, led the impact of BuHV1 infection in IBR control program difficult to establish. A new commercial discrimination kit, based on BoHV1 and BuHV1 gE antigen secreted protein, is now available for differentiation between the two infections. In this study, preliminary data of seroprevalence of BuHV1 and BoHv1 in southern Italy are presented.

MATERIALS AND METHODS:
619 serum samples from 9 buffaloes herds were collected. Herd size ranged from 50 to 600 animals. 588 sera were from buffaloes >12 months, 18 were from animals between 7 and 12 months, and 13 were < 7 months. An indirect ELISA (Eradikit® BoHV1-BuHV1 Discrimination test) was employed (Fig.1). Microplates were coated with BoHV1 gE recombinant protein (even wells) and an equal amount of BuHV1 gE recombinant protein (odd wells). Sera (1/20) was added and plate incubated for 1 h at RT. After washing step, peroxidase labeled antibody anti - IgG was added and plates incubated as above. Reaction was developed with TMB and stopped with H2SO4. Reactivity of each serum against both antigens was measured and compared: the antigen for which the serum showed increased reactivity ( > 40% of the same samples in the other well) indicates the circulating infection.

RESULTS:
Overall, 448 out of 619 serum (p= 72,3%) reacted to ELISA test. Among these, 381 (p=85%) were reactive to BuHV1, 60 sera (p=13%) showed high absorbance values for both wells and were classified as doubtful (indeterminate), while 7 samples (p=2%) were more reactive to BoHV1 antigen. Farms resulted positive were 8/9 (p=88%). All the sera tested positive for BoHV1 were from > 12 months and were distributed in 4 herds. Furthermore, most of sera resulted positive for BoHV1 (4 of 7) clustered in one herd which also displayed the higher number of doubtful samples (14 out 72, p=19%), compared to an average of less than 10% in the other herds, leading to suppose co-circulation of both viruses. Epidemiological investigation revealed that the presence of beef cattle, farmed in the same herd could be an important associated risk factors for the co-circulation of both viral strains.

Table 1 Results of serological analysis

<table>
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<tr>
<th>Age</th>
<th>Dead/Alive</th>
<th>Indeterminate</th>
<th>Positive for BuHV1</th>
<th>Positive for BoHV1</th>
<th>Overall samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 6 months</td>
<td>4</td>
<td>6</td>
<td>-</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>&gt; 12 months</td>
<td>52</td>
<td>2</td>
<td>133</td>
<td>7</td>
<td>164</td>
</tr>
<tr>
<td>Overall</td>
<td>56</td>
<td>2</td>
<td>139</td>
<td>7</td>
<td>160</td>
</tr>
</tbody>
</table>

DISCUSSION AND CONCLUSIONS:
By date, seroprevalence rate of BuHV1 and BoHV1 was widely documented according cross-neutralization test, or a method combining blocking ELISAs, producing conflicting results. To our knowledge, this is the first application of a discrimination indirect ELISA based on both gE antigens. Preliminary data shows that BuHV1 infection is widespread in southern Italy. Animal age was not statistically associated with the risk of serological positivity but this needs to be weighed against the low sample size. Further studies are needed, aimed to confirm the isolation of BoHV1 from buffaloes tested positive to serological test.

REFERENCES:
DETECTION OF HERPESVIRUS IN DONKEYS (EQUUS ASINUS)

Chiavassa E. [1], Caruso C.[1], De Somma D.[1], Modesto P.[1], Peletto S.[1], Paschero M.[2], Baracco G.[3], Acutis P.L.[1], Masoero L.[1]


Keywords: Herpesvirus, Donkey, Nested PCR

INTRODUCTION:
The Herpesviridae family of viruses contains a broad range of pathogens that have been grouped into the subfamilies Alpha-, Beta- and Gammaherpesvirinae based on both biological and genomic characterization. A number of herpesviruses (HV) have been isolated or detected from a range of equine species, including donkeys. Herpesviruses that infected donkeys include two alphaherpesviruses and three gammaherpesvirus: asinine herpesvirus type 1 and 3 (AHV-1, 3) and asinine herpesvirus type 2, 4 and 5 (AHV-2, -4, -5), respectively. Molecular diagnosis techniques provide a rapid and efficient detection of HV. A PCR assay developed for the general screening of HV, based on regions of highly conserved DNA sequences 1, was used to detect HV in nasal, vaginal swabs and tissues from donkeys.

MATERIALS AND METHODS:
Between March and May of 2014 a total of 26 donkey samples comprising 9 nasal and 7 vaginal swabs, 9 aborted foetuses and 1 foal dead at 40 days were analyzed in this study. Abortions and stillbirths were a prominent aspect of the clinical history of several animals in the same donkey farm. No evidence of respiratory diseases were reported. DNA was extracted from swabs, lung and spleen of foetuses and foal using the ReliaPremTM gDNA Tissue Miniprep System according to the manufacturer’s instructions. Amplification of a region of the HV DNA polymerase gene was obtained by nested PCR using degenerate primers (Table 1)1. PCR conditions are summarized in Table 1 2. Amplification products of the expected size were loaded and checked on 2% agarose gel, purified with a PCR Clean-Up System and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit following manufacturer’s protocols. After purification the labeled amplicons were run on a 3130 Genetic Analyzer (Life Technologies). The sequences were manually edited and aligned using the Seqman software of the Lasergene package (DNASTAR Inc.). The sequences were compared with those available on GenBank using BLAST search.

Table 1: Primers and PCR conditions

<table>
<thead>
<tr>
<th>Primer sequence (5’-3’)</th>
<th>Amplification program</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA – GATCTGGACGGGGYTTATACCC</td>
<td>93°C, 12 min</td>
</tr>
<tr>
<td>E.K – TCTGGAGCAAGGACAGACAGYCTGCTC</td>
<td>94°C, 30 s, x 10</td>
</tr>
<tr>
<td>RGL – GATCTGGTCACGGACAGACACTGCTYCTT</td>
<td>50°C, 30 s, x 45</td>
</tr>
<tr>
<td>TGY – TGTATCTGGTCAYGNTYACNGNNGT</td>
<td>72°C, 60 s</td>
</tr>
<tr>
<td>TGY – CACAGAGTTCGCRTNCRTATGAT</td>
<td>94°C, 30 s</td>
</tr>
<tr>
<td>TGR – GGTGTCGCTGGATTTAGGTT</td>
<td>50°C, 30 s</td>
</tr>
<tr>
<td>TGR – GGTGTCGCTGGATTTAGGTT</td>
<td>72°C, 60 s</td>
</tr>
</tbody>
</table>

RESULTS:
Two nasal and 1 vaginal swabs of 2 female donkeys and lung and spleen samples of the foal resulted positive for HV by nested PCR. Sequences obtained from PCR products showed maximum similarities (>94%) with Nucleotide sequences of the HV PCR AHV-5 and EHV-5. Similarly, both EHV-2 and EHV-5 are ubiquitous in healthy horses, but have occasionally been associated with various syndromes. EHV-2 and EHV-5 were detected in aborted equine foetus, but there is no evidence that gammaherpesviruses has abortigenic potential and that it could be transmitted from mare to foal via vaginal secretion and colostrum3. In summary, gammaherpesviruses were detected in 2 female donkeys (nasal and vaginal swab) and in the lung and spleen of a foal without clinical signs of respiratory disease, further investigation on a larger number of samples are needed to evaluate the real role of gammaherpesvirus as abortigenic agent.

REFERENCES:
INTRODUCTION:
In the Danish SPF system PRRSV surveillance is based upon the ability to differentiate between the American (US, Type 2) and the European (EU, Type 1) strain of PRRSV. Danish swine herds are declared either free from PRRSV or positive to either PRRSV EU or PRRSV US – or both strains. The blocking ELISAs used in this surveillance are only validated for serum (Sørensen et al. 1998). Based on the same antigens (supplied by B. Strandbygaard and A. Bøtner, National Veterinary Institute, Denmark) as in the blocking ELISAs, indirect ELISAs for PRRSV EU and US were optimized for analysis of oral fluid (OF) samples.

MATERIALS AND METHODS:
Samples for validation were obtained from PRRSV positive and negative Danish herds in collaboration with Practitioners from Odder Svinepraksis. OF pen pools were collected by hanging a rope in selected pens. For comparison, blood was drawn from all pigs in each OF-sampled pen. A total of 2551 sera and 281 OF pools were sampled, representing pigs from 15-100 kg. All sera were tested in the PRRS blocking ELISAs used in the SPF surveillance, and these results were used as a gold standard for the novel OF indirect ELISA: A PRRSV-positive pen was defined as a pen with at least 50% pigs positive in the blocking ELISA.

RESULTS:
Performance of the OF tests is plotted in Fig. 1. In the novel US OF ELISA, choosing a pen specificity of 0.97, and a cut off value of 84 (calibrated OD value), the herd sensitivity with 10 pens sampled and a within herd pen prevalence of 0.2 would be 0.83. Likewise in the EU OF ELISA, with a pen specificity of 0.97 and a cut off value of 219 (calibrated OD value), herd sensitivity would be 0.78. This implies that if you take 10 rope samples, i.e. sample 10 pens, in one herd, the herd specificity will be 0.74 for both ELISAs.

As expected, a slight cross reactivity was found between the EU ELISA and the US ELISA. However, use of the abovementioned cut offs results in a reasonable specificity towards the heterologous strain in the two ELISAs. Thus specificity to the US strain in the EU-positive herds, is 74% and specificity to EU in the US herds, is 90%.

REFERENCES:
DEVELOPMENT AND VALIDATION OF AN IMMUNE PEROXIDASE MONOLAYER ASSAY FOR THE DETECTION OF ANTIBODIES AGAINST AFRICAN SWINE FEVER

Loeffen W., Moonen Leusen B., Quak S., Weesendorp E.

CVI ~ Lelystad ~ Netherlands

Keywords: African swine fever, IPMA, Serology

INTRODUCTION:
African swine fever (ASF) is currently a serious threat to the pig population in the EU, after the disease was introduced in Georgia in 2007 and subsequently became endemic in Russia (1). Serological testing is an integral part of the surveillance and diagnosis of ASF. In most laboratories, ELISAs, either commercially available or developed in-house, are used as first method for serological testing. While these ELISAs are mostly highly sensitive and specific, both false positives and false negatives may occur. False positives are usually dealt with through confirmation tests, which combine a very high sensitivity with a very high specificity, but can be laborious or take several days to carry out (e.g. virus neutralisation tests for classical swine fever). At CVI in the Netherlands an in-house immuno peroxidase monolayer assay (IPMA) was developed, based on cell lines and use of viruses that are not adapted to cell culture systems. The development and initial validation of this IPMA is described here.

MATERIALS AND METHODS:
The IPMA is based om MARC-145 cells that are infected with either the Malta ’78 or Brazil ’78 strain, but other strains could be used, according to the local epidemiological situation. The MARC-145 cells were selected after an extensive comparison of several cell lines because of their susceptibility to ASF virus (ASFV) and high-contrast immuno-staining of infected cells. MARC-145 cells are diluted to 2x10^5 cells/ml and inoculated with 0.025 MOI ASFV. Monolayers in 96 wells are prepared by adding 100 μl of this mixture to each well and incubating for 40-48 hours at 37°C. Plates are frozen and stored at -20°C until they are used. Before use, the plates are thawed and fixed with 4% paraformaldehyde. The IPMA as then carried out by adding serum samples in 4-fold dilutions, starting at 1:10, to the monolayers. Antibodies binding to the virus are detected by an anti-swine conjugate. The titre is the reciprocate of the highest dilution in which antibodies can be detected. For initial validation of the test, sensitivity and specificity were determined.

RESULTS:
ASFV is able to infect MARC-145 cells. Transcription of mRNA and production of immunogenic proteins will take place in the cytoplasm, and later-on in the virus factories in the cytoplasm. Apparently there is no assembly of virus particles and/or release from the infected cells though, as only individual cells become infected from the initially inoculated virus and no infectious virus can be detected in the supernatant. Figure 1 shows staining of individual cells. In the picture on the left, the cytoplasm is evenly stained, resulting in a clearly infected cell, surrounded by negative cells. The picture on the right shows staining of virus factories in the cells, which could be mistaken for non-specific staining of debris on the monolayer by less-experienced technicians. To avoid this, virus and cells should be inoculated for a maximum of 48 hours.

The cut-off of the test was initially determined by testing negative serum samples and set to a titre of 10. The sensitivity of the test was determined by testing 224 samples from pigs experimentally infected with different ASFV-strains. From day 9 after infection, the sensitivity was 100%. Very quickly after the first detection of antibodies, the titre usually rises to values of >1,000,000. The specificity was determined by testing a total of 370 pigs from an ASFV-free population and was 100%.

REFERENCES:
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USING ORAL FLUID SPECIMENS FOR SURVEILLANCE OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME: A PRACTICABLE OPPORTUNITY?

Miceli I.[1], Monnier M.[1], Marro S.[2], Zoppi S.[1], Dondo A.[1], Faccenda M.[2], Goria M.[1]


Keywords: PRRS, saliva, RT-nested PCR

INTRODUCTION:
Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases in the swine industry. The control of this disease is being implemented either through vaccination and adopting strict bio-security measures inside and outside farm. Current methods of PPRS surveillance in swine population are primarily based on specific antibodies and viral genome detection in serum specimens. However, blood collection is a labor-intensive and invasive procedure that could be cause of stress to animals. Previous studies evaluated the use of oral fluid sampling as a more efficient, welfare-friendly and cost-effective approach to detect PRRSv circulation in swine populations (1). While the detection of viruses in oral fluid is an attractive possibility, there are concerns about the stability of viral RNA due to the rich presence of several degradative enzymes (Protease, Ribonuclease). To assess the potential diagnostic performances of salivary matrices, we tested different handling methods and nucleic acid extraction procedures to identify the optimum combination for maximizing PRRSv detection.

MATERIALS AND METHODS:
The experimental protocol included 412 individual samples: 206 of saliva and 206 of serum. Samples were collected every 2 weeks, for 6 times, from 35 pigs in a farm with a proven PRRSv infection. In addition, in one shot way, in 7 boxes were placed ropes and pigs actively sough out and chewed the rope, leaving the strands moistened with oral fluid. Oral fluid were extracted from the rope by mechanical compression, held on ice and processed the same day as arrival. Handling procedures of saliva specimens included the use of a stabilizer (RNAlater, Qiagen, added to saliva at the time of sampling) and cooling conditions for transfer in combination with Rneasy kit micro kit (Qiagen); the same set of samples was also collected without RNA stabilizer, same cooling condition, while “MagVet ™ Universal Purification Kit” was applied with semi-automatic KingFisher Extractor (Thermo Fisher Scientific Inc.). Oral fluids specimens obtained from the ropes were collected with and without the RNA stabilizer, at cooling condition for lab transfer and submitted to RNA extraction with the semi-automated MagVet method only. Processed samples were then amplified by RT-nested PCR technique according to Monnier et al. (2).

RESULTS:
The first protocol (RNA later + Rneasy kit micro kit) applied to 206 saliva specimens led to detected 59 samples positive to PRRSs; the second protocol (no RNAlater + semi-automated MagVet extraction kit) recovered 67 PRRSv positive samples out of the same 206. The PRRSv was recovered in 62 serum specimens. The oral fluid specimens, collected from ropes (from 7 boxes) and extracted by semi-automated method, were all PRRSv positive for both handling conditions (w-w/o RNA stabilizer).

DISCUSSION AND CONCLUSIONS:
In this study several combinations of sample handling conditions and RNA extractions were evaluated. The method that has provided, preliminarily, the best performances for extraction and amplification of genomic RNA was the MagVet method combined with RT-nested PCR. The use of the ropes results the best procedure of collecting the liquid of buccal cavity because the fluid is composed of saliva produced by salivary glands and transudate that originates from the circulatory system (3). Our results demonstrated also the importance of maintaining the oral fluid samples in cold and it is recommended an immediate analysis for preventing the degradation of RNA. The results of current study clearly showed improvements in PRRSv diagnosis from swine oral fluid in PRRS surveillance procedures.

REFERENCES:
DEVELOPMENT AND VALIDATION OF A REAL TIME PCR FOR THE DETECTION OF ALL SUBTYPES OF PRRSV TYPE 1 (EUROPEAN GENOTYPE)

Faccini S. [1], Podgórska K. [2], Tomasz S. [3], Boniotti M.B. [4], Lelli D. [4], Dottori M. [5], Nigrelli A.D. [1], Bonilauri P. [5]


Keywords: PRRSV Type1, Real Time PCR, Validation

INTRODUCTION:
Porcine reproductive and respiratory syndrome virus (PRRSV) is a major threat to swine production worldwide and causes huge economic losses in the pig industry. PRRS European (EU, type 1) viruses are currently divided in 3 subtypes: subtype 1 (Lelystad virus-like), 2 and 3 and tentative evidence was found for potential additional subtypes [1]. Divergent subtypes of Type 1 PRRSV have caused high rates of false-negative RT-PCR results in diagnostic tests [2]. Aim of this study was to develop and validate a new Real Time PCR able to detect all known Type 1 PRRSV strains.

MATERIALS AND METHODS:
A library constituted by a 495bp region, covering entire PRRSV ORF7 and 5’ and 3’ bordering regions, of 306 Italian subtype 1 field isolates and subtypes 1, 2, 3 and 4 strains available on GenBank was analyzed to check candidate conserved sequences. The detection limit of the method was assessed by testing serial dilutions of two plasmids, containing the artificially synthetized (MWG, Eurofins Genomics) target region of reference sequences PRRSV Lelystad strain (M96262.2, subtype1, pLely) and Lena strain (JF802085.1, subtype3, pLena). Moreover two cultures of PRRSV type 1 field isolates, with known viral titer, were serially diluted in PRRSV negative sera and tested. A total of 292 sera coming from 8 seronegative farms, 25 sera and tissues coming from 12 artificially infected animals, 16 reference positive Type 1 PRRSV cDNA coming from EPIZONE ring trial, 4 cDNA of Eastern Europe strains (Bor and LT3 subtype 2; Sza subtype 3; Okt subtype and 5 cultures of Italian Type 1 field strains were analyzed in order to assess diagnostic sensitivity (DSe) and specificity (DSP). For testing the field application of the method 97 sera samples collected in two conventional farms during the probable viremic stage of the animals (between 7 and 8 weeks of age) were examined.

RESULTS:
One forward primer, 2 reverse primers and a probe (sequence available on request) were selected as virtually able to detect all subtypes of Type 1 PRRSV strains included in our library. Analytical sensitivity (ASe): the viral LOD of the method, estimated by viral culture dilution, was equal to 3.9 Log(10) TCID50/50μl. Linearity was found (R2=0.99) across seven 10 fold dilutions of the 2 viruses in negative porcine serum. The LOD estimated with plasmid dilution was equal to 10 copies/reaction for pLena and 100 copies/reaction for pLely. Diagnostic sensitivity (DSe) and specificity (DSP): none of the 292 sera coming from 8 seronegative farms gave positive amplification. This confers 100% (CI95% lower limit 98.38%) of DSP. All known positive samples were found positive before Cq38 by the method, conferring 100% (CI95% lower limit 92.87%). Field application: 92 over 97 sera from probable viremic animals were tested positive by the method.

DISCUSSION AND CONCLUSIONS:
This new method can be considered fit for the purpose of Type 1 PRRS strains detection. The application of this methods in field samples from European countries, will improve the validations of this new Real Time PCR developed. Acknowledgements: The work was funded by Italian Ministry of Health research project PR201001.

REFERENCES:
DETERMINATION OF TELOMERASE ACTIVITY AND HTERT EXPRESSION IN BOVINE LEUKEMIA VIRUS (BLV) INFECTED CATTLE

Szczotka M., Iwan E., Kuzmak J., Szczotka A.

National Veterinary Research Institute ~ 24-100 Pulawy, al. Partyzantów 57 ~ Poland

Keywords: bovine leukemia virus (BLV), telomerase, telomeres, Real-Time PCR, FISH

INTRODUCTION:
Bovine leukemia is characterized by a persistent lymphocytosis and B cell malignant lymphosarcoma development after extended latency periods. Telomerase catalytic subunit (hTERT) has been shown to play a critical role not only in telomere homeostasis but also in cellular survival, DNA repair, and genetic stability. The aim of the studies was determination of hTERT expression and telomerase activity in BLV infected animals

MATERIALS AND METHODS:
Telomerase activity was analyzed by Real-Time PCR in blood, lymphatic organs and dendritic cells of leukemic and healthy cows. In the assay cells or tissues (with enzymatic activity of telomerase) were lysed and the telomerase activity was determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate in vitro upon the addition of the appropriate buffer conditions and dNTPs. The PCR products were visualized using sensitive DNA fluorochromes SYBR Green. Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double-stranded DNA. The telomere length and fluorescence intensity was determined with the use of fluorescence in situ hybridization (FISH). The hTERT expression was investigated in immunofluorescence (IF) test with the use of monoclonal antibody.

RESULTS:
In all samples from BLV infected cows high activity of telomerase and expression of hTERT was found. The highest telomerase activity was detected in spleen and bone marrow. In cow with persistent lymphocytosis telomerase activity was the highest in lymph node. The level of telomerase activity showed correlation with hTERT expression and telomere length. Studies have demonstrated that human tumor cell lines and bovine leukemic cells have shorter telomeres than normal tissues and that telomerase was activated in up to 90% of all investigated animal and human leukemic cells, while it was present only in a limited range (very low values) of normal fetal and adult tissues. The role of telomerase in the extension of the cellular replicative lifespan has recently been shown by ectopic expression of the enzyme, being consistent with the oncogenesis model whereby the acquisition of an ‘immortal’ phenotype is a requirement for advanced tumor progression.

DISCUSSION AND CONCLUSIONS:
Telomerase - a telomere – synthesing reverse transcriptase compensates the loss of telomere associated with cell division. hTERT encodes the catalytic subunit of telomerase and is present in most immortalized and cancer cells. Telomeres are important structures for the correct function and stability of chromosomes. Telomerase activity is expressed in most human tumor tissues, but not in normal tissues, except for those of the germline. Telomerase activity and hTERT expression almost always correlate with disease severity in lymphoproliferative disorders. Immunohistochemical detection of hTERT will facilitate exact diagnosis of the telomerase positive cells and expand the application of telomerase in leukemia/lymphoma diagnosis. Immunotherapy of human leukemias and tumors, directed against telomerase-positive cells as well as strategies using a suicide gene promoter targeted to hTERT-expressing cells are still under active investigation and in some instances in early-stage clinical trials.

REFERENCES:
INTRODUCTION:
Herpesvirus of turkey (HVT) belongs to genus Mardivirus of Alphaherpesviridae. In spite of its close relationship to Gallid herpesviruses, chicken can be infected by HVT only artificially (vaccination) and it replicates without causing any lesions. The genome of HVT was found to be ideal for inserting specific protein coding genes of other viruses. Several recombinant HVT-based (rHVT) vector vaccines have been developed (e.g. NDV, AIV, IBDV, ILTV). Its widespread use demands practical and efficient tools for monitoring rHVT vaccine-take, and thus the adequate vaccination coverage of a flock. Feather pulp is the favoured sample for detection of MD viruses; however, we found spleen more reliable for HVT measurement. Handling and transportation of spleen samples from farm to laboratory need special care and cold chain to avoid deterioration. Whatman FTA™ Card has been shown to be suitable for sample storage and transportation without cooling due to its nature of preserving the integrity of nucleic acid. For real-time PCR it is mandatory to extract DNA from the card (in contrast pieces of card can be directly used as template for classical PCR), but it is a challenging task to do it efficiently due to its strong binding to the paper and low copy number of HVT. In this study we compared methods for DNA extraction from chicken spleens containing rHVT printed onto FTA™ Card. Comparative analyses to different organ samples were carried out to evaluate the sensitivity of the method.

MATERIALS AND METHODS:
Spleens of 21-33 days old broiler chickens vaccinated s.c. at day old with a rHVT-ND vaccine (Vectormune® ND, Ceva Animal Health) were bisected and section surface were printed onto FTA Card. Cards were stored at room temperature and transported via mail from different countries. Remaining part of the same spleen samples were stored and transported frozen. In the laboratory, DNA was extracted from cards with two different methods: (i) 3 pieces (approx. 4 mm2 of each) were excised from each spleen print and were pooled. It was digested by proteinase K during shaking at 1000 rpm, 56°C for 1 h, then DNA was purified using QIAmp DNA Mini Kit (Qiagen); (ii) other pool of 3 pieces of the same print was digested by GenSolveTM (GenTegra) reagents (65°C, 1400 rpm, 1 h) according to the manufacturer’s protocol. For comparative analyses spleen samples were homogenized in PBS with antibiotics and digested with proteinase K (1000 rpm, 56°C, 1 h), then DNA was purified as above. Detection and quantification of rHVT vaccine in the samples was determined with insert (NDV F gene) specific real-time PCR assay. All samples resulting specific signal were considered as positive. Ct values of amplification curves were compared statistically (paired T test).

RESULTS:
Ct values of FTA card samples found to be slightly, but significantly higher (1-2 in average) than the ones measured from the organ (spleen) samples, meaning that lower quantity of target DNA could be detected from the same organ samples than from the FTA prints. There was no significant difference between the two methods tested for extraction from FTA cards. Although all of the frozen spleens were found to be positive, 5-10% of the same samples printed on FTA cards were found negative by one or both of the extraction methods.

DISCUSSION AND CONCLUSIONS:
FTA cards provide a convenient and safe tool for diagnostic sample storage and transportation. The advantages of this technology are: (i) the bound pathogens are non-infectious anymore (but it also excludes isolation of them); (ii) no need for cooled transportation; and (iii) saves considerable storage space. However, it has been observed that the detection limit (the amount of target DNA required for positive signal) from FTA-stored nucleic acid samples is higher than those directly from the organs. Therefore, the use of FTA cards for diagnostic purposes needs careful considerations of all the factors. Vaccine take monitoring needs sensitive detection, especially in case of a non-spreading vector as HVT in chicken, because the ratio of properly vaccinated animals has a profound effect on the flock immunity both against the Marek’s disease and insert-related virus. The loss of sensitivity observed in this study can be adjusted by the proper timing of sample collection or repeated sampling.
EQUINE VIRAL ARTERITIS PREVALENCE IN ALGERIA


Keywords: Equine Arteritis Virus, Algeria, Epidemiology

INTRODUCTION:
Equine arteritis virus (EAV) is a single-stranded positive RNA virus that belongs to the Arteriviridae family. EAV is one of the major causes of respiratory diseases in horses [1]. Clinical signs associated to the diseases ranged from hyperthermia to oedema and rash [2]. EAV has been first isolated in 1953 in Bucyrus city, Ohio, USA [3]. In order to determine equine viral arteritis (EVA) prevalence in Algeria, 268 sera from non-vaccinated horses have been collected from the western and eastern regions.

MATERIALS AND METHODS:
Serological analysis of equine sera, collected from 2009 and 2011, was performed using virus neutralization test (VNT) as described by the World Organization of Animal Health (OIE) chapter 2.5.10.

RESULTS:
Overall, twenty sera (7.46%) were detected seropositive, one hundred and fifty two (56.71%) were negative and ninety six sera (35.82%) were cytotoxic. EAV prevalences were significantly higher in the Western region (Tiaret) compared to the Eastern region (Barika and El Eulma). Interestingly, more than 20% of the tested horses over 16 years old were seropositive for EAV. However, EAV prevalence did not depend on either horse breed or horse gender.

DISCUSSION AND CONCLUSIONS:
This study is the first to describe the circulation of EAV in the Algerian horse population. Next step will be to collect semen samples from non-vaccinated seropositive stallions to study virus population circulating in the Algerian horse population.

REFERENCES:
INTRODUCTION:
Pancreatin is a type of digestive enzyme combination produced by pancreas’ exocrine cells from either pigs or cows, used in human to treat pancreatic exocrine insufficiency. A feature common to all biological products obtained from any material of animal origin is the risk of viral contamination. To assure virological safety of biological therapeutics, an approach in which virus control occurs at various stages of the drug manufacture is required with the aim to test the capacity of the production process to remove or inactivate viruses. This can be achieved by spiking the material to be purified with significant amounts of virus, carrying out the step in defined scaled down conditions and determining the virus elimination or inactivation during the concerning step. In this study, the viral removal or inactivation achieved by the final step of drying under vacuum (5 hours at 60°C ± 1°C corresponding to the worst case scenario), during the manufacture of the pancreatin is evaluated.

MATERIALS AND METHODS:
Pathogens have been selected by taking into account the adventitious viruses and because of the necessity to cover a large spectrum for their resistance to the physical - chemical treatments. Pseudorabies virus (PRV), Encephalomyocarditis virus (EMCV), Bovine Viral Diarrhea Virus (BVDV) and Porcine parvovirus (PPV) were tested. Pancreatin was obtained by extraction from activated frozen pancreas. Because of very high cytotoxicity of the product, 0.67 g of dried pancreatin was resuspended in 13.5 ml of culture medium followed by a dilution 50 fold in culture medium. 8 ml of the spiked suspensions were placed in an oven under vacuum at 700 mmHg and the heating was carried out for 5 hours at 60°C ± 1°C. Titration was calculated on cell monolayer by plaque assay method. As a part of the virus validation protocol, cytotoxicity and interference were performed. Reduction factors (log10 of the ratio of the virus load (Li) in the pre-purification material and the virus load (Lf) in the post-purification material) was calculated for each virus.

RESULTS:
Results obtained during the drying under vacuum of the starting material prepared as described above showed that for PRV, EMCV, BVDV and PPV all infectious particles were inactivated in the spiked suspension of pancreatin after 5 hours at 60°C ± 1°C and no plaque were detected after step of heat treatment. Even if PPV was the most resistant virus, the log reduction factor achieved during heat treatment was ≥ 5.59 (Table 1). The unspiked starting material showed no toxicity on the cells used during the titration assays. Test article (pancreatin diluted as previously described) showed no interference on virus replication.

DISCUSSION AND CONCLUSIONS:
On the basis of the reduction factor, it can be concluded that dry-heat treatment was an effective process for inactivating viruses and it could be effective in reduction of viral contamination (Fig.1). Treatment at 58–60°C for 10 hours generally provides, for PPV, a log10 reduction that ranges from a low of 1.0 to at most 3.9. In one case, PPV was not inactivated by treatment at 60°C for 15 hours but literature also reported that treatment at 74°C for 90 minutes provided a log10 reduction of 8.4 with a complete inactivation[2]. Even if in the past pancreatin was a source of hospital acquired salmonellosis[3] to our knowledge no case has been reported where patients have been actually affected by pancreatin contaminated by any virus. However, companies producing animal derived pharmaceutical products should increase the level of safety of their products by reducing contamination for endogenous, non endogenous and adventitious virus, above all against zoonotic viral agent, such as Hepatitis E virus (HEV) and Norovirus (NoVs) considered emerging pathogens.

REFERENCES:

Tab.1. Reduction factor obtained for each virus
INTRODUCTION:
Ostreid Herpes virus 1 μναν is a DNA virus, not pathogenic for humans, which is part of the family Herpesviridae. Since 2008 in Europe and particularly in France, cases of unexplained mortality were recorded in farms of Crassostrea gigas due to this virus which in some cases have involved 100% of the farm (Final Report OsHV-1 μναν, International Workshop). The aim of this work was to implement the method in PCR provided by Regulation 175/2010 issued by the European Commission, and acquire information on the presence of factors that may cause mortality in oysters reared in marine areas of the Ligurian Sea. In particular, the project aims to collect and organize information about the possible correlation between the presence of OsHV-1 μναν, environmental parameters and shellfish pathogens isolated from juvenile oysters collected in the breeding areas.

MATERIALS AND METHODS:
For the implementation of the PCR method, two extraction methods and two Taq Polymerase enzymes of different commercial brand were compared. Moreover from May 2011 to February 2014, 205 oysters samples were collected monthly, 149 of French and 56 of Italian origin, recording the values of the environmental parameters and of toxic algae in the farming area. All samples were subjected to morphological survey and analyzed for OsHV-1 μναν, V. parahaemolyticus, V. cholerae, V. algynolitucus, V. aestuarianus, V. Harvey, V. splendidus, Salmonella and E. coli. At the end of the analysis statistical tests were performed to correlate all the data of the environmental parameters, algae and Vibrio with OsHV-1 μναν in the presence and absence of die-off.

RESULTS:
46.8% (96/205) of the samples tested positive for the presence of the genome OsHV-1 μναν; 8.8% (18/205) of the oysters were positive for the presence of the genome of V. aestuarianus and 0.97% (2/ 205) for V. harveyi. 100% of the samples were negative for the presence of V. splendidus, V. cholera, V. parahemolyticus while the values of Salmonella and E. coli are always below the limits stes for class B breeding water.

DISCUSSION AND CONCLUSIONS:
The extraction method most appropriate that has ensured the highest yield was found to be the commercial kit based on ion-exchange resins, and the PCR method used has proved to be robust as the two commercial Taq Polymerase showed no significant differences. Regarding monitoring, the only one die-off occurred in a sample of juveniles from France in November 2013, in the days immediately following the placing in the farming area. This sampling was simultaneously positive for both OsHV-1 μναν and Vibrio aestuarianus; statistical analysis showed a positive correlation between the presence of Vibrio aestuarianus, OsHV-1 μναν and die-offs. On the contrary, in the other oysters from France albeit positive to the genome of OsHV-1 μναν, only one sample was positive at the same time for Vibrio aestuarianus and has not occurred any outbreak episode. The survey results show that the simultaneous presence of OsHV-1 μναν and Vibrio aestuarianus in oysters can become an economic problem for local farmers and here comes the need to monitor the juveniles coming from endemic areas and to establish a zone of containment for juveniles imported.

REFERENCES:
IN VITRO EVALUATION OF ANTIVIRAL ACTIVITIES OF THERMOPSIS TURCICA AND LIMONIUM ICONICUM

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Keywords: Thermopsis turcica, Limonium iconicum, MTT

INTRODUCTION:
The aim of present study was to examine the anti-herpes simplex virus type 1 (HSV-1) activity of methanolic and aqueous extracts from Thermopsis turcica and Limonium iconicum growing in Turkey.

MATERIALS AND METHODS:
Both methanol extracts and aqueous extracts were tested by means of the MTT assay (tetrazolium-based colorimetric assay). The EC50 was defined as the concentration required to achieve 50% protection against virus-induced cytopathic effects, and the selectivity index (SI) was determined as the ratio of CC50 (concentration of 50% cellular cytotoxicity) to EC50.

RESULTS:
Results showed that the aqueous extract of T. turcica and methanolic extract of L. iconicum possessed weak anti-HSV activity (EC50 = 1116 µg/ml, SI = 3.13 for the aqueous extract of T. turcica; EC50 = 14529 µg/ml, SI = 1.31 for the methanolic extract of L. iconicum) when compared to acyclovir (ACV) using as positive control (EC50 = 0.749 µg/ml, SI = 14.69).

DISCUSSION AND CONCLUSIONS:
The selectivity index of this extracts were low, due to their high toxicities. In contrast, the methanolic extract of T. turcica and aqueous extract of L. iconicum were not possessed anti-HSV activity.

REFERENCES:
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HERPESVIRUS DETECTION IN SEA TURTLES: PRELIMINARY DATA


Keywords: sea turtles, Caretta caretta, herpesvirus

INTRODUCTION:
Many important diseases of sea turtles are associated to infection by herpesviruses. This study was aimed to investigate the presence and diversity of herpesvirus in Mediterranean sea turtles (Caretta caretta) stranded on Sicilia.

MATERIALS AND METHODS:
A total of 100 tissues belonging to thirty-two sea turtles (Caretta caretta) stranded during May and August of 2014 on Sicilia were analyzed by nested consensus polymerase chain reaction (PCR) for herpesvirus (HV) (1). It has been used in different classes like mammals ( human (1), porcine (2), dolphin (3)), avian (4) and reptile (5). DNA was extracted from tissue samples (liver, lung, brain, and intestine) and swabs (cloaca and mouth) by DNeasy Blood & Tissue Kits (Qiagen) following the manufacturer’s instructions. This one is a consensus primer PCR method which amplifies a region of herpesviral DNA-directed DNA polymerase and which uses degenerate primers in a nested format was developed. Consensus primer PCRs (50 µl) contained 10 µl of viral template DNA, 1 mM (each) PCR primer, 200 mM (each) deoxynucleotide triphosphate, and 1.25 U of Taq polymerase and 10x Buffer from Taq PCR Core Kit (Qiagen). The reactions were started with 3 min at 94 °C, followed by 45 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 46°C, and 60 s of strand extension at 72°C in a Veriti® Thermal Cycler (Applied Biosystems). After cycling, the reaction mixtures were incubated for 5 min at 72 °C and were then held at 4°C. Primary PCR mixtures contained two upstream primers (DFA, 5'-GAYTTYGCNAGYYTNTAYCC-3'; and ILK, 5'-TCCTGGACAAGCAGCARNYSGCNMTNAA-3') and one downstream primer (KG1, 5'-GTCTTGCTCACCAGNTCNACNCCYTT-3') in a multiplex format. Secondary PCRs were performed on 10 µl of the primary PCR mixture in a 50-µl volume with upstream primer TGV (5'-TGTAACCTCGGTGTAYGGNTYACNGGNGT-3') and downstream primer IYG (5'-CACAGAGTCCGTRTCNCCRTADAT-3') under the same conditions used for the primary reaction. Secondary PCR products were analyzed on a 2% agarose gel made up in 1x TBE and containing 0.05 µg of ethidium bromide per ml. The amplified region of 190 to 250 bp was confirmed by sequencing and BLAST was used to compare Herpesvirus sequences in the GenBank.

RESULTS:
Six samples were found positive for Herpesvirus within these 100 samples, confirming this methodology works even for sea turtles.

DISCUSSION AND CONCLUSIONS:
More herpesvirus infections have been documented in chelonians (turtles and tortoises) than any other taxa of reptile. The herpesvirus polymerase is the gene for which the most comparative sequences from multiple herpesviral species are available. These data suggest that consensus primer PCR targeted to herpesviral DNA polymerase may prove to be useful in the detection and identification of known herpesviruses in samples from sea turtles and the initial characterization of new herpesviral genomes.

REFERENCES:
POSTER PRESENTATIONS

OTHER ANIMAL DISEASES
INTRODUCTION:
Members of the genus Echinococcus are helminth parasites (phylum Platyhelminthes, class Cestoda) that cause echinococcosis, a zoonosis with a worldwide distribution that can be transmitted through a variety of domestic, synanthropic, and sylvatic cycles. The adult worm of the genus Echinococcus lives in the small intestine of suitable canids. The red fox (Vulpes vulpes) is widely distributed and abundant. This generalist carnivore predates upon a wide range of wild and livestock species and plays an important role in disease transmission. In particular red foxes are known to harbour parasites of zoonotic importance (1). The wolf (Canis lupus) is a wild carnivore species present in Italy. The habitats of this predator overlap rural human communities, where wolves have access to domestic ungulates, their main prey. On such occasions with a close contact between wolves, domestic animals and humans, transmission of pathogens, especially those whose life cycle is based on predator-prey interactions, might occur (2). Susceptible intermediate host (ungulate and rodent) species that accidentally ingest infective eggs will develop the parasite’s larval stage or metacestode. Humans are dead-end hosts that do not play a role in the natural cycle of the parasite (1). The aim of this study was to evaluate the involvement of the population of fox and wolf as definitive host of Echinococcus spp. through the development of an integrated diagnostic approach consisting of manual method, Sedimentation and Counting Technique (SCT) and Real time PCR.

MATERIALS AND METHODS:
During the period 2009-2013, carcasses of red foxes hunted or found dead (n = 70) sourced from Valle d’Aosta and Liguria regions were submitted to necropsy. In most cases, during the examination the entire intestinal tract was removed and stored at -80°C for 7 days in order to prevent any risk of infection. Occasionally faeces were collected. Moreover, in 2013-2014 three intestines of wolf from Liguria, one sample of faeces from a wolf sourced from Puglia and 50 stool samples of wolf collected in Piemonte region, were delivered to the laboratory. All the intestines were subjected to SCT, the gold standard method for determination of E. multilocularis in definitive host (3). The obtained sediment fractions and faeces were subjected to DNA extraction with QIAamp DNA mini kit. On the nucleotide sequence of EG95 gene, the software Primer Express has identified a pair of primers and a TaqMan probe optimal for the development of a Real time PCR, that was performed to establish the presence of Echinococcus spp. Specificity and sensitivity of this method were evaluated respectively on negative controls (Taenia multiceps, Mesocestoides spp.) and positive controls (E. multilocularis, E. granulosus).

RESULTS:
All examined sample resulted negative at SCT, but parasite taxa were recovered from red foxes (Strongylidae, Ascaridae, Trichuridae, Taeniidae, Capillariidae, Ancylostomatidae, Coccidia) and wolves (Taeniidae). Real time PCR showed a good sensitivity (limit of detection 5.95 pg DNA) and specificity (it do not recognised genus other than Echinococcus). Real time PCR has given a total concordance of results with SCT (all intestines from Valle d’Aosta and Liguria resulted negative). Only the fecal sample sourced from Puglia was diagnosed Echinococcus spp.-positive.

DISCUSSION AND CONCLUSIONS:
In conclusion, the adopted diagnostic approach is a valuable way to improve the understanding of the parasite circulation in wild canids. Further research on wild hosts will be useful in clarifying the transmission patterns of Echinococcus spp.

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MOLECULAR SCREENING OF ANIMAL TISSUES FOR THE DNA DETECTION OF COCCIDIAN PARASITE BY NESTED AND MULTIPLEX PCRS

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Keywords: Nested PCR, Coccidian parasites, Multiplex PCR

INTRODUCTION:
In Sicily, abortion episodes occur each year with economic loss up to 40% in some farms. The rural farm management and shared pastures make difficult the infection control. Multiplex PCR can be a useful tool in molecular diagnosis of infectious diseases for the simultaneous detection of different pathogens in a single reaction tube. The aim of this work was the comparison of results obtained by nested PCRs with a multiplex PCR (MPX-PCR HNT) for Hammondia, Neospora and Toxoplasma in a molecular screening of fetal tissues from abortion cases. Additional tissue and food samples, positive by Toxoplasma specific nested PCR, were included in the comparison of analysis.

MATERIALS AND METHODS:
DNA extraction was performed after tissue homogenization using the kit GenElute (Sigma), following manufacture's instruction. Single nested PCR for Hammondia and Toxoplasma (Sreekumar et al., 2005), and for Neospora and Toxoplasma (Vitale et al. 2012) were performed. Multiplex PCR MPX-HNT Hammondia, Neospora, Toxoplasma, was performed according to Tramuta et al. 2011. DNA sequencing was performed with the kit Big Dye Terminator V3.1 and loaded in 3130-4 Genetic Analyzer (Applied Biosystems).

RESULTS:
Analysis for Toxoplasma and Hammondia: The DNA from a swine brain that presented a cyst by histology analysis, resulted positive to Toxoplasma by nested PCR(Fig. 1). The same sample analyzed by multiplex HNT resulted negative for both parasites.

Analysis for Toxoplasma and Neospora: in the first PCR an amplicon of almost 1000bp is obtained. Enzymatic restriction distinguishes between the two parasites (Vitale et al. 2013). In figure 2 is shown the analysis of fetal tissues from ruminant abortion cases by nested PCR. The results' comparison was performed on positive and negative fetal tissues. 30 Toxoplasma and 10 Neospora positive plus 50 negative samples were assayed with MPX HNT. All results on fetal tissues were confirmed. 10 positive tissues from adult animals, plus a sausage sample that had caused human acute toxoplasmosis (Vitale et al. 2014), resulted negative in MPX HNT.

The results obtained on ruminant abortion cases in Sicily for the three parasites were as follows: on a total of 30 bovine samples 1 Toxoplasma and 1 Neospora positive were detected. 5/42 sheep and 3/19 goat samples were positive for Toxoplasma. No positive samples for Hammondia were detected.

All positive samples were confirmed by sequencing.

DISCUSSION AND CONCLUSIONS:
The comparison of results obtained by nested PCR for each specific parasite, Hammondia, Neospora and Toxoplasma with a multiplex PCR (MPX-PCR HNT) in a molecular screening of animal tissues, showed a higher sensitivity of nested PCR in the chronic stage of infection. On fetal tissues the same results were obtained by both PCR methods probably because the abortion is a consequence of active parasitic multiplication (the acute stage). For this reason MPX PCR, can be a useful tool for a more rapid diagnosis and a better management of the abortion episodes.

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Acknowledgement: Grants RCIZSi 08/10 ed RCIZSi 15/11 to M. Vitale from the Ministry of Health. Dr. A. Altavilla and Dr M. La Giglia are acknowledged for scientific and technical support.
IMPROVED DIAGNOSTIC PERFORMANCE OF A COMMERCIAL ANAPLASMA ANTIBODY COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY USING RECOMBINANT MAJOR SURFACE PROTEIN 5–GLUTATHIONE S-TRANSFERASE FUSION PROTEIN AS ANTIGEN


Keywords: Anaplasma antibody, competitive blocking enzyme-linked immunosorbent assay, diagnostic specificity and sensitivity

INTRODUCTION:
Anaplasmosis is a tick-borne disease of ruminant livestock in tropical and subtropical regions caused by rickettsia of the genus Anaplasma, including A. marginale, A. centrale, A. ovis, and A. phagocytophilum. As Anaplasma invades and multiplies within mature erythrocytes, acute disease is manifested with anemia, weight loss, abortion, and death in infected cattle. In animals that survive acute disease, Anaplasma causes life-long persistent infection. These persistently infected animals are clinically healthy, but serve as reservoirs for continued transmission of the pathogen to other animals. Therefore, control of Anaplasma infection is enhanced by identification of carrier cattle using a specific and sensitive serodiagnostic assay. In the current study, the hypothesis was tested that removal of MBP from the recombinant antigen used for plate coating in commercial cELISA would further improve the specificity.

MATERIALS AND METHODS:
Sera from Anaplasma noninfected cattle (n = 358) were collected as true negative set from dairy herds maintained in barns free of ticks that transmit Anaplasma. Anaplasma-positive sera (n = 135) were obtained as true positive set from cattle with positive results by both serology and nested PCR assays. An additional 163 sera were selected as possible false positive set from diagnostic samples submitted to the Washington Animal Disease Diagnostic Laboratory. Commercial rMSP5-MBP cELISA and novel rMSP5-GST cELISA were evaluated using three sets of sera in relative diagnostic performance.

RESULTS:
The number of 358 sera with significant MBP antibody binding (≥30%I) in Anaplasma-negative herds was 139 (38.8%) when tested using the rMSP5-MBP cELISA without MBP adsorption. All but 8 of the MBP binders were rendered negative (<30%) using the rMSP5-MBP cELISA with MBP adsorption, resulting in 97.8% specificity. This specificity was higher than some previous reports, so to improve the specificity of the commercial cELISA, a new recombinant antigen designated rMSP5–GST was developed, eliminating MBP from the antigen and obviating the need for MBP adsorption. Using the rMSP5-GST cELISA, only 1 of 358 Anaplasma-negative sera, which included the 139 sera with significant (≥30%I) MBP binding in the rMSP5-MBP cELISA without MBP adsorption, was positive. This resulted in an improved diagnostic specificity of 99.7%. The rMSP5-GST cELISA without MBP adsorption had comparable analytical sensitivity to the rMSP5-MBP cELISA with MBP adsorption and had 100% diagnostic sensitivity when tested with 135 positive sera defined by PCR. Further, the rMSP5-GST cELISA resolved 103 false-positive reactions from selected sera with possible false-positive reactions obtained using the rMSP5-MBP cELISA with MBP adsorption and improved the resolution of 29 of 31 other sera.

DISCUSSION AND CONCLUSIONS:
The rMSP5-GST cELISA resolved 3 types of problems observed in the rMSP5-MBP cELISA, including MBP binders, nonspecific binders of unknown mechanism, and sera with %I near the cutoff (25–35%). The improved cELISA maintained reliable analytical sensitivity and specificity in addition to producing 100% diagnostic sensitivity and 99.7% diagnostic specificity using the cutoff of 30%I determined by ROC analysis. Based on the high diagnostic performance demonstrated in the current study, the rMSP5-GST cELISA appears to be a simpler and more reliable serodiagnostic tool for bovine anaplasmosis with various applications including epidemiological monitoring and disease/disease-free certification.

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PERFORMANCE EVALUATION OF THE ELISA KIT PRIONICS PRIOCHECK® BESNOITIA AB 2.0 FOR SEROLOGICAL DIAGNOSIS OF BOVINE BESNOITIOSIS

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Keywords: Besnoitia, ELISA, Diagnostics

INTRODUCTION:
This publication describes the performance evaluation of the PrioCHECK® Besnoitia Ab 2.0 realized by the French Departmental Veterinary Laboratory of Ariège (LVD 09) using on one side, samples from the national serum bank established by the National Steering Committee for bovine Besnoitiosis and on the other side, samples selected by LVD 09. The LVD 09 is accredited by Cofrac for analysis in animal immune serology with a scope of accreditation available on www.cofrac.fr. Until now, Besnoitiosis is not part of its scope of accreditation, but LVD 09 applies the same procedures and the same validation criteria. Due to their respective expertise in the field and their geographic proximity, the LVD 09 and the National Veterinary School of Toulouse (ENVT) are combined to form a laboratory expert group under the umbrella of the National Federation of Sanitary Defense Groups (FNGDSB).

MATERIALS AND METHODS:
The performance measurement of the PrioCHECK® Besnoitia Ab 2.0 was made from 524 serum samples, in which 452 are from the national serum bank established by the France National Steering Committee for bovine Besnoitiosis and 72 around the threshold of the ELISA kit are chosen. Together 196 positives and 328 negatives confirmed by Western Blot, which performed by ENVT are used for the evaluation. The results are interpreted as indicated in the technical insert.

RESULTS:
Diagnostic Sensitivity(DSe) was calculated from known infected animals whose Western Blot analyzes showed a positive result. For the calculation of DSe, 196 cattle were selected. Out of the 196 Western Blot positive samples, the PrioCHECK® Besnoitia Ab 2.0 ELISA kit detected 166 animals as positive, 21 as doubtful and 9 as negative. To calculate the DSe, doubtful results were considered as positive results. DSe is equal to: 187/196 x 100 = 95.4% [92.5-98.4%]. Diagnostic Specificity(DSp) was calculated from 328 animals recognized as free and whose Western Blot analyzes showed a negative result. Out of the 328 Western Blot negative sera, the PrioCHECK® Besnoitia Ab 2.0 ELISA kit detected 288 animals as negative, 27 as doubtful and 13 as negative. To calculate the DSp, doubtful results were considered as negative. DSp is equal to: 315/328 x 100 = 96.0% [93.9-98.1%]. Negative Predictive Value (NPV) and Positive Predictive Value (PPV)
The NPV is the probability that a negative ELISA defines an animal as noninfected with Besnoitiosis. The PPV is the probability that a positive ELISA defines an animal as infected with Besnoitiosis. The NPV is calculated by TN/(FN+TN)*100= 97.2%, PPV TN/(FP+TP)*100= 93.5% (TN/TP: “true negative/positive; FN/FP: “false negative/positive”).
The repeatability of the ELISA kit was performed by analyzing 92 samples of internal reference material, where the PP is close to the doubtful area of the ELISA kit. The repeatability test was also used to evaluate the effect of edges. The calculated CV% of repeatability appears acceptable as in all cases lower than 10%. The reproducibility of the ELISA kit was performed by analyzing 3 samples on different microplates at different positions and different days. Samples with PP around the doubtful area of the ELISA kit were selected. The obtained CV% values of reproducibility around 5% (4.6%, 5.1% and 5.5%) suggesting the kit reproducible.

DISCUSSION AND CONCLUSIONS:
In this study, the PrioCHECK® Besnoitia Ab 2.0 showed a slight lack of sensitivity (9 false negatives) and a negative predictive value (NPV) of 97.2% and a positive predictive value (PPV) of 93.5%. It is important to note that this kit, in addition to its good sensitivity and specificity, is repeatable and reproducible.
INTRODUCTION:
Systemic response to heavy exertion that has been identified in sport horses is hypothesized to result from exercise-induced injuries within musculoskeletal system. This response usually does not manifest clinically, unless, unless the effects of microinjuries accumulate or worsen with further exertion (1,2). The major equine acute phase protein, serum amyloid A (SAA) has been proposed as blood marker of exercise-induced acute phase response (APR) in endurance horses (2,3). Accuracy of SAA in the evaluation of APR following racing effort remains unclear, as total course of the response has not been investigated, as well as the dynamics of APR in horses with clinical post-exercise orthopedic disorders. The aim of this study was to determine changes in SAA concentration following exercise of high intensity in healthy Thoroughbred racehorses and horses that sustained clinical orthopedic injuries. Relevance of SAA as a potential marker of orthopedic injury was discussed.

MATERIALS AND METHODS:
Routine hematological parameters and SAA concentration were measured in blood samples collected daily during 6 days after race or speed training session from 15 horses diagnosed with acute orthopedic injuries and 10 healthy control horses. Routine hematological analyses were performed with automated analyzer (Horiba, Japan). SAA concentrations were measured using enzyme linked immunosorbent assay (TRIDELTA Ltd., Ireland).

RESULTS:
Typical post-exercise changes in hematological parameters were observed in both groups. There were no significant differences among mean SAA levels determined for respective days in each group and between the groups, probably due to large individual variations and subsequent high SD value. All detected SAA levels remained within reference range. However, in 8 healthy and 9 injured horses SAA decreased 2-25 times between the peak level observed on the 2nd day and 5th day after race, resembling the pattern typical for APR. SAA concentration at the 3rd-4th day after the race seems to be a fairly accurate diagnostic test for injured horses. Area under receiver operating characteristic curve (AUC) for this test is 0.767 (CI 95%: 0.574, 0.959) and significantly outweighs AUC for the test based on random selection (p=0.027). At the cut-off of 2000 ng/ml sensitivity and specificity of the test are 67.7% (CI 95%: 41.7%, 84.8%) and (CI 95%: 49.0%, 94.3%), respectively.

DISCUSSION AND CONCLUSIONS:
Exercise-induced APR in racehorses is reflected by minimal changes in SAA serum concentration and lasts up to 5 days. There are no significant differences in the changes of SAA level in healthy horses and horses with clinical orthopedic injuries, although response observed in injured horses appears to be more pronounced. SAA level on a 3rd and 4th day after high-intensity exercise may be investigated as the indicator of damage of musculoskeletal system caused by heavy loading.

REFERENCES:
INTRODUCTION:
Halicephalobus gingivalis is a free-living nematode belonging to the order Rhabditida affecting equidae and humans. Commonly it is found in association with soil, manure and decaying organic matter. It is characterized by rhabditiform esophagus, dorsoflexed ovary and ventroflexed vulva. Opportunistic infections are not frequent but they were reported in different countries. The etiopathogenesis is unknown; probably it penetrates through oral and nasal mucosa or skin wounds, although a possible prenatal or perinatal transmission was suggested in two foals. The most affected organs are oral and nasal cavities, brain, kidney, lymph nodes and adrenal glands. A definitive diagnosis is possible in vivo by biopsy in the accessible nodular lesions or post-mortem by histology. The present study show the first case of H. gingivalis infection in Piedmont, Italy.

MATERIALS AND METHODS:
In August 2012, a thirteen-year-old Koninklijk Warmbloed Paard Nederland stallion, residing in a farm in the Province of Turin, was submitted to clinical examination after a two days history of a severe and rapidly progressive neurological disorder. The horse presented with right head tilt and circling, depression alternated with excitability, fever and lateral strabismus. Drug treatment was performed, but the status of the horse continued to deteriorate. The stallion, suspected of West Nile virus infection, was euthanized and necropsy was performed. At postmortem examination all organs appeared normal on gross evaluation and only head and blood samples were sent to Neuropathology Laboratory of IZS in Turin for investigations. The brain was sampled: one portion was frozen at -20°C for biomolecular analyses and the other one was fixed for histology. The blood sample was assayed with an ELISA kit competitive IgM trade to exclude an early infection of the equine. To isolate and identify the parasite, portions of frozen brain, were digested at 37° C, for 48-72 hours with artificial gastric juice. To obtain DNA, same parasites were subjected to three cold-heat treatments in order to break their cuticle. The DNA was extracted and amplified using primers previously reported by Nadler et al (2003) targeting a 675 bp fragment of the LSU rDNA. Amplicons were sequenced and MEGA 6 was used for phylogeny inference according to the maximum likelihood criterion using the newly generated and reference sequences. Nucleotide substitution models were evaluated using jModelTest2 and the robustness of the hypothesis was tested in 1000 non-parametric bootstrap analyses.

RESULTS:
Neuropathological findings were consistent with a verminous meningoencephalitis predominantly affecting the basal ganglia and thalamus. A large numbers of macrophages, lymphocytes, eosinophils and multinucleated giant cells formed perivascular cuffs surrounded by malacic areas infiltrated with many gitter cells and with evident axonal spheroids. Several larvae and eggs of H. gingivalis are dispersed throughout affected tissue. The blood resulted negative for WN virus. The molecular analysis and phylogeny are in progress and the results will be presented at the Congress.
WHAT ARE THE MAJOR RESPIRATORY PATHOGENS DETECTABLE BY REAL-TIME PCR ON DEEP NASOPHARYNGEAL SWABS OF CLINICALLY HEALTHY CALVES?

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Keywords: Real-time multiplex PCR, Respiratory Pathogens, Nasopharyngeal swabs, Clinically Healthy Calves

INTRODUCTION:

Since 2010, we use in our laboratory in-house molecular tools (multiplex real-time PCR) (1) associated with a supplier kit (LifeTechnologies) for a rapid and simultaneous diagnosis of 7 major pathogens involved in acute Bovine Respiratory Disease (BRD) of young cattle: BoRSV, parainfluenza-3 virus, bovine respiratory coronavirus (BRC), Pasteurella multocida, Mannheimia haemolytica, Mycoplasma bovis and Histophilus somni. Improving the diagnosis has contributed to increase the apparent prevalence observed for each pathogen in diseased calves compared to the prevalence obtained with more traditional analysis techniques (1). Moreover, the apparent prevalence obtained by PCR for Pasteurella multocida and BRC appear higher on deep nasopharyngeal swabs (NS). (Figure 1).These results, combined with the fact that NS remains the most simple sampling procedure on live animals, led us to question the diagnostic value of a positive PCR result for a sample of upper respiratory tract: detection of commensal bacteria and healthy carriage of BRC or actual involvement in the pathology observed?(2).The aim of our study was then to determine the nature of the pathogens that could be detected by PCR in clinically healthy calves to help the practitioner with the interpretation of a positive PCR result on NS of diseased calves.

MATERIALS AND METHODS:

We performed a field study in collaboration with ZOETIS. NS were performed by two veterinarians to ensure reproducibility. After sampling, the swabs were placed in positive cold and frozen within 24 hours. 54 calves of beef breeds, aged on average 60 days, without clinical signs of respiratory disease (absence of hyperthermia, dyspnoea, cough or nasal discharge) were collected in July 2013 (30 calves taken in meadow, in 5 different herds) and January 2014 (24 calves sampled in stable in 2 herds). Real-time PCR were performed simultaneously as described previously (1,2)

RESULTS:

The 54 NS were all found negative for BoRSV, PI-3, BRC and M.bovis apart one NS found positive for BRC in herd n°6. Positive results were obtained for Pasteurellaceae for all herds except herd n°3 (2 NS). (Table I)

DISCUSSION AND CONCLUSIONS:

None of the pathogens described to initiate BRD in young cattle (BoRSV, PI-3 virus, M.bovis) was found in this study. Similarly, BRC was detected on only one NS. These results are in agreement with the “clinically healthy” status of the selected calves and seem to confirm the diagnosis value of a positive result for BRC on NS of diseased calves. The 3 members of Pasteurellaceae family, commensal bacteria of the nasopharynx, were detected. The frequency of detection of Pasteurellaceae with a strongly positive result (in our laboratory Ct values <35) is generally higher in winter than in summer. The contained atmosphere promotes bacterial multiplication in the naso-pharynx and persistence in the environment. Furthermore, P. multocida was found more frequently in this study, with higher positivity levels than M.haemolytica and H.somni. As the PCR used have comparable efficiencies (1), the explanation for this difference lies either in the difference of the quantity of each species actually presents in the naso-pharynx, or in a herd effect, or in the nature of PCR target. In conclusion, the detection of commensal bacteria in the naso-pharynx of clinically healthy calves is not surprising (3) but these results lead us to recommend deeper samples like TA and to express reserves when P.multocida is detected by PCR on NS of diseased calves.

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INTRODUCTION:
Exercise-induced acute phase reaction (APR) is the systemic response to heavy exertion that involves release of acute phase proteins (APPs) into the blood. Although these changes are analogous to typical APR observed in inflammation, the number of APPs involved and changes in their concentrations differ between these two reactions. Serum amyloid A (SAA) is the only APP reflecting APR resulting from strenuous exercise in horses. Our previous studies indicated that in endurance horses after completion of long-distance (120-160 km) rides SAA increases significantly about 10 times when comparing to the pre-exercise values. Such reaction did not occur in horses competing regularly on limited distances (60 km and shorter). The aim of this preliminary study was to investigate effect of moderate distance (80 km) ride on SAA concentration in endurance horses.

MATERIALS AND METHODS:
6 healthy Arabian horses were tested for routine hematological parameters and SAA concentration before and after completion of 80-km (N class and 1*) endurance ride. Hematological analyses were performed with automated analyzer (Horiba, Japan) and SAA concentrations were measured using enzyme linked immunosorbent assay (TRIDELTA Ltd., Ireland).

RESULTS:
In all horses before and after the competition SAA level remained within reference range (0-20 mg/l). After the ride, moderate increase (up to 3x) in the mean SAA concentration was observed, however, the reaction varied substantially among the individuals. Typical exercise-induced changes in leucogram parameters were reported in all horses.

DISCUSSION AND CONCLUSIONS:
Our results indicated the possibility of exercise-induced APR after competing at 80 km endurance ride, however the pattern of the reaction largely differed among individuals. It has been proven that horses prepared for longer distances do not express exercise-induced APR when performing below their athletic capability. Then, competing on the same distance may elicit different reactions depending on the level of fitness. Horses participating in this competition came from several training centers and had various experience in the rides. It seems likely that post-exercise SAA concentration may be related to the fitness level of the horse. To confirm this hypothesis the studies on larger and homogenous groups will be performed.

REFERENCES:
5-LOX EXPRESSION IN THE BRAIN TISSUE OF STRANDED STRIPED DOLPHINS (STENELLA COERULEOALBA) AND BOTTLENOSE DOLPHINS (TURSIOPS TRUNCATUS), WITH OR WITHOUT INFECTIOUS ENCEPHALITIS/MENINGOENCEPHALITIS

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Keywords: 5-LIPOXYGENASE, STRANDED DOLPHINS, MORBILLIVIRUS, TOXOPLASMA GONDII, BRUCELLA CETI

INTRODUCTION:
Dolphin Morbillivirus (DMV), Toxoplasma gondii and Brucella ceti are regarded as pathogens of major concern for both free-ranging striped dolphins (Stenella coeruleoalba) and bottlenose dolphins (Tursiops truncatus) (1). Although a more or less severe encephalitis/meningo-encephalitis is known to occur in striped dolphins and, to a lesser degree, in bottlenose dolphins infected by the aforementioned agents, very little information is available on the neuropathogenesis of brain lesions, including the neuronal and non-neuronal cells targeted during infection and the molecular mechanisms leading to neurodegeneration (2,3).

MATERIALS AND METHODS:
With this in mind, we investigated the Western blot (WB) expression of 5-lipoxygenase (5-LOX), a key-enzyme for mammalian infections, in the brain tissue from 11 striped dolphins and 5 bottlenose dolphins, with 3 of the striped dolphins and 2 of the bottlenose dolphins under study showing no morphologic evidence of central neuropathies. The remaining 8 striped dolphins and 2 additional bottlenose dolphins exhibited encephalitic/meningo-encephalitic lesions of various degree, associated with DMV (1 striped dolphin), T. gondii (5 striped dolphins and 2 bottlenose dolphins) and B. ceti (1 striped dolphin) infection, as well as with DMV-T. gondii coinfection (1 striped dolphin). The ImageJ software was used for densitometry imaging to analyze the intensity of WB bands.

RESULTS:
All the 8 striped dolphins affected by encephalitis/meningoencephalitis showed an intensity of 5-LOX WB bands which was more pronounced than that observed in the 3 dolphins without any morphologic evidence of brain lesions, with the most prominent band intensity being detected in the B. ceti-infected animal. The same was not true for T. gondii-infected as compared to T. gondii-uninfected bottlenose dolphins, 1 of which had the most consistent 5-LOX band intensity. Malacic areas, associated or not with cholesterol clefts, were seen scattered throughout this animal's brain.

DISCUSSION AND CONCLUSIONS:
Based upon the results presented herein, the finding related to a higher expression of 5-LOX enzyme in the brain tissue of the 8 striped dolphins affected by infectious encephalitis/meningo-encephalitis appears to be of interest. The fact this was not paralleled by a simultaneous increase of 5-LOX expression in the brain from T. gondii-infected in comparison to T. gondii-uninfected bottlenose dolphins is also of concern, likely reflecting the mutual host-parasite adaptation of the latter (“inhore”) in comparison to the former (“offshore”) species (4). Since 5-LOX is a putative neurodegeneration biomarker in human patients (5) as well as in experimental animal models (6), further investigation on this challenging issue is needed.

REFERENCES:
FIRST RATE DRINKING WATER: NOT ONLY A BENEFIT, A REQUIREMENT ON-FARM BIOSECURITY

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Keywords: drinking water, biosecurity, coliforms

INTRODUCTION:
Farm biosecurity is an important part of disease prevention and control. The importance of farm biosecurity has been highlighted during the last decade, in fact the European Union (EU) proposed a new animal health law focusing on biosecurity [1]. In Campania Region, it was implemented a biosecurity plan developed in cooperation with Istituto Zooprofilattico Sperimentale del Mezzogiorno, to prevent and control diseases such as brucellosis, tuberculosis, paratuberculosis, bovine rhinotracheitis etc. During monitoring of several farms we have particularly focused on high calves mortality event due to colibacillus bacteria. So we investigate every probable sources of contamination such as feed, personnel, equipment. All the samples didn’t result contaminated. Finally, we analyze drinking water considering it as the probable sources of contamination.

MATERIALS AND METHODS:
Water samples were analyzed for detecting coliforms, E. coli, enterococcus, Pseudomonas aeruginosa, using a membrane-filtration method. Briefly, 100 ml of water sample were filtrated through 0.45μm cellulose acetate membrane filters using a vacuum pump. Filter membranes were incubated on TTC-Tergitol 7 agar (Biolife) at 37°C for 24 h to detect total coliforms and at 44° C for 16-24 h to detect E. coli. Colonies were analyzed with confirming test such as oxidase and indole test. To detect Enterococcus, filter membrane was incubated at 37°C for 48 h on Slanetz Bartley agar, only membrane with suspected colonies was incubated at 44 °C for 2 on Bile Aesculin Azide Agar ISO formulation. To detect Pseudomonas, filter membrane was incubated at 37°C for 24-48 h on CN (Cetrimide, Nalidixic acid) Pseudomonas agar.

RESULTS:
Drinking water showed high positivity to coliforms. Particularly, drinking water resulted 300 CFU/100 ml in September and 70 CFU/100 ml in October.

DISCUSSION AND CONCLUSIONS:
Coliforms contamination decreased in October probably because of seasonal trend weather conditions but only after filter system installation it was completely eliminated. Coliforms contamination of the well water denotes the occurrence of faecal contamination and it may also increase exposure of colibacillosis of cattle. Despite therapy, treatment and cleaning stable, cattle continue to fall ill and die: these microorganisms persisted into the water generating vicious cycle. Only after installation of water filters there was a decrease of sick animals.

Water quality is one of the crucial point in biosecurity because drinking water could be a major source of exposure of cattle to enteric bacteria, including a number of food borne pathogens. It can have a considerable impact on animal health. Routine monitoring of water sources and appropriate intervention can provide farmers with a desirable return on investment.

REFERENCES:
INTRODUCTION:
In buffaloes, the increment productive performance increases the risk of the onset of conditioned diseases (1). Nevertheless, the winter season and lack of space engender stress, the non-lactating phase being a critical period. In particular in buffaloes in healthy condition during dry period (DP), levels of haptoglobin which has been identified as the parameter most sensitive to stress in the buffalo, were found to be up to 8 times higher than the mean value (2). The aim of the present study was to evaluate by means of a retrospective analysis the state of innate immunity of pluriparous buffaloes during the DP between healthy buffaloes vs buffaloes with health disorders in the first week after delivery.

MATERIALS AND METHODS:
The study was conducted from November to March on a semi-intensive dairy farm with an indoor space of 32 m² per head and an outdoor grazing area of 5.2 hectares on 10 pluriparous Buffaloes (Bubalus bubalis) in healthy clinical status whit a partum-conception period of 52.40 ± 8.80 days, milk yield of 2433.50 ± 231.60 kg in 291.80 ± 3.46 days. Weekly blood samples were taken starting from the 14th day after the beginning of the DP to 7 days after parturition. The samples were centrifuged (2.500 rpm/15') and the serum was stocked at -80°C until analytical tests were carried out. Serum samples were tested for the following parameters of innate immunity: hemolytic complement (Alternative Complement Pathway) (3), lysozyme (4), bactericidal activity (5) and haptoglobin by means of the Phase Haptoglobin Colorimetric Assay kit (Tridelta Development LTD). Furthermore, metabolic parameters (uric acid, amylase, bilirubine, ALP, CK-NAC total, cholesterol, creatinine, γGT, glucose, AST, ALT, ALP, LDH, Mg, P, triglycerides and urea) were evaluated. After parturition, buffaloes were divided in two groups based on healthy clinically condition in the first week after parturition. By retrospectively analyses data recovered were compared between animal with (Group NH, n=4) and without (Group H, n=6) healthy disorders. Statistical analysis were performed by Student’s t-test.

RESULTS:
No differences were observed between two groups in length of lactation, milk yield, length of dry period, metabolic parameters and hemolytic complement, lysozyme, bactericidal activity values. Haptoglobin value showed a higher value in group NH starting to 21 days before calving to 7 days after parturition (fig.1).

DISCUSSION AND CONCLUSIONS:
Analysis of these data suggests that during dry period, in addition to physiologically consumption of hemolytic complement, lysozyme and bactericidal capacity, haptoglobin values could be used as predictive index for healthy disorders risk. Further studies are required to improve the understanding of the timing of these indexes and their correlation with different healthy postpartum disorders.

REFERENCES:
PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST SWINE IMMUNOGLOBULINS

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Keywords: Monoclonal antibodies, Swine IgG, ELISA

INTRODUCTION:
Many tests for the diagnosis of porcine diseases presently in use are based on polyclonal antibodies (PAbs) as secondary antibodies. PAbs vs immunoglobulins of common animal species, easily available in the market, often lack of homogeneity between different lots and may also react in a non-specific way in serological tests. Conversely, monoclonal antibodies (MAbs) have a better specificity and batch to batch homogeneity (1). The aim of the present study was the production and characterisation of MAbs anti-swine IgG, useful for the diagnosis of swine diseases, as Brucella suis infections in pigs and boars (2).

MATERIALS AND METHODS:
MAbs production
MAbs were produced by fusion of Sp2/O-Ag-14 mouse myeloma cells with spleen cells of Balb/c mice immunized with purified swine IgG. Fifteen MAbs, showing in i-ELISA strong reactivity in terms of optical density (OD450nm > 1.500), were isotyped and further characterized in i-ELISA with purified immunoglobulins from buffalo, chicken, cow, donkey, horse, human, mouse, rat and sheep. MAbs that reacted only with swine immunoglobulins were characterized by immunoblotting (IB) against purified swine IgG as antigen. Selected clones were amplified in vitro by serial cultures; supernatants were collected, purified by affinity chromatography, labeled with horseradish peroxidase (HRP) and used to develop an i-ELISA for the diagnosis of porcine brucellosis.

i-ELISA-Brucella suis
Microplates NUNC-Polysorp, coated with purified sLPS from B. abortus S99 produced according to OIE Manual (2), were incubated for 30 minutes at room temperature (RT) with samples and controls, both diluted 1/10 in PBS-Tween. Then, MAb-HRP 10C2G5 was added at RT for 30 minutes. After incubation, plates were washed and TMB substrate was added for 30 minutes. The reaction was stopped by adding H2SO4 0.5N; OD450nm was expressed as percentage of positivity (PP%) with the following equation: PP% = [(OD sample – OD negative control)/(OD positive control – OD negative control)]x100. The optimal cut-off value was determined using ROC curves (3, 4), using the PP% values of tested sera (80 positive sera and 472 negative sera).

RESULTS:
Five out of 15 MAbs reacted only with swine IgG and IgM; characterization by IB indicate that MAbs 10C2C11 and 10C2G5 reacted with both heavy and light chain of IgG, while MAbs 8E10B9, 8E10C9 and 8E10D9 reacted only with the light chain (Fig.1). Among the 5 MAbs selected (class IgG1, kappa light chain), MAb 10C2G5 was used to standardize and validate the i-ELISA. The optimum i-ELISA cut-off value, for which the sensibility and specificity resulted 100% (CI Se = 96.4-100, CI Sp = 99.4-100), was 20 PP% (Fig.2).

DISCUSSION AND CONCLUSIONS:
In this work, we obtained 5 MAbs specific for swine immunoglobulins. MAb 10C2G5 was used in i-ELISA for the detection of antibodies vs Brucella suis in pigs and wild boars sera with good results (sensitivity and specificity equal to 100%). All the MAbs selected could be tested for their use in diagnosis of pigs diseases by several techniques (ELISA, immunofluorescence, immunohistochemistry), in substitution of commercial polyclonal anti-swine immunoglobulins. This would result in improvement of diagnostic methods involving PAbs anti-IgG; in fact MAbs, having greater specificity for the antigen compared to PAbs, show fewer cross-reactions and provide more accurate and reproducible results due to lack of variability between batches.

REFERENCES:
INTRODUCTION:
Parturition, changes in homeostasis, metabolic and physiological challenges occurring in the period after calving may contribute to the activation of host immune system, including the initiation of the acute phase response. This study was aimed at the evaluation of the relationships between the activated immune response, characterized by the presence of acute phase proteins, and altered energetic metabolism in dairy cows after calving.

MATERIALS AND METHODS:
Into the evaluation we included 195 clinically healthy dairy cows of a Slovak spotted breed from a conventional dairy farm. The monitored cows were in a period of 1 – 2 weeks after parturition, and showed no health disorders during the observation. The laboratory analyses were performed in blood samples collected by direct puncture of v. jugularis. Blood serum was analyzed for major bovine acute phase proteins – haptoglobin (Hp, mg/ml) and serum amyloid A (SAA, μg/ml), and selected variables of energetic profile – glucose (Glu, mmol/l), total cholesterol (TCH, mmol/l), total lipids (TL, g/l), triglycerides (TG, mmol/l), non-esterified fatty acids (NEFA, mmol/l), and β-hydroxybutyrate (BHB, mmol/l). Hp and SAA were assessed using commercial diagnostic kits (Tridelta Development, Ireland). The concentrations of Glu, TCH, TG, and BHB were determined using commercial diagnostic kits (Randox, United Kingdom) on automatic biochemical analyser ALIZE (Lisabio, France). Total lipids and NEFA were analyzed by spectrophotometric method. The obtained results from the evaluated cows were divided into two groups according to the measured concentrations of NEFA: Group A (n=108) – cows with serum concentrations of NEFA below 0.35 mmol/l; Group B (n=87) – cows with serum concentrations of NEFA above 0.35 mmol/l. The significance of differences in values between the groups of animals was evaluated by Mann-Whitney Test. Relationships between the concentrations of evaluated variables were calculated by linear regression and Spearman (R) correlations coefficient.

RESULTS:
Analyses showed significantly higher mean serum concentrations of Hp and SAA in cows with concentrations of NEFA above 0.35 mmol/l compared with those with serum NEFA concentrations below 0.35 mmol/l (P<0.001 and P<0.001, respectively). In serum concentrations of BHB we found a similar trend of significantly higher values in cows with NEFA concentrations above 0.35 mmol/l (P<0.001). On the other hand, cows with higher values of NEFA showed significantly lower mean concentration of glucose (P<0.001). In mean concentrations of TCH, TL and TG we observed no significant differences between the two groups of cows. The concentrations of both measured acute phase proteins – Hp and SAA significantly positively correlated with the values of NEFA (R=0.716, P<0.001; R=0.710, P<0.001, respectively), as well as BHB (R=0.291, P<0.001; R=0.300, P<0.001, respectively).

DISCUSSION AND CONCLUSIONS:
Hardardottir et al. (1994) reported that the acute phase response initiated by processes occurring around parturition is associated with numerous changes in lipid and glucose metabolism. Investigations in human medicine showed that altered lipid metabolism, increased concentrations of NEFA in blood serum are directly associated with increased systemic inflammatory conditions with adverse health effects on the transition cows (Sordillo et al., 2009).

Presented results indicate strong relationships between the variables of energetic metabolism, and between indicators of acute phase response in cows shortly after parturition. Understanding how all these metabolic factors interact with the immune system may help in developing disease control strategies that may aid in maintaining good health in dairy cattle.

REFERENCES:
INTRODUCTION:
Bovine neosporosis is a worldwide spread disease and Neospora abortion has been reported in most countries of the world including Australia, New Zealand, Europe, Japan, Korea, Taiwan, Thailand, Vietnam, some African countries such as South Africa and Zimbabwe and North and South America. In Italy, several surveys have been conducted to assess the spread of this infection; in 1998, a survey on 5912 dairy cattle showed overall prevalence of 24.4%, and 58.3% of the farms had at least one positive animal (Magnino et al., 1998). In 2003, a serological survey conducted on 111 farms and 1140 animals showed the presence of at least one seropositive in 39.7% of the farms in Southern Italy and in 54.5% of the herds in Northern Italy (Otranto et al., 2003). During the years 2005-2008 in Brescia Province (Italy) a survey was conducted to identify the most frequent abortion agents in bovine fetuses, and Neospora caninum revealed to be one of the most important of them (Giovannini et al., 2009).

This study describes further results of tests performed over the years 2010-2013 on bovine aborted fetuses and bovine serum samples from farms with infertility problems; some of the tests on fetuses and all tests on bovine sera were carried out as part of a Control Program of Bovine Abortion conducted in collaboration with the Local Health Authority (A.S.L.) during the period 2012-2013, in order to identify the main abortion agents in dairy herds of Brescia Province.

MATERIALS AND METHODS:
During the years 2010-2013, 366 bovine aborted fetuses and 690 sera from 69 dairy cattle farms were tested in Brescia Diagnostic Section Laboratories of Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (IZSLER) for the most important bovine abortion agents. Concerning Neospora caninum infection, on fetuses brains a PCR test was performed; this test allows to detect the presence of DNA of the parasite through the amplification of a part of the region Nc5. Sera were tested with a commercial kit based on an indirect ELISA immunoenzymatic technique; sera with S/P value ≤ 40% were considered negative, sera with 40% < S/P value < 50% were considered doubtful and sera with S/P value ≥ 50% were considered positive.

RESULTS:
122 foetuses out of 366 resulted positive at PCR test for the identification of Neospora caninum DNA (33%), and 257 sera out of 690 resulted positive at ELISA test (37%). Of the 69 tested farms 41 (59%) showed at least one seropositive animal.

DISCUSSION AND CONCLUSIONS:
These data confirm the relevance of Neospora caninum as abortion agent in dairy cattle of Brescia Province and the percentage of positivity detected in fetuses confirms previous observation in 2005-2008. Nowadays there are no effective tools for the management of bovine neosporosis (antibiotics, vaccines, etc.), so the control of this infection in the herds is mainly based on animal monitoring (clinical symptoms, serological tests, etc.) and on control of sources of infection, that can be managed through implementation of effective and specific biosecurity programs.

REFERENCES:

Keywords: Bovine, Abortion, Neospora
MOLECULAR TEST SYSTEM FOR THE EXPRESS DETECTION OF HONEYBEE ASPERGILLOSIS


Keywords: stonebrood, Aspergillus flavus, Aspergillus fumigatus, PCR

INTRODUCTION:
Due to the peculiarities of the climate of central Russia (up to 6 months of winter) mycosis of honeybees cause serious damage to beekeeping. Among these diseases Ascospherosis and Aspergillosis are most often registered (1-4), and also called as chalkbrood and stonebrood, respectively. These two diseases have similar symptoms, however for treatment and prevention different fungicides are needed. The main etiological agents of stonebrood are fungi A.flavus, A.fumigatus and A.niger. The aim of this work was to create a diagnostic test system for indication of Aspergillosis pathogens using molecular genetics approach.

MATERIALS AND METHODS:
Pathogenic isolates of Aspergillus flavus, obtained from the aspergillosis infected apiaries of Republic of Tatarstan and collectible Aspergillus strains were used, such as: A.flavus (Link.), A.fumigatus (Fres.), A.niger (v. Tiegh), A.nidulans (Eidam, Wint.), A.terreus (Thom), A.ochraceus (Wilhelm), A.sulphureus (Fres. Thom et Church), A.candidus (Link), A.wentii (Wehmer), A.clevatus (Desm), A.giganteus (Wehmer). Isolation of DNA from fungal cultures and pathological test material was performed using affinity sorption on silica gel, which was preceded by lysing samples in guanidinium thiocyanate. In parallel investigation of pathological material by conventional microbiological methods conducted. Selecting and computer design of primers was performed on the basis of data on the sequence of microscopic fungi in the Genbank.

RESULTS:
The investigations conducted allowed to select two pairs of specific primers, while using one of them proved possible to conduct a rapid detection of microscopic fungus Aspergillus flavus in the cultures and the pathological material by PCR, and another pair was specific for the genome of the strain Aspergillus fumigatus (Fres.). A pair of primers AFL1-AFL2 identified specific fungal DNA from both collectible culture A.flavus and the material obtained from the apiary. The diagnosis of Aspergillosis caused by A. flavus was subsequently confirmed by a microbiological method. As a result of PCR reaction mixture, wherein the DNA A. flavus, isolated from pathological material, pure or mixed culture of the fungal specific fragment formed 250 bp presented, while in samples obtained from pure cultures of other fungi of the Aspergillus genus synthesis of the fragments was not observed. The second pair of primers, conventionally called AFU1-AFU2, identified specific DNA of the fungus A.fumigatus (Fres.) forming a fragment of 380 bp.

DISCUSSION AND CONCLUSIONS:
These results indicate that PCR with primers AFL1-AFL2 and AFU1-AFU2 is an effective test indicating major pathogens of bees Aspergillosis (A.flavus, A.fumigatus), allowing in a short time to establish the etiology of mycosis and to identify the type of pathogen.

REFERENCES:
INTRODUCTION:
Tuscany coast is included in the Pelagos Sanctuary for Mediterranean Marine Mammals preservation, a Specially Protected Area of Mediterranean Importance (Mangos and André, 2012). Along the Tyrrhenian coast of Italy between January and March 2013, a cetacean unusual mortality event occurred (Casalone et al., 2014). In the present work we describe the cetacean stranding occurred along Tuscany coast in 2013.

MATERIALS AND METHODS:
In 2013, a total of 244 stranded cetaceans were reported, among them 43 animals stranded on Tuscany (http:// mammiferimarini.unipv.it/index_en.php). Anatomo-phathological investigation of 17 specimens (11 striped dolphins Stenella coeruleoalba, 4 bottlenose dolphins Tursiops truncates, 2 fin whales Balaenoptera physalus) were performed whenever possible, according to the preservation conditions (Geraci & Loundsbury 2005) by Diagnostic Laboratory of Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT), Pisa. Tissues were immediately analysed or sampled frozen for bacteriological, parasitological, and virological investigations and preserved in 10% neutral-buffered formalin for histopathological investigation. The examinations were performed as previously described by Casalone et al. (2014) by IZSLT, in collaboration with Padova University.

RESULTS:
The average body condition score of analysed animals (17) resulted 2.94. The most frequent lesions evidenced were hyperaemia and haemorrhage of meninges (in 12 animals), intestinal inflammation (in 8 animals), and lymph nodes lesions (in 6 animals). In seven subjects, the stomach resulted empty. Several potential pathogens for aquatic organisms and some contaminant bacteria were identified (Figure 1). Brucella ceti and Salmonella spp., previously detected in a dolphin in the same area (Alba et al. 2013), were not found. Virological examination evidenced Morbillivirus in 8 animals, while 5 animals resulted positive for Herpesviruses and one bottlenose dolphin presented a Parvo-virus like in the intestine. The results of parasitological investigation were reported in figure 2. Toxoplasma gondii was detected by PCR in the brain of one striped dolphin and five subjects resulted serologically positive.

DISCUSSION AND CONCLUSIONS:
Due to the high number of animals that presented a bad body condition score, no definitive conclusions on the causes of the cetacean stranding occurred can be drawn. Data obtained allowed to formulate hypotheses about the possible causes of this mortality outbreak, although certain conclusions cannot be drawn, in particular due to the high number of animals that presented a bad body condition score. Dolphin Morbillivirus may have caused an impairment of the immune system and other pathogens like Photobacterium damselae damselae, Herpevirus and T. gondii may have played a role in this mortality event. Further analyses are in progress to clarify the role of the pathogens here reported.

Acknowledgments
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REFERENCES:
POSTER PRESENTATIONS

FOOD SAFETY AND ZOONOSES
INTRODUCTION:
Verocytotoxin producing E. coli (VTEC) O157 is a zoonotic pathogen, with cattle being its natural reservoir. The main virulence genes of VTEC O157 are those encoding the Verocytotoxins (VT), conveyed by bacteriophages. Recently a VT-phage termed ϕ-8, associated with VTEC O157 isolated from human disease and influencing the assembly of the adhesion machinery encoded by the locus of enterocyte effacement (LEE), has been described (1). A similar effect has been reported for the product of rpoS gene (2), a crucial regulator of the stress response. We investigated the ability of ϕ-8 positive VTEC O157 to adhere to intestinal cell lines in comparison with VTEC O157 strains possessing different VT-phages and determined the expression levels of rpoS in the two groups.

MATERIALS AND METHODS:
VTEC O157 strains from different sources, either possessing ϕ-8 (4 strains) or different VT-phages (4 strains), were analysed for their ability to adhere to human (Caco-2) and bovine (CIEB) cultured epithelial cells. Bacteria were incubated onto cells monolayers for 1 hour and washed with PBS. The attached bacteria were collected using PBS/Triton 1%, plated onto TSA in decimal dilutions, and counted. The sequence of the rpoS gene was determined as previously described (2). The expression of rpoS gene was evaluated by Real-Time PCR by calculating the ratio between the Ct corresponding to the rpoS mRNA and that of the housekeeping gene gapA. The VTEC O157 strains were exposed to UV light and the RNA extracted at 0, 30, 60, 90 and 120 minutes intervals. The cDNA has been produced with the QuantiTect Reverse Transcription Kit (Qiagen, USA). The primers and probes used are listed in Table 1.

RESULTS:
The adhesion assays showed that the ϕ-8 positive strains adhered to bovine intestinal cells more efficiently than the other VTEC O157 (p<0.01). Accordingly, one of the strains, from which the ϕ-8 phage was experimentally removed, showed impaired adhesion to epithelial bovine cells compared to its wild-type parent strain. Finally, the two groups showed no differences in the adhesion efficiency to the human cells. Interestingly, the rpoS gene sequences of VTEC O157 carrying ϕ-8 clustered apart from those of the strains possessing different VT-phages, when clustalW analysis was performed. However, we did not observe differences in the rpoS mRNA levels in the two groups.

DISCUSSION AND CONCLUSIONS:
The results of the adhesion assays suggest that ϕ-8-positive strains may attach to the bovine gut more efficiently, supporting the proposed influence of ϕ-8 in the colonization mechanism (1). Our results also show, in ϕ-8-positive strains, the presence of an rpoS allele, a crucial regulator of the stress response, whose DNA sequence clustered in a group distinct from the one containing the rpoS sequences from the control group (ϕ-8-negative strains). We observed that the level of expression of the two rpoS alleles did not differ significantly in the two groups, suggesting that the reason for such an association could be in the protein function. The observed increased adherence of ϕ-8 to the bovine epithelial cells and the presence, in these strains, of a peculiar rpoS allele suggests that VTEC O157 might have undergone a selective process based on the stress response, favouring those strains capable of prolonged persistence into the farm environment, which in turn increases the risk of human exposure.

REFERENCES:
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INTRODUCTION:
Introduction of novel fish species in the European fish markets can increase the risk of commercial and/or sanitary frauds (i.e., introduction of species with potential toxic effects on humans (1)). Identification of fish species is mandatory; however, traditional identification methods (external morphological features and IEF) can fail in case of processed products subject to filleting, beheading, skinning and/or cooking. The DNA-based identification system has become a powerful option to accurately identify the fish species on the basis of the presence of species-specific polymorphisms.

A novel and powerful DNA-based technology with a potential for species identification is pyrosequencing (2). In this study, we evaluated the application of PCR followed by pyrosequencing as an alternative to the DNA barcoding for the rapid identification of two groups (Clupeidae and Pleuronectidae).

MATERIALS AND METHODS:
One-hundred and sixteen (57 Clupeiformes and 59 Pleuronectiformes) specimens consisting of whole fish (skinned, filleted, eviscerated, etc.) and processed seafoods (dried, salted, marinated, smoked, etc.) were collected. Scientific and common names (10 Clupeiformes and 15 Pleuronectiformes) were assigned following identification by species-specific morphological traits and/or Sanger sequence DNA analysis. Genomic DNA was isolated from all the specimens and three different PCR targeting conserved regions of mitochondrial DNA (16S rRNA, NADH dehydrogenase subunit II gene (ND2) and cytb gene) were performed. The sequences originated by pyrosequencing analysis were automatically compared with a sequence library containing all the reference sequences of the species of interest.

RESULTS:
20 out of the 25 targeted species were correctly and unambiguously identified based on the PCR targeting of the 16S rRNA gene (phase 1), followed by the pyrosequencing analysis. For sequences not uniquely identified in phase 1, two other PCR followed by pyrosequencing were performed (phase 2), specifically targeting the group of Clupeiformes (1 species) and the Pleuronectiformes (4 species) The proposed workflow is schematically illustrated in Figure 1.

DISCUSSION AND CONCLUSIONS:
The pyrosequencing technique provides a short sequence (30-40 nt) with a number of polymorphisms sufficient enough to enable differentiation of the species under investigation. The developed protocol allowed an efficient, accurate and unambiguous discrimination of Engraulis encrasicolus (Clupeiformes), Pleuronectes platessa and Solea solea (Pleuronectiformes) (high-value species) and of other low-value species (i.e. Sprattus sprattus, genus Limanda and Lepidopsetta). This is relevant in the framework of the official control of commercial fish fraud. A major point of interest is that the method developed in this study has proven to be highly efficient on processed fish products, as well. Phase 1 can be considered as a primary screening test. Phase 2 acts as a fine tuned identification step. If necessary phase 1 and 2 can be performed simultaneously to reduce the turn-around-time. Compared to the classical sequencing system (Sanger method) the pyrosequencing pipeline is more time-efficient and certainly cheaper.

REFERENCES:
INTRODUCTION:
Helicobacter pylori infection is a common bacterial infection and approximately 50% of the world’s population has been estimated to be infected. Humans are the principal reservoir, but the previously reported detection of H. pylori in raw bovine milk raised the concern that cattle may act as source of human infection. With the exception of H. pylori, few studies on the presence of other Helicobacteraceae in the milk and gastrointestinal tract of cattle have been so far performed. Wolinella succinogenes, which belongs to the family Helicobacteraceae, was originally isolated from cattle rumen, and “Candidatus Helicobacter bovis” was described in the pyloric portion of the abomasums. The aim of this study was to investigate the prevalence of Helicobacteraceae in raw milk and gastrointestinal tract of cattle in order to assess their potential zoonotic role in the transmission to humans of these bacteria.

MATERIALS AND METHODS:
The study was performed in the Lodi Province (located in the northern Italy). The bulk milk from 163 dairy cattle herds and the content of rumen, abomasum, and rectum, and the fundic and antral mucosa of the abomasum from 25 bovines sampled at slaughter were investigated. All samples were cultured onto DENT Selective Medium (Oxoid) and subjected to a screening PCR for Helicobacteraceae; the samples positive for Helicobacteraceae were further tested with specific PCRs for H. pylori, “Candidatus Helicobacter bovis” and Wolinella spp.

RESULTS:
Three out of 163 bulk milk samples tested positive for Helicobacteraceae (1.8%; C.I. 95%: 0-3.9), but not for the subsequent specific PCRs. All bovines sampled at slaughter tested positive for Helicobacteraceae PCR (100%), and by using specific PCRs H. pylori, “Candidatus Helicobacter bovis”, and Wolinella spp. were detected in 0%, 68%, and 48% of animals, respectively. The distribution of the different Helicobacteraceae throughout the gastrointestinal tract is shown in Figure 1. In any case it was possible to isolate Helicobacter spp., but given the similar growth conditions we isolated Campylobacter jejuni from 5 bovine antral samples, and Arcobacter butzleri and A. cryaerophilus from raw milk.

DISCUSSION AND CONCLUSIONS:
The prevalence of Helicobacteraceae in the raw milk was negligible (1.8%), and H. pylori was not identified in any of the positive samples. On the contrary, Helicobacteraceae were identified in all the bovines (100% of prevalence) sampled at slaughter, indicating a wide distribution of these organisms in the bovine gastrointestinal tract. As regards the spatial distribution of these organisms, W. succinogenes was most prevalent in the content of rumen and rectum, while “Candidatus Helicobacter bovis” was almost exclusively identified in the abomasum. Despite the high prevalence of Helicobacteraceae in the bovine gastrointestinal tract, H. pylori was never detected. On the basis of our results, H. pylori was not found in either raw milk or gastrointestinal tract of cattle, indicating that bovines represent not a potential source of human infection of this microorganism.

REFERENCES:
INTRODUCTION:
In the European Union ten to twenty percent of human Salmonella enterica infections may be attributable to pig source, as reported by EFSA (1). Salmonella contamination may occur, directly or indirectly, throughout the whole production chain. Carcasses contamination at the abattoir is considered to be an important source of Salmonella in pork products (2). Thus, the objective of this study was to evaluate Salmonella contamination of swine carcasses in some slaughterhouses in Umbria.

MATERIALS AND METHODS:
A total of 150 carcass sponge swabs have been collected between March 2013 and March 2014, from 150 randomly selected pigs in five different abattoir in Umbria, proportionally to the slaughter capability of each facility. Carcasses were tested by swabbing four high-risk contamination areas for a total area of 400 cm² (3). The detection of Salmonella spp. was performed by VIDAS® assay (AFNOR BIO 12/16-09/05). Each ELFA-positive broth was confirmed in accordance to UNI EN ISO 6579:2008 and serotyped, according to the Kauffmann-White scheme. To establish relatedness among most common isolated serotypes, Pulsed Field Gel Electrophoresis (PFGE) was performed in accordance with the CDC’s PulseNet protocol (4) and analysed by Tenover criteria (5).

RESULTS:
Salmonella spp. was isolated from 54 of 150 samples (36%). Figure 1 shows the detail of Salmonella serotypes isolated from swine carcasses per abattoir and sampling day. Isolates belonged to 6 different serotypes: S. Derby, S. Typhimurium, S. London, S. Bredeney, S. Rissen and S. 4,[5],12:i-. Genomic DNA of 23 S. Derby, 11 S. Typhimurium and 6 S. Bredeney was analysed by PFGE. Salmonella strains, which belonged to the same serotype and were recovered during the same sampling day, showed uniform PFGE profile, even if collected from pigs of different farms. Instead strains which belonged to the same serotype and were collected from the same slaughterhouse but on different sampling days, showed different restriction patterns.

DISCUSSION AND CONCLUSIONS:
Our study shows high Salmonella isolation rates in samples collected from different abattoirs. However, in tested facilities is observed a large variation in the levels of Salmonella contamination that does not reflect a seasonal trend. The different hygiene performances may depend on various practices in slaughter. The combined results of serotyping and PFGE indicate the important role of carcasses cross-contamination during slaughter process. In fact, high levels of carcasses contamination due to the same Salmonella serotype (Fig. 1 - SD1) and also due to the same pulsotype (Fig. 1 - SD6, SD12, SD14 and SD15) are observed. Carcass contamination during slaughter process could originate from the animal itself and other animals or through several transmission routes such as manual handling and equipment. These data underline the importance of Good Hygiene Practices and HACCP programmes to ensure the microbiological quality of carcasses. Nevertheless, to improve safety of pork products, more information is needed to understand at which step of the slaughter process swine carcasses undergo the contamination by Salmonella.

REFERENCES:
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ALKALINE PHOSPHATASE ACTIVITY IN CHEESE MADE WITH PASTEURIZED MILK

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Keywords: cheese, alkaline phosphatase, pasteurization

INTRODUCTION:
An adequate heat treatment inactivates the alkaline phosphatase (ALP) in milk, bringing it to a level below the legal limit of 350 mU/L (Commission Regulation (EC) N. 1664/2006); ISO 11816-1:2013 describes the fluorometric method to define ALP activity. Actually, ALP determination is focused also on milk products. ISO 11816-2:2003 describes the procedure to define ALP in cheese and European Union Reference Laboratory (EU-RL) has recently proposed both a revision of the in force ISO normative (ISO/CD 11816-2: 2010) and a tentative new limit of 10 mU/g for cheese made with pasteurized milk (M. Nicolas and L. Pellegrino, 2011).

The main objective of this study is to perform a preliminary evaluation of ALP activity in Italian cheese made with pasteurized milk in order to guarantee their safety for the national and international markets.

MATERIALS AND METHODS:
Due to the very high amount of different cheeses produced in Italy (more than 600, with a huge number of variants and subtypes, often produced in very little dairy factories), we defined a classification of Italian cheese based on all the composition and production elements that could theoretically affect the ALP values. By this approach, we tried to define clusters of similar products and to select the “reference cheese” within each group for ALP determination. More than 450 samples, corresponding to 59 different types of cheese, were analysed with the fluorometric method (Fluorophos®, Advanced Instruments, Inc. Norwood, USA) indicated in ISO/CD 11816-2: 2010. Cheese made with raw milk, “pasta filata” cheese like mozzarella and blue cheese were excluded.

RESULTS:
The main factors of cheese classification together with the respective ALP value observed for each group are listed in table 1. Our preliminary observations demonstrated that the majority of controlled Italian cheeses, made with pasteurized milk, respect the tentative limit of 10 mU/g. The overall results are summarized in table 2. The difference between industrial and artisanal production is evident: in many cases the first respects the “tentative limit”, the second often exceeds it.

DISCUSSION AND CONCLUSIONS:
Handmade products are often less standardized and controlled if compared to industrial products; part of artisanal cheese declared as “made with pasteurized milk” resulted simply “thermized”, with obvious effects on the geometric mean ALP value of the total group. We observed two kinds of possible non-conformity: the first, often observed in artisanal products, is simply related to the correspondence between the heat treatment, indicated on the label, and that really applied. In other cases the results should be better investigated by a “step by step control plan” to identify which phase of the production process may be responsible. The principal steps are:
1- Control of pasteurization treatment
2- Comparison between different batches of the same cheese
3- Control of the effects of added ingredients on the ALP content
4- Monitoring of ALP activity during ripening period
5- Determination of reactivated ALP
6- Control of the cheese portioning

This control plan suggests the approach to check the “critical points” for cheese that exceeds the tentative limit. The final objective is to distinguish between products that need changes in the production process from products that result non-compliant for their intrinsic characteristics. In this last case, specific derogations could be proposed on the basis of technical and scientific evidence of their specificity.

REFERENCES:
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3. ISO 11816-2:2003 (IDF 155-2)
4. ISO/CD 11816-2:2010

INTRODUCTION:
Celiac disease (CD) is an immune-mediated enteropathic condition triggered in genetically susceptible individuals by the ingestion of gluten. The disease is associated with human leukocyte antigen (HLA) DQ2 and DQ8 haplotypes. In presence of gluten, CD is self-perpetuating. Given the role of gluten in causing inflammation and autoimmunity, CD represents a unique example of an immune-mediated disease for which early serologic diagnosis and dietary treatment can prevent severe, sometimes life-threatening, complications (1). Celiac disease is the most common food intolerance in humans with an estimated prevalence of about 1% (2). According to the 2012 ISTAT data the theoretical number of people with celiac disease should be about 600,000, but 148,662 are actually diagnosed. A comparison of the data for the years 2010, 2011 and 2012, shows a steady increase in the number of people affected of CD in all regions of Italy (2). Since the Italian diet is mostly based on processed grains, especially bread and pasta (Mediterranean diet), and since many Food Business Operators (FBOs) produce both gluten containing and gluten-free food, it is important to control that the products labeled as “gluten-free” are in compliance with the regulatory framework. EC Regulation No. 41/2009 applies to food products, infant and follow-on formulas excluded, and regulates the composition and labeling of food products suitable for gluten intolerant people. According to this regulation, gluten-free food must contain less than 20 mg/kg of gluten to allow to add, on the label, the “gluten-free” indication; “very low gluten content” is mandatory if the gluten content does not exceed 100 mg/kg (3).

This study reports the results of official Monitoring Plan obtained at the Microbiology Department of IZSLER, Brescia, during the period 2010-2013. The aim was to check that the legal requirements were satisfied to ensure a high level of safety of CD-affected consumers’ health.

MATERIALS AND METHODS:
From January 2010 to December 2013, 396 samples labeled gluten-free or administered as gluten-free in mass catering, were analyzed. The analyzed samples were divided into 7 food groups: cereals and bakery products; meat and fish products; soups, broths and sauces; fruits and vegetables; ready to eat meals; additives; others. Samples were analyzed by ELISA using an AOAC validated commercial kit RIDASCREEN® gliadin r-Biopharm (Darmstadt, Germany) able to detect and quantify gluten in foods, through the specific monoclonal antibody R5. The limits of quantification were validated between 5 mg/Kg and 80 mg/Kg.

RESULTS:
Among the 396 analyzed samples, the gluten value was >5 mg/Kg in 15 tested samples, 6 of which were between 5 and 19.9 mg/Kg (and therefore comply with the law); 3 samples had a value between 40-60 mg/kg, while the remaining had a value of gluten content >80 mg/Kg. The prevalence of positive samples on the total results was 2.3% (95%CI 1.2-4.3). Among the 9 positive samples, 8 was cereals and bakery products, and 1 was a cocoa-based preparation.

DISCUSSION AND CONCLUSIONS:
Celiac disease is a gluten-sensitive enteropathy that develops in genetically susceptible individuals by exposure to cereal gluten proteins (4). The study of the non-compliant food prevalence was important to provide an overview on the current situation in Lombardy and Emilia Romagna regions. Results show that the incidence of positive samples was low, and this proved that FBOs and mass catering serving gluten-free food are careful to satisfy the law requirements. In conclusion, the safety level of CD-affected consumers’ health is considered high, demonstrating that the HACCP adopted practices are effective. The fact that positive samples are cereals and bakery products is due to the fact that they were the most analyzed specimens.

REFERENCES:
(2) Direzione generale per l’igiene e la sicurezza degli alimenti e la nutrizione, Relazione annuale al Parlamento sulla celiachia, 2012
INTRODUCTION:

The animal by-products (ABP) are biodegradable residues that include: animal carcasses, parts of animal carcasses, animal origin products not intended for human consumption, including collective diet supply residues, residues of the leather industry, etc... Animal products wastes derived from animal parts deemed unfit for human consumption, as doomed to destruction because they cannot be used either for the livestock industry, either as a fertilizer, nor suitable for biogas production or composting, go to build the complex landscape of ABP.

In the following work the ABP Category 1 and 3 (Reg.CE 1069-2009, and amended) will be considered as indicators of Salmonella spp., Enterobacteriaceae, Clostridium perfringens and Escherichia coli beta glucuronidase + microbial activity in by-products intended for the production of protein meals.

MATERIALS AND METHODS:

From 2011 to 2014, under the “Monitoring Plan for the verification of the requirements of the by-products in ABP processing plants and storage”, 610 laboratory tests corresponding to 118 sampling activities were carried out. Of this analysis, 267 corresponding to 54 samples, each divided into several sample units were intended to detect Salmonella spp, 272 on 55 for Enterobacteriaceae, 36 on 36 for Clostridium perfringens, 5 to 1 for Escherichia coli beta glucuronidase +, and the remaining 30 for other analytes (heavy metals, PCB, GHT, etc...). The analysis were performed by Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM) laboratories with specified methods aimed for the “Monitoring Plan for the verification of the requirements of the products in ABP processing plants and storage” and the data statistical analysis was performed with IBM SPSS ver.21 software.

RESULTS:

Regarding the detection of Salmonella spp. 13 sample units of protein meals were found positive, whereof 7 through real-time PCR screening method and 5 by isolation showing the presence in 25 g of tested product. On latter reisolation, typing and antibiogram was performed, confirming a Salmonella cerro resistant to Trimethoprim / sulfamethoxazole and sensitive to most antibiotics presence. The search for Enterobacteriaceae, Clostridium perfringens and E. coli did not produce any positive results in protein meals.

DISCUSSION AND CONCLUSIONS:

This survey does not reveal a Public Health warning but provides a constant monitoring of those pathogens as microbial contamination indicators in by-products. These in fact, as a waste product of what is consumed on our tables, give us the power to reveal, parallel, the various microorganisms presence and activity and to take appropriate countermeasures. It is also conceivable that the presence of Salmonella spp. is showed due to contamination in the product processing, and then still harmless for the human being. However, the low percentage of positivity (Salmonella spp. 4.85%) indicates a good security level and promotes the control methods dictated by the Monitoring Plan.

Our results stand apart from those found in the United States (Beilei et.al, 2013) where, in a 2003 sampling, 34.4% positivity vs Salmonella spp. in 201 samples was found. The present work aims to promote the perpetuation of these controls to compare them over the years and expose more and more critical issues of food security.

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Beilei et. al, 2013 - Retrospective Analysis of Salmonella, Campylobacter, Escherichia coli, and Enterococcus in Animal Feed Ingredients. Foodborne Pathogens and Disease, 2013, Volume 10, Number 8


STRONTIUM-90 ACCUMULATION IN ANIMAL BONES: RADIOCHEMICAL DETERMINATION BY LIQUID SCINTILLATION COUNTING (LSC)

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Keywords: 90Sr, LSC, Animal Bones

INTRODUCTION:
90Sr is a radiotoxic isotope due to its long physical and biological half-life. It is produced during nuclear fission processes and then it decays to its radioactive daughter nuclide 90Y, emitting high energy (546 keV) β particles [1]. Animal feeding in polluted soils and with contaminated water is the largest source of exposure to 90Sr, which may be absorbed by following metabolic pathways similar to those of Ca. Once 90Sr enters the blood, it may be accumulated in bones where it is retained, so, it is very important to control the radiocontamination level in the environment and in food. A direct correlation between the activity of 90Sr in soil sample and site altitude was demonstrated [2] and 90Sr content increases also with the age of the animal. The radiochemical determination of 90Sr represents a challenge for Organisms in charge of analytical controls. The most complex analytical step is the separation of 90Sr from other alkaline earth elements, such as Ca and Ba. In this work, a radiochemical procedure using ultra low level liquid scintillation counting (LSC) for the monitoring of 90Sr in animal bone samples, after achieving 90Y secular equilibrium condition, was implemented and validated. The validation parameters, such as trueness, precision, counting efficiency, and measurement uncertainty were determined using samples fortified with known activities of 90Sr (at 10 and 100 Bq kg-1). In order to verify the method applicability for routinely controls, it was then employed for the analyses of 10 cow bone samples.

MATERIALS AND METHODS:
50 g of bone sample was ashed at 1000 °C, dissolved in HNO3 8M+0.5 ml of HF at 50%. The mixture was treated by leaching at 320°C and filtered. Oxalic acid (~20 g) and sodium acetate (~7 g) were added and strontium oxalate was precipitated at pH 4.5. The precipitate was filtered and dissolved in 35% H2O2 and HNO3 8M and then dried. The residue was dissolved in HCl 0.1 M. Then the interferences (210Pb e 210Bi) were removed as sulphides, the solution was placed in a separatory funnel with 200 ml of 20% Bis-(2-ethylhexyl) phosphate (HDEHP) in toluene and was mixed. The acid phase (200 ml) was maintained at 25 °C for two weeks until achievement of 90Sr/90Y secular equilibrium. The solution at pH 1.0 was placed in a separatory funnel with 200 ml of 5% HDEHP in toluene and mixed vigorously. The Y presents in the organic phase was extracted two times with 150 ml of HNO3 3M. 50 ml of 8% oxalic acid were added and yttrium oxalate was precipitated at pH 2.5. The residue obtained after filtration was dissolved in HNO3 and H2O2 and was dried. 8 ml of 0.1M HCl and 12 ml of scintillation cocktail were added and the solution was used for to determination of 90Sr by LSC. The validation parameters were determined using cow, pork and chicken bone samples fortified with known activities of 90Sr (10 Bq kg-1) and two replicates of each species were carried out. Decision threshold and detection limit were also calculated, in compliance with ISO 11929:2010, by analysing six blank samples.

RESULTS:
Method precision and trueness were demonstrated evaluating CV% values and mean recovery, equal to 10% and 98.7% respectively (Tab.1). No interfering radionuclides were observed in spectra analysed, as shown in figure 1. Detection limit and decision threshold, correspond to 8 and 3 mBq kg-1 respectively (α=β=0.05). Quantitative analyses on real samples were carried out, by the above described method, on 10 cow bone samples. In all samples 90Sr was quantified at appreciable levels in the range 5.22 and 20.61 Bq kg-1.

DISCUSSION AND CONCLUSIONS:
The new analytical procedure allows the selective extraction of the desired radionuclide, without interferences, and it is suitable for radiocontamination surveillance programs. In particular, considering that analyzed samples are coming from significant farm animals, this method is an important improvement in food safety controls.

REFERENCES:
INTRODUCTION:
In 2013 RASFF has notified an alert for contamination of food with horse meat; initially categorized as a health alert due to the suspicion of the presence (subsequently not detected) of phenylbutazone, the problem has since evolved into an investigation focused on commercial fraud. EU had established a monitoring plan (3) that included a large-scale control of food, and the laboratories involved used methods from the reference center EURL-AP (5,1) or in-house techniques validated and accredited. Some of them were able to detect only horse DNA, but others verified presence of multiple species simultaneously. For this reason it was possible to verify that there were many non-compliance, respect as stated on the label, for many other species as well as for horse DNA. Consequently local competent authorities, independently by the new 2014 monitoring plane for meat control (4) started to control different type of materials to verify no-compliances, in particular food declared to contain fine and little-used meats like for example donkey meat. Methods to detect species that are based on DNA amplification are generally set-up with mitochondrial targets, because it’s more resistant to the transformation processes and present in multiple copies within cells, but in some situations this determination is not sufficient to obtain complete informations. The mitochondrial DNA is transmitted exclusively maternal, so samples declared as containing donkey meat and positive for horse DNA, it has to be considered not-compliant and may contain meat mule (horse mother/father donkey). It is evident the necessity in these complex cases to develop methods which, side by side with the PCR target mitochondrial, can allow to clarify these situations.

MATERIALS AND METHODS:
Analysis commissioned by authorities to verify donkey origin were performed on 28 samples, from raw and complex food: meat 8, salami 10, ragù 4, food preparation 6. Horse DNA was detected by two PCR methods: 1) Real-time PCR (5,1) 2) an in-house validated PCR method, approved by Reference laboratory and Ministry of Health (2). For the detection of donkey DNA PCR end point was applied (primer sequence: F 3’ – TCATTCCCCCACCCTAATGGCT – 5’, R 3’ – GGGAGGAGTAGGGAAGATGCGATC – 5’; thermal profile 95 °C x 10’, 35 cycles 95 °C x 15’ - 61 °C x 30’ – 72 °C x 30’, 72 °C x 5’). A PCR based on genomic targets for equine and horse (7) and a PCR for identification of Y chromosome genetic variations (with final sequencing) were performed (8) on meat samples from horse, donkey,mule and hinny; different amplification conditions were applied for both PCR.

RESULTS:
In Fig. 1 results from field samples analysis are showed: there are 78.6% of samples not-compliance (respect to label declarations: donkey meat). In Fig. 2 are reported results from preliminary standardization of genomic target and Y chromosome target: for equine genomic targets PCR all samples tested were positive (data not shown). Horse genomic target PCR showed expected results (donkey samples negative and other samples positive); Y chromosome PCR gave in general not satisfactory results.

DISCUSSION AND CONCLUSIONS:
Results obtained from multi-target analysis showed that in particular product prepared with donkey meat can be differences between real composition and label declaration; it is possible to suppose that this situation is extended also to other type of particular food, like dishes prepared with other wild animals meat. Preliminary studies on horse/donkey DNA detection can be used as bases to standardized detection methods and to improve and set-up a panel of analysis to distinguish that species (genomic and Y chromosome PCR).

REFERENCES:
A COMPARATIVE STUDY OF THE APPLICABILITY AND LIMITATIONS OF ETHIDIUM MONOAZIDE AND PROPIDIUM MONOAZIDE TREATMENTS FOR A SELECTIVE DETECTION OF VIALBE CAMPYLOBACTER CELLS BY qPCR

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Keywords: Campylobacter, viability, EMA and PMA, qPCR

INTRODUCTION:
The differentiation between viable and nonviable cells is a crucial point that limits the implementation of PCR-based methods in food diagnostic applications. To circumvent this disadvantage, combinations of quantitative real-time PCR (qPCR) assays and ethidium monoazide (EMA) or propidium monoazide (PMA) pretreatments of cells have been proposed.1 The dyes EMA and PMA permeate membrane damaged bacteria and covalently bind to DNA after light exposure. Regarding the suitability of this approach for the detection of viable Campylobacter spp. cells, conflicting results have been reported.2 Therefore, the purposes of this study were to comparatively analyze the suitability of EMA and PMA in various concentrations for a selective detection and quantification of viable Campylobacter cells by qPCR and to correlate the results to the culture-based enumeration. In addition, we drew attention to methodological limitations and suggested an option for improvement.

MATERIALS AND METHODS:
The reference strain C. jejuni DSM 4688 and 16 field isolates belonging to the species C. jejuni and C. coli were included. EMA and PMA were added to samples to a final concentration of 10 µg/ml EMA and 100 µg/ml EMA or 25.55 µg/ml PMA and 51.10 µg/ml PMA. The signal reduction from nonviable cells was determined by using tenfold dilution series of heat-inactivated C. jejuni starting from approximately 3 x 10^7 cells. Furthermore, mixed samples of viable and nonviable cells were tested in ratios up to 1:1000. Chicken leg quarters were spiked with different quantities of the reference strain and field isolates and comparatively analyzed by EMA-qPCR and microbiological cell enumeration. The Pearson regression analysis and the Bland-Altman analysis were used to calculate a relation between qPCR results and microbiological cell counts and for method comparisons, respectively. Mathematically calculated and experimentally obtained values were compared by ANOVA Sidak multiple comparison test.

RESULTS:
Results from qPCR experiments revealed concentration dependent shifts towards higher Ct values after application of the intercalating dyes EMA and PMA, especially after EMA treatment. Thus, an internal DNA standard was used along with the adapted standard curves for the experiments. A comparison of Campylobacter counts determined by qPCR and culture-based enumeration achieved high correlation coefficient (R2) values of 0.99 (EMA) and 0.98 (PMA). In mixed samples up to 1:1000 ratios, EMA (10 µg/ml) and PMA (51.10 µg/ml) led to a detection of viable cells only in qPCR experiments and the experimentally determined signal reductions corresponded to the mathematically expected values. Nevertheless, larger amounts of heat-inactivated cells (>10^6) remained detectable by qPCR, although the signals were reduced considerably. As possibility of a solution, a double staining of EMA was performed and clearly improved the signal reduction from nonviable cells. The optimized EMA protocol was successfully applied to 16 C. jejuni and C. coli field isolates from poultry, indicating the applicability for field isolates as well. Analysis of viable cells from food samples (spiked chicken leg quarters) resulted in comparable bacterial cell counts when using EMA-qPCR and culture-based enumeration. The correlation coefficient between both analytical methods was 0.95 and a high agreement between the results from both methods was achieved by Bland-Altman analysis.

DISCUSSION AND CONCLUSIONS:
For Campylobacter spp., the application of EMA and PMA prior to qPCR analysis shows promise as a rapid method for a selective detection of viable cells. However, the signal reduction in samples containing more than 104 inactivated cells was incomplete, but results could be improved by a double staining with EMA.

REFERENCES:
ANALYSIS BY MULTIPLEX PCR FOR BACTERIA IN SMALL RUMINANT ABORTION CASES WITH PARTICULAR REFERENCE TO ZOONOSIS AND FOOD SAFETY, IN SICILY

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Keywords: Abortion, Zoonosis and Food safety, Multiplex PCR, , Zoonosis

INTRODUCTION:
In animal farms, the occurrence of several abortion episodes represent big problems for both animal health and economic loss. At least 40% of abortion cases in Sicilian farms are due to bacterial infection and Brucella spp. is the main species involved; however, other bacteria of the genera Coxiella, Campylobacter etc. are also circulating in small ruminant farms and they can be transmitted to humans for direct contact or inhalation. The presence of zoonotic agents in livestock farms represents also a food safety concern for the possibility of related food products contamination. If dairy products or meat, are contaminated, a consequent increase on the number of people exposed to pathogens occurs. The results’ comparison of simplex and multiplex PCR for zoonotic bacteria, reveal the high prevalence of both Brucella and Campylobacter spp in Sicily.

MATERIALS AND METHODS:
128 fetus were collected from small ruminant farms in Sicily. Clinical and anatomo-pathology observations were performed. The DNA extraction from fetal tissues was performed after homogenization using the kit GenElute (Sigma), following manufacturer’s instruction.

Simplex PCR (Masala et al 2007, D’Andrea et al. 2012) were compared with the following two Multiplex PCR: MPX-CCC: Chlamydophila abortus, Chlamydophila pecorum, Coxiella burnetii (Berri et al 2009), MPX-BLC: Brucella, Leptospira e Campylobacter fetus (Tramuta et al. 2011) DNA sequencing was performed with the kit Big Dye Terminator V.3.1 on 3130-4 Genetic Analyzer (Applied Biosystems).

RESULTS:
The screening on the abortion cases by clinical observation, serology, bacterial isolation, molecular diagnosis showed that Brucella spp. is the main responsible for abortion in Sicily but an important role of Campylobacter spp. was also shown. In the table 1 results regarding zoonotic bacteria are reported.

Results comparison between simplex and multiplex PCRs: 50 abortion samples positive for Brucella by isolation were all confirmed by MPX BLC. However 4/40 negative samples resulted positive by MPX and simplex PCR. It is possible that PCR detected dead bacteria and original samples were not idoneus for isolation. 20 Leptospira positive and 30 negative samples in simplex PCR were all confirmed with MPX BLC. Eight samples positive for C. burnetii by simplex PCR and 20 positive for Campylobacter spp. together with 50 negative samples were all confirmed with MPX CCC. All positive samples were confirmed by DNA sequencing.

In Figure 1 an example of BLC is shown. No abortion cases due to Leptospira spp was found in small ruminant farms except for one farm in which the coexistence with Coxiella burnetii was detected. However an abortion case due to L. bratislava was found in a horse farm.

DISCUSSION AND CONCLUSIONS:
The lack of brucellosis eradication in Sicily exposes humans to Brucella spp. infection not only through the direct contact with animals but also through the consumption of contaminated dairy products (Graziani et al., 2013). Other zoonotic agents like Coxiella burnetii , Campylobacter spp. are circulating in small ruminant farms. The results of the molecular screening in abortion cases, showed a high prevalence for Brucella spp. and Campylobacter spp. that can both contaminate food products (dairy, milk , meat). The switching from simplex to multiplex PCRs could be very useful for a more efficient molecular diagnosis in terms of cost and time. Early identification of infectious agents in abortion episodes can give a valid support in the control and management of infections.

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Table 1: Infectious agents were detected in 63 od 123 fetus. Only zoonotic bacteria are reported.
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PREVALENCE AND CHARACTERIZATION OF VEROTOXIGENIC ESCHERICHIA COLI IN SWINE FAECES AND CARCASSES IN UMBRIA AND MARCHE REGIONS

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Keywords: Escherichia coli STEC, pig, faeces, carcasses

INTRODUCTION:
Shiga toxin-producing Escherichia coli (STEC) can cause severe clinical diseases such as haemorrhagic colitis (HC) and haemolytic-uremic syndrome (HUS). Although cattle have been suggested to be typical reservoir of STEC, many food products from other origin, including pork products, have been confirmed as vehicles of STEC transmission (1). This study aims to assess the prevalence of STEC in slaughtered pigs in Umbria and Marche regions and to characterize isolates.

MATERIALS AND METHODS:
A total of 420 samples, including 210 colon content specimens and 210 carcass sponge swabs have been collected from 210 randomly selected pigs, slaughtered in 11 different facilities in Umbria and Marche. After enrichment in mTSB and incubation, each sample was subjected to DNA extraction, and analyzed by Real time PCR for the detection of stx1, stx2 and eae genes (2). Samples positive for the stx genes were screened by using multiplex PCR (3) for the detection of O:157, O:26, O:103, O:111, O:145 serogroups specific genes. One loopful of the stx-positive enrichment culture was directly streaked onto McConkey agar. Samples that gave positive results by multiplex PCR for serogroups were subjected to immunomagnetic separation technique (IMS) and cultured onto SMAC agar and TBX agar. Up to 20 colonies, grown on each agar plate, were confirmed for the pathogenicity genes by multiplex PCR (4). STEC isolates were subjected to slide agglutination, subtyping of the stx genes (5) and tested for aaiC and aggR genes. PFGE was performed in accordance with the CDC’s PulseNet protocol, using conditions specified for E. coli:O157.

RESULTS:
Different stx-positive rates were observed in carcass sponge swabs and faecal specimens (Figure 1); 38.5% (81/210) of faecal samples and 13.8% (29/210) of carcass sponge swabs were stx-positive. A total of 33 STEC isolates was obtained from 30 samples, giving a culture positive rate of 7.1 % (30/420). The faecal samples gave a higher culture positive rate (12.4%; 26/210) than carcass sponge swabs (1.9%; 4/210). All strains were negative for eae, aaiC and aggR genes and for “top five” serogroups. The isolates were positive for stx2 and belonged to stx2e subtype, except one strain, from a carcass, that was stx2d positive and two isolates, obtained respectively from the faeces and carcass of the same animal, that were negative for all stx2-subtypes tested. Figure 2 shows the detail of STEC pulsotypes isolated from swine carcasses and faeces; five clusters are identified (100% of identity) collected from two different abattoirs.

DISCUSSION AND CONCLUSIONS:
Our results indicate a prevalence rate of 12.4% in faeces and 1.9% in carcasses. Since characterization of virulence genes revealed that the majority of STEC isolates lacked factors correlated with severe human disease, the association between swine STEC and human illness needs to be further investigated. Nevertheless, because many of STEC virulence genes are located on mobile elements, the possibility that STEC from pigs may represent an emergent source of human infection cannot be neglected (1).

REFERENCES:
INTRODUCTION:
Listeria monocytogenes is a Gram-positive, psychrophilic, facultatively anaerobic and it is an important foodborne pathogen that can be transmitted from the food-processing environment to food. In recent years several alert notifications were sent in RASFF system as a consequence of L. monocytogenes dairy products contamination. According to Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, for research of L. monocytogenes in ready-to-eat foods UNI EN ISO 11290-1:2005 method must be utilized in official control. Could necessary carry out a large number of microbiological analyses in short time, such as during a alert or performing a environmental monitoring plan. The aim of this work was to compare results of UNI EN ISO 11290-1:2005 with a rapid test performance.

MATERIALS AND METHODS:
N. 842 samples of dairy processing plant were analyzed: n. 177 dairy products, n. 665 environmental sponge. All the samples were analyzed for the detection of L. monocytogenes using VIDAS® LMO2 test and the UNI EN ISO 11290-1:2005. VIDAS® LMO2 test is an automated enzyme-linked fluorescent immunoassay for the detection of L. monocytogenes. The kit VIDAS® LMO2 is composed of a cone SPR® (Solid Phase Receptacle) with immobilized anti-L. monocytogenes antibodies and a strip consists of 10 wells in which are contained the washing solution, anti-L. monocytogenes antibodies conjugated to alkaline phosphatase and the substrate Fluorescent 4-methyl-umbelliferin phosphate. For the food sample preparation were weighed 25 g of sample and diluted with 225 ml of Half-Fraser Broth (bioMérieux), while for the environmental sponge were added 90 ml of Half-Fraser Broth. Primary enrichment was incubated for 24 ± 2 h at 30 °C and later 0.1 ml were taken and transferred into 10 ml of Fraser Broth (second enrichment broth) and incubated for 24 ± 2 h at 37°C. After incubation 500 μl of secondary enrichment were added into the sample well. Then samples were analyzed using mini-VIDAS apparatus. The result is expressed as “positive” or “negative” on the basis of the comparison between the value of RFV (Relative Fluorescent Value) of the sample and the standard fluorescence value. Samples were also analyzed using a microbiological method in according to UNI EN ISO 11290-1:2005.

RESULTS:
N. 8 samples of environmental sponge and n. 6 of dairy products resulted positives. We obtained the same results utilizing both the reference method and the rapid tests.

DISCUSSION AND CONCLUSIONS:
In this study we compare the alternative VIDAS method for the detection of L. monocytogenes in environmental samples and dairy products with the reference method UNI EN ISO 11290-1:2005. The isolation and identification of L. monocytogenes from food and environmental samples require very long time because is necessary the use of selective agents, enrichment procedures and incubation temperatures to support multiplication of L. monocytogenes and reduce the levels of contaminating microorganisms. As the data shows, the results were the same utilizing the reference method and VIDAS® LMO2 rapid test. In conclusion, the use of VIDAS method would be particularly useful like screening test in investigations of food-borne outbreaks and for the implementation of effective hazard analysis critical control points (HACCP) programmes in food production and processing establishments.

REFERENCES:
DETECTION OF SWINE MEAT AND BONE MEAL IN ANIMAL FEED BY REAL-TIME PCR

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Keywords: PAPs, MBM, real-time PCR

INTRODUCTION:
A new European Commission Regulation (1) amends the EU Regulation No. 999/2001 concerning the use of processed animal protein (PAPs) in feed for farmed animals. The Regulation reforms the stringent rules on the use of PAPs from non-ruminants (e.g. pigs and poultry) in feed, permitting their use in farmed aqua feed only. The availability of robust methods for species-specific PAPs detection in feed is an important prerequisite to enforce the current and upcoming European legislation, so that intra-species recycling can be excluded. In accordance with the Regulation No. 56/2013, the European Reference Laboratory for animal proteins has validated a qualitative real-time PCR (RT-PCR) for the detection of ruminant in feed for aquaculture, to flanking the official microscopic method (2). In this work we study analytical methods to detected non-ruminant PAPs in swine meat and bone meal (MBM) and in feedstuffs contaminated with MBM, by RT-PCR.

MATERIALS AND METHODS:
A tissue sample was prepared from muscle minced, homogenised, while swine MBM was prepared from rendering, processed in accordance with the current European Legislation and then lyophilized. Negative feedstuffs were contaminated by mixing MBM to 100g of raw material, at final percentage from 2% up to 0.01%. PCR assay was performed on samples from tissue, commercially rendered MBM, experimentally MBM, MBM contaminated animal feed and animal feed from interlaboratory study. Genomic DNA from tissue of bovine, chicken, ovine, fish and a maize were used as negative controls. DNA was extracted by using CTAB method for all samples analyzed and DNA concentration was measured with a spectrophotometer. Species-specific primers and probe were used to detect the ryanodine receptor gene (108bp) (3). TaqMan PCR was performed in a final volume of 25 µl, 50-100 ng DNA and the PCR conditions were optimised empirically. The primers and probe were tested for specificity. Sensibility was verified testing feedstuffs contaminated experimentally with MBM at final percentage from 2% up to 0.01%. The determination of the limit of detection (LOD) was carried out with dilution series of DNA, tested in ten replicates, starting from percentage 1%.

RESULTS:
Primers species-specific for swine showed very high specificity when tested DNA of other species. PCR assay performed on samples from feed samples contaminated showed a high sensitivity, detecting contaminations at 0.1% with Ct value among 31-36 (Tab 1). The LOD was calculated at percentage 0.03 %, containing about 3ng DNA (Fig. 1).

DISCUSSION AND CONCLUSIONS:
The main difficult to comply the current and upcoming European Regulation is to find analytical methods able to detect heat-treated PAPs from feedstuffs. The specificity and sensitivity of PCR method can be used in combination with microscopic method to develop a correct analysis of different PAPs species-specific in animal feed. Our results show a good specificity of species-specific primers on all samples analyzed (experimentally and commercially rendered MBM), with a good sensitivity detecting contaminations at 0.1%. This study confirms that PCR real-time can be considered a powerful tool for the identification of PAP in animal feed (4) but its successful is related to many factors as molecular target, DNA degradation, DNA extraction methods and so on.

REFERENCES:
INTRODUCTION:
Recent outbreaks associated with the consumption of fresh produce highlighted the need to integrate the regulatory framework to increase the safety of food categories as sprouted seeds. A study of EFSA Biohazard group concluded that seeds can be contaminated by pathogenic bacteria through multiple factors. Temperature and moisture can promote the growth of bacteria and therefore should be considered as the main risk factors. The limitations in the safety of sprouts are also due to the lack of traceability. The safety, can be managed only by the strict application of the HACCP principles through the entire process of production, distribution and consumption. Even if the presence of pathogenic bacteria in the buds is relatively rare, although being a serious health hazard, this work considered the need to have innovative methods for rapid identification and traceability, to facilitate the risk assessment.

MATERIALS AND METHODS:
In 2013, 223 packs of different sprouted seeds species were analyzed. 147 specimens were collected at the market, 76 directly at production. Label information indicated that most of samples came from Italy (9 producers, 33 from the Netherlands (1 producer); 16 were not labeled.

The following analyses were carried out:
• Listeria monocytogenes (screening by Real Time PCR (RTPCR) BioRad-iQ Check L. m. II kit), Baseline method by RTPCR (1) and confirmation by ISO 11290-1:1996amd/1:2004
• Salmonella spp. (screening by RTPCR BioRad-iQ Check S. II kit), Baseline method by RTPCR (2) and confirmation by ISO 6579:2002cor/1:2004
• E. coli STEC (ISO/TS 13136:2012 and EU-RLVTEC for O104:H4), E. coli O157:H7 (screening by RTPCR BioRad-iQ Check O157:H7 II kit); numbering by TEMPO
• Yersinia enterocolitica (screening by RTPCR and UNI EN ISO10273:2005)
• Norovirus (RTPCR) and hepatitis A virus (seminested PCR) by internal accredited IZSLER methods
• serotyping
• challenge test according to the AFFSA Guide Shelf-life studies of L. monocytogenes on leafy salad.

RESULTS:
Results showed that Salmonella was detected in 2 unlabeled samples, and that the strains were not isolated. 3 samples were positive for L. monocytogenes by qPCR Check L. m. II kit and all the samples were confirmed by ISO; 2 of these sample were also positive for Baseline method, and a further sample, negative for qPCR Check L. m. II kit and ISO, was found positive. These, were from the Netherlands: red beet sprouts (serotype 4b/4e); radish sprouts (serotype 1/2b); bean sprouts (serotype 4b/4e, only microbiological positive) and red beet sprouts (Baseline positive). One red chard sample was positive for vt2 gene, but O104 negative, while a second case was positive for the eae gene (STEC negative). Only 1 sample was positive for E. coli O157: H7 with the IQ screening test. The E. coli numbering value by TEMPO was >10 cfu/g in 5 tested samples, 1 of which was >100 cfu/g.

Of these, 3 were not labelled and 2 were received already open. The isolation was unsuccessful and all samples tested negative with the ISO/TS 13136:2012. No positives were observed for Y. enterocolitica. For virological tests, 1 sample was positive for hepatitis A virus. The challenge test showed that the Pasqualina, Novella and Tricolore salad, were not susceptible for the growth of L. monocytogenes.

DISCUSSION AND CONCLUSIONS:
The aim of this work was to evaluate the level of contamination of sprouts in different Italian regions. Results showed that pathogens were present in low percentages. 9/223 samples were positive in RTPCR, even if the pathogens were not isolate in all the positive samples by ISO. These data confirmed the higher sensitivity of molecular methods respect to microbiological approaches. However, it is unclear whether it was correlated with the inability of RTPCR to distinguish infectious and non-infectious bacteria, or to the inefficiency of cultural methods to detect pathogens in environmental samples. Another important objective was to verify the applicability of shared molecular methods between different labs: the operating protocols were easy to use and applicable to routine analyses.

REFERENCES:
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2. (Delibato et al., 2014)
INTRODUCTION:
Food-borne diseases have enormous impacts on the health and livelihoods of people in Georgia and are of great concern to consumers, Producer Farmers and policymakers. The most risky food chains are animal source foods and fresh vegetables contaminated with human or animal waste, and these are a major focus of the program. Another focus is the impact of fungal toxins on livestock and on the people who eat livestock products. Mycotoxins are produced by fungi which infest staple crops in tropical countries and which have human health, trade and livestock sector impacts. Mycotoxins, such as aflatoxin, are toxins found in food that can cause illness and be lethal in high doses. Aflatoxin is a type of mycotoxin produced by Aspergillus molds. Aflatoxin is probably the most well known mycotoxin, besides trichothecene, and the most researched. This is because aflatoxins are very toxic and highly carcinogenic. There are three main types of aflatoxin mycotoxins:

- Aflatoxins B: This group includes aflatoxin B1 and B2.
- Aflatoxin B1 is the most common aflatoxin, as well as the most toxic and carcinogenic.
- Aflatoxins G: This group includes aflatoxin G1 and aflatoxin G2
- Aflatoxins M: This group includes aflatoxins M1 and M2.

These aflatoxins are metabolic products which are found in the urine and milk produced by animals which have been given feed with aflatoxins in it. These toxins are formed by strains of moulds that infest susceptible grains such as maize and sorghum. Dairy cows that eat contaminated feed can yield contaminated milk. Aflatoxins are a group of about 20 chemically related toxic chemicals produced primarily by the foodborne mold Aspergillus flavus and A. parasiticus. Aflatoxins contaminate a variety of staple foods including maize, peanuts, and tree nuts; they cause an array of acute and chronic human health disorders. Aflatoxin B1, the most toxic of the aflatoxins, is a potent liver carcinogen, causing hepatocellular carcinoma (HCC) in humans and a variety of animal species.

MATERIALS AND METHODS:
There is also an increasing body of evidence that aflatoxins modulate the immune system (number of macrophages, lymphocytes and erythrocytes) reducing the animal’s response to challenges. The presence of mycotoxins in feed can hit all animal producers hard. Loss of productivity, and sometimes loss of the finished product can result from feeding grains with high levels of mycotoxins. Among the most affected species are high producing dairy cattle. The importance of quality feedstuffs to producers can mean the difference between profit and loss.

REFERENCES:
http://www.rightdiagnosis.com/intro/underdiag.htm
http://www.highfiber.com/~galenvtp/vtlafltx.htm

RESULTS:
Down on the dairy farm, the incidence of diseases such as displaced abomasum, ketosis, retained placenta, metritis, mastitis and fatty livers increases with mycotoxin exposure. Mycotoxin induced diseases seldom respond if at all to veterinary therapy and result in increasing losses if only veterinary solutions are pursued.

DISCUSSION AND CONCLUSIONS:
Furthermore, ration adjustments and management changes (grouping, cow movement, stall allotment, etc.) are of little value although they may be a factor in predisposition to mycotoxicoses.
INTRODUCTION:
Equine infectious anemia (EIA, swamp fever) is a contagious worldwide viral disease of the equines. It is characterized by intermittent fever, anemia, chronic loss of weight, and dysfunction of the cardiovascular system [1,3]. The causal agent of equine infectious anemia is an RNA virus of the genus Lentivirus of the family Retroviridae [2,3]. Standard diagnostic approaches for EIA include virus isolation in cell culture, serological methods for antibody detection, and amplification of viral sequences by PCR. The latter allowing for accurate diagnosis of early times post infection.[2,3]

The aim of this work was to develop a test system to detect EIA virus genomic sequences by real-time quantitative reverse transcription PCR (RT-qPCR).

MATERIALS AND METHODS:
In our work we used different EIAV strains and isolates, heterologous viruses, and samples from normal animals. Extraction of viral RNA was performed using a modified method of Boom et al. RT-qPCR was carried out on the thermocycler Rotor Gene 6000 (Corbett Research, Australia).

Cloning of the amplified EIA virus genome’s fragment was performed using the "Ins Taclone PCR cloning kit"(Fermentas, Latvia). The synthesis of RNA from cloned DNA inserts was performed using the kit "RiboMAX Large Scale RNA Production System T7" (Promega, USA).

RESULTS:
The specific primers and oligonucleotide probe complementary to a region of the EIA virus genome were designed following analysis of nucleotide sequences of different strains and isolates available in Gen Bank. The selected primers flank 99 b.p. fragment within the gag-gene of EIA virus genome.

To assess the specificity of the developed test-system we performed amplification of different strains and isolates of EIAV, blood and organs samples from infected animals, heterologous viruses, and nucleic acids extracted from horse leukocyte culture and blood samples from normal animals. Test system has identified viral RNA and proviral DNA of EIA virus. With any of the examined normal samples and heterologous viruses no false-positive results in RT-qPCR were obtained.

The analytical sensitivity of the method was determined using in vitro transcripts generated from a plasmid in which the amplicon was inserted. Ten-fold serial dilutions of in vitro transcribed RNA (of known concentration) were used to determine analytical sensitivity of RT-qPCR. The limit of sensitivity was the maximum dilution at which a positive result was obtained. The calculated value of analytical sensitivity of RT-PCR in real-time was 1,18*10 RNA copies / ml and 0,77 *10 DNA copies / ml.

DISCUSSION AND CONCLUSIONS:
The designed test-system is suitable to detect the EIA virus sequence in blood samples and organs of infected horses. This proves the specificity of the primers and the probe included in the test. The analytical sensitivity of the developed test - system is 1,18*10 RNA copies / ml and 0,77 *10 DNA copies / ml.

REFERENCES:
CONTROL OF LISTERIA MONOCYTOGENES IN TRADITIONAL FERMENTED SARDINIAN OVINE SAUSAGE

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Keywords: L.monocytogenes, Ovine Sausage, Molecular subtyping, Sardinia

INTRODUCTION:
Listeriosis is a serious infection as a consequence of ingestion food contaminated with L.monocytogenes (1). In traditional fermented sausages, realized with raw meat, without any heat treatment, the particular conditions due to fermentation may inhibit the growth of most pathogens as a consequence of several factors such as; Ph, lactic acid, water activity. Several “Mediterranean style” dry fermented sausages could be included in categories of RTE, according to current regulations (2). Despite the remarkable variety of operating modes according to different local traditions that determine variety of fermentation and ripening, there is scarce epidemiological evidence about the involvement of fermented sausages in listeriosis outbreaks. This work is a report of L.monocytogenes control in food processing managements and in traditional fermented sardinian ovine sausage.

MATERIALS AND METHODS:
Following measures were performed, during three years
- Were monitored 11 production batches of fermented sardinian ovine sausages (total 150 samples); for each production batch have been submitted to control 10-20% of the sheep carcasses used for processing (total 200 samples)
- Environmental monitoring was performed on all production environments, equipment and operators’ hands
- Molecular Identification and characterization of the strains isolates: PFGE,MLST, VNTR, PCR, according to previous published protocols (4) (5)(6)(7)
- Antibiotic-resistance of the strains isolates: Sensititre - Trek diagnostics, Cleveland, OH
- Serotyping of the strains isolates: antisera DenkaSeikenCo Ltd Tokio Japan
- Production process analysis and development of the flow chart by identifying CCP for revision and rationalization of the HACCP plan
- Accurate training of operators in terms of hygiene and Good Manufacturing Practices
- Improvement of hygiene measures of work surfaces and equipment

RESULTS:
The adoption of control measures, during our research, has limited the presence of L. monocytogenes in this product (<100 ufc/g according to RTE current legislation).

DISCUSSION AND CONCLUSIONS:
The implemented actions after the revision of the haccp plan, permitted the rational control of L.monocytogenes in the working environment. The identification of L.monocytogenes with additional investigation as molecular subtyping have increased the biological and epidemiological knowledge and consequently contributed to create a better conditions to control the pathogen. Fermentation, biocompetition, drying sausages during ripening, may limit the L.monocytogenes growth. This pathogens may be found at the end of the production cycle if all production chain isn’t well checked. This paper demonstrate that a correct application of GMP and HACCP plan may manage the L.monocytogenes presence.

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1) EFSA Journal 2013;11(4):3129 [250 pp.]
2) EC Regulation 2073/2005
ASSESSMENT OF RISK RELATED TO THE CONSUMPTION OF FRESH FRUIT AND VEGETABLES DISTRIBUTED AT EDUCATIONAL INSTITUTES

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Keywords: Fruit, vegetables, risk assessment

INTRODUCTION:
In recent years the problem of food-borne infections has become increasingly important, as demonstrated by the numerous controls carried out in different types of foods. This is an important issue for the Food Safety also because the consumers put more attention to the quality of foodstuffs.

The Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna (IZSLER), through a collaboration between the laboratories of Piacenza, Brescia and Parma, started a project with the aim to evaluate the risk associated to the consumption of fresh fruits and vegetables in educational institutes. In fact, many reported outbreaks in literature are caused by enteric microbiological and virological pathogens due to the crowded environments and the habits of children (1; 2). The survey involved different types of ready to eat (RTE) fruits (strawberries, apples, oranges) and vegetables (carrots and tomatoes) for the detection of pathogenic and spoilage bacteria, molds, viruses and parasites. The aim of this work was to evaluate the prevalence of these microorganisms in fresh produce distributed among educational institutes canteens, in order to understand the health risk and monitor the trend of contamination in foodstuffs.

MATERIALS AND METHODS:
From April 2014 to June 2014, 32 samples of fresh fruit and vegetables, collected from three different educational institutes were analyzed.

In detail, the samples collection provided 2 withdrawals of apricots, 5 of oranges, 3 of carrots, 3 of cherries, 3 of strawberries, 2 of fruit salads, 9 of apples, 3 of peaches, and 2 of tomatoes. Each sample was analyzed for bacteria, viruses and parasites, with different protocols:

MICROBIOLOGICAL METHODS: Bacillus cereus, mesophilic lactic acid bacteria, Enterobacteriaceae, Yeasts, coagulase-positive Staphylococci, ß-glucuronidase positive Escherichia Coli, molds, Aw.

MOLECULAR METHODS (PCR - Real Time PCR): Campylobacter spp. (RT PCR), Cryptosporidium parvum (nested PCR), Giardia lamblia (RT PCR), Listeria monocytogenes (RT PCR), Norovirus G1 and GII (RT PCR), Salmonella spp. (RT PCR), Toxoplasma gondii (PCR), Hepatitis A Virus (hemi-nested PCR) MICROBIOLOGICAL AND MOLECULAR METHODS: Escherichia coli STEC.

To confirm the outcomes, positive PCR products were purified with QiAquick® Gel Extraction Kit (Qiagen) and then cycle sequenced using the BigDye® Terminator Cycle Sequencing kit (v3.1, Applied Biosystems) and an ABI3130 genetic analyzer (Applied Biosystems). The sequences were assembled using SeqMan (Lasergene program package, DNAStar Inc., Madison, WI, USA) and aligned by using the Clustal W program.

RESULTS:
These preliminary results obtained from the microbiological and molecular methods showed that there were no positive samples for bacteria or for viruses. The analyses for the parasites detected 2 PCR positive samples for Toxoplasma gondii; these two samples were fruits salad from two different schools. However, the outcome of the sequencing method stated that it was not T. gondii DNA.

DISCUSSION AND CONCLUSIONS:
According to a recent survey, more than a third (36%) of Italian children are either overweight or obese by the age of eight (3). For this reason, in the last years, primary schools started to improve the alimentary habits serving more fruits and vegetables. In this contest, the food safety is important, especially when concerning children. Only two samples were detected positive for Toxoplasma gondii, but amplicon sequencing revealed a different target DNA sequence, probably due to the use of non-specific primers in PCR. With the preliminary data obtained in this work it was possible to observe that vegetables and fruits were not a probable source of pathogens contamination, also as a result of a good policy of the HACCP practices employment by the producers. However, it is necessary to continue the survey in order to obtain a complete overview, supported by a high number of samples, about the circulation of microorganisms in fresh products for children consumption.

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2. Wikswo M.E. et al., 2012.
3. www.salute.gov.it
IDENTIFICATION OF ANIMAL SPECIES BY MICROARRAY AND REAL TIME PCR TOOLS APPLICATION IN FOOD SAFETY

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Keywords: Microarray, Real Time PCR, Food safety, Consumer, diagnostic tool

INTRODUCTION:
With the circular April 9, 2003, published in OJ n. 93 of 22.04.2003, the Ministry of Agriculture and Forestry provides some clarification on the application procedures improving the European Regulation on the labeling of beef and beef products and beef. In terms of food safety, for the control of animal species in counter products, molecular techniques are currently the methods of choice for the guarantee of the consumer. However when the cutting commercial no longer allows the recognition of species or in the case of complexes prepared, the only way to recognize the species remains the examination of DNA. In the first case is used generally to the technique of DNA barcoding, while in the second case, it is necessary to apply methods as microarray and real-time PCR able to discern between the various target animals. Hybridization tools permit to reveal various species with only one step PCR; real-time PCR specifically amplifies DNA fragments very small, no more than 100-150 bp, and is applicable even if the procedures of processing of the food are harmful to the DNA. In this paper we present preliminary data on the development of microarray and real-time PCR method to determine the contents of beef products, using screening and confirmation tools applied on crude extracted DNA.

MATERIALS AND METHODS:
The DNA sample is extracted using special kits that are based on the use of affinity columns or resins consist of silica-magnetite. As screening the chipron technology was applied. Chipron LCD Array Kit Meat 4.0 is a DNA-based identification kit of animal species. With its sophisticated design of primer and probe, Meat 4.0 provides user a fast and reliable screening method to identify 24 animal species in a very short protocol. Special designed software let user receive identification report with just few clicks. For Real time amplification was employed a mix comprises: 1X Master Mix (Life Tech.), 0.5 pmol / ul of each primer, 0.25 pmol / ul of each probe, 20 ng of extracted DNA and water up to 20 ul. The amplification cycles were made up as: 50° C for 2’ and initial denaturation at 95° C for 10’; 45 repetitions of denaturation at 95° C for 20’ and polymerization at 60° C for 1’. The results were automatically processed by the dedicated software. The worksheet automatically also calculates the load of any dilution tested. The detection limit was measured in the order of 0.1 pg/ul However, the limit beyond which the food is considered to be merely influenced by a meat species is 1%.

RESULTS:
The results of the experiment are given by the optimization and validation of the method in terms of screening and quantitative analysis for the determination of meat species in complex food mixtures prepared. In our experience we analyzed 40 food samples for specie detection. The results showed in same cases not declared species. We demonstrated as the screening and confirmation tol application is a good tool useable on field. In any case the above information to the consumer, so ‘as the information contained in labels should be designed to highlight the link between the meat offered for sale on the counter and the individual animal or group of animals from which they come. It is therefore necessary that the operator puts in place appropriate systems to create on the sales counter is a direct correlation between the origin of the meat exposed individual and the corresponding information to the consumer.

DISCUSSION AND CONCLUSIONS:
If the majority of the analysis are based on food chemistry methods, more or less sophisticated, or microbiology, also from time DNA analysis have taken a prominent place. To determine the authenticity, or figure out if a product is what it claims to be, the DNA, as conclusive evidence of animal or plant species used, has a central role.

REFERENCES:
INTRODUCTION:
Over the past few decades, the occurrence of harmful algal blooms has increased both in frequency and in geographic distribution in many areas of the world. This resulted in negative effects both on public health and on economy. Several algal species, in fact, can produce potent toxins which affect human health through the consumption of contaminated seafood or aerosol exposure. Palytoxin (PITX) is one of the most potent marine toxins known and is produced, together with its analogues (PITXs), by benthic dinoflagellates belonging to the genus Ostreopsis, a genus recently found also in the Mediterranean Sea. Occurrence of Ostreopsis spp. may therefore result in PITX presence in seafood (250 µg/kg proposed regulatory limit) and, in order to prevent sanitary risks, rapid and sensitive monitoring methods for PITX-group toxins are needed.

MATERIALS AND METHODS:
We recently developed an electrochemical biosensor method for PITX detection (1). The method is based on a mediated amperometric measure of the lactic dehydrogenase (LDH) released by the PITX-induced hemolysis of sheep erythrocytes. In method development different extraction procedures from mussels were compared (1, 2, 3) and some of them showed a strong matrix effect on the assay, with an evident inhibition of the PITX-induced hemolysis even in presence of high concentrations of toxin. In this work we assessed the performance of a different, simple extraction procedure for shellfish testing, evaluating specifically its efficiency in reducing the matrix effect on the bioassay. The procedure included the following steps: blending of 10 g of whole shellfish tissue with 90 ml of PBS, centrifugation (4800 ×g at 4°C for 20 min), filtration through a 0.22 μm filter, shaking with chloroform (1:1 v/v) for 15 min followed by centrifugation (12500 ×g for 5 min) and collection of the aqueous phase and, finally, dilution 1:50 of the retained suspension in PBS. Analysis was performed on two different shellfish species (mussels and clams) to assess the response of the extraction to variations of tissue composition. Samples were prepared by spiking the shellfish extracts with different concentrations of PITX (0.2, 0.4, 0.8, 1.6, 3.12 ng/ml) and were compared to same toxin concentrations in PBS.

RESULTS:
The results of the experiments showed that the proposed extraction procedure allowed an almost complete removal of the matrix effect. The amperometric signal obtained by the haemolytic-enzymatic assay on shellfish extracts showed a significant agreement with the results of the blank sample (Fig. 1). The concordance of the results on mussels and clams confirmed the performance of such procedure on different kinds of shellfish species.

DISCUSSION AND CONCLUSIONS:
The proposed extraction procedure, by reducing the matrix effect, allows a significant improvement of the samples preparation for PITX detection by electrochemical sensor. Additionally, the procedure is simple to perform, rapid and cost-effective, therefore showing fitness for the use in a rapid detection method. Further experiments are ongoing to quantitatively assess the recovery of PITX from matrix.

REFERENCES:
INTRODUCTION:
Verotoxin-producing Escherichia coli (E.coli VTEC) is an emerging cause of foodborne disease in human and this infections are typically associated with transmission through animal products. At the time PCR technique is consider as an alternative to standard culture methods for E.coli detection in foods. Molecular methods for pathogenic E.coli detection in food comprise in particular qualitative methods in which the bacterial targets is identified based on its virulence factors. E.coli O157:H7 serotype has been identified as main causative agent of foodborn diseases and of sporadic cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Bhong et al. 2008). Cattle are known reservoirs of VTEC strains (Hussein et al., 2005), and their incidence, also that E.coli non-VTEC infections, is difficult to estimate since these serogroups are not routinely screened (Bettelheim, 2007). Molecular technique has been standardized by ISO/TS 13136:2012 for the detection so called “top-five” serogroups. In 2009, indeed, the European Food Safety Authority (EFSA) published Technical specifications for harmonized monitoring and reporting of verotoxigenic Escherichia coli (VTEC) in animals and foodstuffs by the European Union Member States in accordance with the Directive 2003/99/EC. In this study we reported the data analysis of the monitoring plan on prevalence and distribution of E.coli VTEC in foodstuffs collected in the Sardinia region of Italy, as determined by a qualitative TaqMan™ Real time PCR protocols and immunomagnetic-separation techniques (IMS). The aim of the present study was to apply a molecular screening test for the detection of E.coli VTEC in foodstuff, collected in retail, trade market, cash and carry market and to define the virulence profile of the isolates in case of positive samples establishing a potential correlation with the culture method.

MATERIALS AND METHODS:
This study was based on investigations carried out from January 2012 to May 2014 on n.308 products of animal origin and ready-to-eat foodstuffs produced and marketed in the Sardinia region of Italy. The food samples were analyzed for the presence of E.coli O157:H7 VTEC and non-O157 VTEC. Upon arrival at the laboratory, samples tested in accordance with ISO 16654:2001 and for the RT-PCR amplification and the determination of the VTEC populations we adopted the ISO/TS 13136:2012. The two methods were performed on the same foods samples.

RESULTS:
We observed that the IMS method not showed no-compliance in all food samples tested, while of the n.308 foodstuff samples collected from Sardinia monitoring plan between twenty nine months, only one Verocytotoxigenic gene (Vtx2) was detected by real-time PCR in one minced fresh meat sample. The remaining samples tested were negative or were inhibited in the TaqManTM based system (Table 1; Table 2). The non analyzable results for Vtx2 associated serogroup-specific assays were because of inhibition of PCR reactions in the Fast real time PCR and to the difficulty of culture isolation. All Internal Amplification Control (pUK19) were positive and in compliance with molecular method in verocytotoxigenic gene detection (Table 2).

DISCUSSION AND CONCLUSIONS:
Molecular technique proved to be suitable for screening purposes, but it must be considered that this does not result in a bacterial isolate. By taking into consideration only the screening results without strain isolation, we will have a distorted consideration on the occurrence of VTEC serogroups in the Sardinia foodstuff. However, fast and reliable isolation of respective strains poses a major challenge.

REFERENCES:
EFFECTS OF THERMAL TREATMENT ON WALNUT DETECTION IN FOOD

Razzuoli E., Migone L., Porcario C., Rubini D., Gennari M., Lazzara F., Vito G., Ferrari A.
IZS Piemonte, Liguria & Valle d’Aosta ~ Genova ~ Italy

Keywords: allergens, walnut, thermal process

INTRODUCTION:
Food allergies, an emergent food safety issue, affect 1-2% of adults and up to 5-7% of children [1]. Walnuts, an ingredient of many pastry products, play an allergenic role for many people. Thermal exposure, which often occurs for walnut products, may induce structural changes in proteins, thus affecting their allergenicity and the performance of the tests used to detect them [2]. Aim of this study was to evaluate thermal treatment effects on walnut allergenicity and on the performance of ELISA kits aimed to detect walnut traces.

MATERIALS AND METHODS:
Walnut samples were exposed to the following treatments: 1) boiling, 2) roasting at 80°C (10 min.), 3) roasting at 180°C (10 min.), 4) roasting at 180°C (30 min.), 5) freezing at -20°C for 7 days, 6) boiling followed by freezing, 7) roasting at 80°C (10 min.) and then freezing at -20°C for 7 days, 8) roasting at 180°C (10 min.) followed by freezing, 9) roasting at 180°C (30 min.) and then freezing. Secondly 20 samples of baked dry biscuits, 20 cookies and 10 dry pastry products were spiked (10 ppm) with walnuts, either raw or treated as describe above. ELISA analysis was performed using 2 different commercial kits (A and B). Data were elaborated by one-way ANOVA (Prism graphPad 5.03) and a p-value ≤0.05 was considered statistically significant.

RESULTS:
Boiling (1, P<0.05), intense and prolonged roasting (4, P<0.0001) showed high influence on the sensitivity of both kits (Fig. 1, panel A). Intense roasting (3) caused significant effect only on kit A performance, while mild roasting (2) did not significantly influence the detection ability (P >0.05) of neither of the two ELISA tests. Freezing process alone (5) revealed a significant improvement only of kit A performance; the association of freezing to heat treatments led to a better performance of kit A than that displayed by the same kit when the singular heat treatments were applied (Fig. 1, panel B). Regarding kit B (Fig. 1, panel B), its performance was significantly decreased (P<0.001) by the association of treatments, particularly in case of the boiling-freezing process(6)and when treatment 8 was applied.

DISCUSSION AND CONCLUSIONS:
Many reports are available in literature on the testing of thermal process effects on walnut immunogenicity. According to such studies the walnut immunogenic potential is little affected by thermal treatments, even if they may cause structural changes in walnut proteins. Moreover, such modifications seem to be protein-dependent. Actually, the five currently known allergenic proteins of walnut - Jug r1, Jug r2, Jug r3, Jug r4 and Jug r5 - have different susceptibility to thermal treatments[3].To date, few studies are available on the effects of these processes on ELISA test performances to detect walnut proteins. In our study different performances of Kit A and B were detected in relation to roasting and freezing processes, considered singularly or in combination. Conversely, in relation to the boiling treatment, both the kits showed a decreased performance,likely because of the water-solubility of walnut proteins, responsible for a heat-induced protein loss. When heating and freezing processes were associated, kit A showed a better performance in detecting walnut proteins than kit B. This was likely due to the different types of antibodies coated on the ELISA plates of the two kits. Results obtained from the prolonged roasting treatment point that both A and B kits are unsuitable to detect walnuts on high temperature processed food. Hence, the evaluation of the most suitable allergen detection method in food, taking into account thermal processing effects, is really demanding to ensure consumer’s safety.

REFERENCES:
3. Masthoff et al., 2013. “A systematic review of the effect of thermal processing on the allergenicity of tree nuts” Allergy. 68:983-93

Figure 1. Effects of thermal treatment on ELISA performance
INTRODUCTION:
Recent studies performed in Europe showed that emerging enteropathogens, such as Arcobacter spp., Escherichia coli O157 and Clostridium difficile, were isolated from shellfish (1,2,3,6).
Of all Arcobacter spp., A. butzleri, A. cryaerophilus and A. skirrowii had been associated with gastrointestinal disease in humans. In particular, A. butzleri is the most important species for human infection and it has been classified as a serious hazard to human health by the International Commission on Microbiological Specification for Food (2, 5).

MATERIALS AND METHODS:
From December 2012 until February 2014, 93 samples of shellfish from different coastal areas of the Adriatic Sea in Region Marche, central Italy, were analyzed. These included 20 samples of mussels (Mytilus galloprovincialis) and 73 samples of clams (Chamelea gallina). All mussels and 13 clams were from Class A areas, while the remaining 60 samples of clams were collected from Class B areas (7). Sample preparation was performed according to the UNI EN ISO 6887-3:2004. Detection of pathogens was performed as follows: Arcobacter spp. according to the method proposed by L. Collado et al. (2009), E. coli O157 according to the ISO 16654:2001 and C. difficile according to the method proposed by V. Pasquale et al. (2012). The identification of Arcobacter spp. was performed with rpoB gene sequencing (1).

RESULTS:
Of the 93 shellfish samples analyzed, 41 (44 %) were positive for Arcobacter spp. The rpoB gene sequencing was performed for 19 samples of which 13 were A. butzleri and 6 A. cryaerophylus. Nineteen of the shellfish (20%) were positive for C. difficile, and all were negative for E. coli O157.
Arcobacter spp. was isolated from 6 of the 20 (30%) samples of mussels and from 35 of the 73 (48%) samples of clams. C. difficile was isolated from 5 of the 20 (25%) mussel samples and from 14 of the 73 (19%) samples of clams. Both pathogens were isolated in 13 of the 93 (14%) samples analyzed, including 3 mussels (23%) and 10 clams (77%). Of the 13 clams from Class A areas, 6 (46%) were positive for Arcobacter spp. only, 1 (8%) for C. difficile only and 1 (8%) for both pathogens. Of the 60 clams from Class B areas, 7 (12%) were positive for Arcobacter spp. and 3 (23%) for C. difficile, while 9 (15%) were positive for both pathogens. Also, 35 of 93 (38%) samples were negative for all pathogens, of these 8 were mussels (23%) and 27 clams (77%).

DISCUSSION AND CONCLUSIONS:
In this study Arcobacter spp. was the most frequently isolated pathogen, followed by C. difficile. E. coli O157 did not seem to represent a risk in the studied production areas. Chamelea gallina seems to be a more hazardous matrix than Mytilus galloprovincialis, especially for B class areas. To our knowledge, this study is the first aimed to evaluate the prevalence of these three emerging enteropathogens in indigenous shellfish in Italy. Although partial, these results suggest the need for further investigations for these foodborne pathogens that are not considered in the legislation, but that can be concentrated in shellfish representing a risk for humans.

REFERENCES:
PRELIMINARY OBSERVATIONS FROM A CROSS-SECTIONAL STUDY OF VEROTOXIN-PRODUCING ESCHERICHIA COLI (VTEC) IN THE PROVINCE OF TURIN (PIEDMONT)


Istituto Zooprofilattico Sperimentale Piemonte Liguria e Valle d’Aosta ~ Turin ~ Italy

**Keywords:** E. coli, VTEC, HUS

**INTRODUCTION:**
Escherichia coli are normal inhabitants of gastrointestinal tract of animals and humans. Some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases (1). The E. coli strains producers of Verotoxins (VTEC), are characterized by the ability to produce potent cytotoxins whose effect on target organs is the basis of serious diseases such as the haemolytic uraemic syndrome (HUS). E. coli O157: H7 is the serotype most frequently isolated from cases of HUS, although other STEC serogroups have been associated with (O26, O103, O111, O145, O45 and O121). Ruminants represent the main host of VTEC and are generally healthy carriers of the organisms.

The presence of VTEC in animal faeces provides the potential to enter the food chain by faecal contamination of animal products, fruit and vegetables. The present work aims to provide preliminary data of a “cross-sectional” study of STEC in dairy farms in the province of Turin involved in human outbreaks of HUS.

**MATERIALS AND METHODS:**
From March to June 2014, 333 environmental fecal samples were collected from 37 dairy farms in the province of Turin. After homogenization, 25 g of each fecal sample were inoculated into 225 ml of buffered peptone water (BPW) and incubated at 37°C overnight. A polymerase chain reaction (PCR) assay was performed on 500 μl of broth-culture in order to determine the presence of vtx1 and vtx2, genes for Vero toxins 1 and 2 and eae, gene for E. coli attaching-and-effacing and serogroups (O26, O157, O145, O103 and O111). To isolate STEC from sample positive by PCR, 100 μl of the enrichment broth-culture was tested with immunomagnetic separation and plating onto selective Sorbitol MacConkey agar added with Cefixime-Tellurite Supplement (CT-SMAC) for the detection of serogroup O157, on Rhamnose MacConkey Agar (RMAC) for the detection of serogroup O26 and on chromogenic agar (STEC) for the detection of the other serogroups. After confirmation with biochemical test and serum agglutination, the selected strains were identified by PCR assay. The colonies positive were subjected to susceptibility test according to Kirby-Bauer method.

**RESULTS:**
16 out of 37 farms were positive to the isolation of one or more VTEC strains, 1 farm to E. coli eae+vt1-vt2- and 2 farms to E. coli O103 eae-vt1-vt2-. Furthermore, 3 farms were positive for two different serogroups (O145/O103, O26/O103, O26/O157). For the remaining farms (13), positivity for O26 (7), O103 (3), O157 (2) and O145(1) was detected. 75 E. coli VTEC have been tested for their antibiotic susceptibility showing resistance to clindamycin(100%), oxacillin(100%), erythromycin(97%), sulphisoxazole(93%), cephalothin(67%), tetracycline(59%),ampicillin(33%),kanamycin(33%),amoxicillin with clavulanic acid(23%),cefazolin(13%). All strains tested were susceptible to chloramphenicol(100%),gentamicin (100%),enrofloxacin(100%).

**DISCUSSION AND CONCLUSIONS:**
The present study is the first systematic investigation of VTEC in cattle in Piedmont. The positivity by biomolecular method is not always confirmed by microbiological culture. The explanation to this phenomenon can be given by pathogenicity factors not related to specific serogroups investigated, bacterial load below the threshold of detection of microbiological method or by a lower competitiveness of VTEC strains than other E. coli. The results obtained seem also to suggest that the antibiotics selection encourage mostly commensal E. coli flora instead of STEC strains that are quite susceptible to molecules used for the treatment of E. coli neonatal enteritis (tetracycline, gentamicin, fluoroquinolones).

**REFERENCES:**
1. Oie Terrestrial Manual; www.oie.int
INTRODUCTION:
Salmonella spp. is one of the major food-borne pathogens and has an importance as a leading cause of food-borne bacterial diseases in humans throughout the world (1). The EFSA report of 2012 confirms the decreasing of salmonellosis cases in humans with a total of 91,034 cases reported. Among the most frequently isolated serotypes in humans the most commonly isolated serotypes are S. Enteritidis, S. Typhimurium and monophasic S. Typhimurium 1,4,[5],12:i (2).

According to the food safety criteria set out by Regulation (EC) 2073/2005 and subsequent amendments and additions, excluding fresh poultry meat, Salmonella spp. has to be absent in 25 g (3).

The aim of this paper is to report the results of the analysis performed for Salmonella spp., under the Regulation (EC) 2073/2005, from 2011 to 2013 and the frequency of the isolation of different serotypes.

MATERIALS AND METHODS:
In 2011-2013 a total of 1,937 food samples collected at retail stores were tested for Salmonella species by Food Control Laboratory of Veterinary Medical Research Institute for Piemonte, Liguria and the Valle D’Aosta. The samples were tested for Salmonella species, using the reference method ISO 6579: 2002/Corr., 2004. The strains isolated were serotyped according to the Kauffmann-White and Le Minor scheme. Moreover the monophasic S. Typhimurium 1,4,[5],12:i:- was confirmed using an end point PCR for the evaluation of the presence of the genes fljA e fljB.

RESULTS:
Thirty one food samples out of 1,937 (1.6%) were positive for the detection of Salmonella spp.. Table 1 shows the prevalence of Salmonella spp. isolated from the food samples tested. Among the foodstuffs, minced meat and meat preparations from other species than poultry accounted for the 58.1% of positive samples (18/31), while meat products intended to be eaten raw accounted for the 25.8% (8/31).

Serotype prevalence and distribution in food samples are reported in Table 2. A total of 14 serotypes were isolated, with monophasic S. Typhimurium 1,4,[5],12:i:- (7/31; 22.6%), S. Typhimurium (6/31; 19.3%) and S. Derby (3/31; 9.7%) being the most frequent.

DISCUSSION AND CONCLUSIONS:
The data indicate minced meat and meat preparations (other species than poultry) as the major source of contamination, with 4.2% of non-compliant samples. In Europe a reduction of Salmonella spp. in poultry and products thereof is reported and can be linked to the on-farm control measures set out against this pathogen, as well as the reduction of S. Enteritidis in humans (5). If the number of reported human cases of S. Enteritidis has dropped, though, on the other hand, monophasic S. Typhimurium 1,4,[5],12:i:- increased (4).

In Italy, monophasic S. Typhimurium 4,[5],12:i:- and S. Typhimurium are the first two serotypes isolated both in humans and food (4). Moreover EFSA suggests, for a correct identification of monophasic S. Typhimurium 4,[5],12:i:-, to proceed with serotyping and then apply a PCR protocol in order to confirm the lack of the second phase antigen (6). The identification of the serotype is a pivotal point to evaluate the distribution and variation of the epidemiological situation as well as the effectiveness of the control program.

REFERENCES:

Table 1: Salmonella spp prevalence

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>Positive samples</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium, monophasic</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>S. Derby</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>S. Enteritidis</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>S. Thompson</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>S. Paratyphi</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Agona</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Hadar</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Stanley</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Virchow</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Newport</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Cappel</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Stanley</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. Hadar</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 2: Serotypes of Salmonella by source of isolation
DETECTION OF VIBRIO PARAHAEOMOLYTICUS AND VIBRIO VULNIFICUS IN SHELLFISH FARMED AND MARKETED IN SARDINIA REGION IN 2013


Istituto Zooprofilattico Sperimentale della Sardegna ~ Sassari ~ Italy

Keywords: Vibrio parahaemolyticus, Vibrio vulnificus, Sardinia

INTRODUCTION:
Vibrio is a genus of Gram-negative halophilic bacteria, that consists of more than 100 species grouped in 14 clades diffused worldwide, widely distributed in coastal and sea-waters, estuarine habitat and sediments (5). In particular, represent a serious hazard for public health infection due mainly to Vibrio cholerae, Vibrio parahaemolyticus e Vibrio vulnificus, since they are cause of foodborne infections consequently to ingestion of raw molluscs and ichthyic products, seawater and other kind of seafood. Main V parahaemolyticus virulence factors are two toxins: the thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH). Different serotypes have been correlated to human diseases, however, in the last 20 years, a specific pandemic strain, the serotype Q3:K6 and its derivatives, O4:K68, O1:K25, O1:KUT (3), has been cause of pandemic spread in the word. In Italy, between 2007 and 2008, three clinical cases have been reported, two TDH-positive with pandemic potential and one TRH-positive V. parahaemolyticus strains. Also, two strains of V. parahaemolyticus, belonging to a pandemic clone, have been isolated in a sample of marine plankton collected in Venetian lagoon (4). V. vulnificus doesn’t cause human disease outbreaks but it is responsible for severe syndromes that are fatal in about 50–60% of cases. Diseases associated with V. vulnificus infection follow two different patterns: primary septicaemia who manifest after eating row seafood, with a mortality rate of over 50%, in the other pattern, wound infections are incurred following exposure to seawater or handling of seafood products, with a death rate of 25% (1).

The aim of the present work is to study the presence of V. parahaemolyticus and V. vulnificus in shellfish farmed and marketed in Sardinia in 2013. Collection of epidemiologic data of molluscs, concerning the contamination levels due to Vibrio parahaemolyticus and Vibrio vulnificus is essential for a correct valuation of vibrio’s risk.

MATERIALS AND METHODS:
During the 2013, the Laboratory of IZS of Sassari, analyzed 1079 samples, collected from ASLs of Sardinia. A number of 754 samples for Monitoring and 325 for the market surveillance. The research of V. parahaemolyticus and V. vulnificus were made using the ISO/TS 21872-1: 2007 and ISO/TS21872-2: 2007 (2).

RESULTS:
Results are shown in table 1 and 2.

DISCUSSION AND CONCLUSIONS:
V. parahaemolyticus and V. vulnificus give origin to emerging public health problems. Reg. CE 2073/2005 does not fix specific criteria, however, recommend the institution of management for good hygienic practices.

According to the international literature the number of outbreaks of V. parahaemolyticus are increasing due to the diffusion of its pandemic strain Q3:K6 TDH-positive, and, also, presence of V. vulnificus in oyster is raising. Despite highlighting about its presence in Italy in the last years, Vibrio is not routinely researched in microbiology laboratories, neither, lows that report legal limits regarding of acceptability of samples are known. For this reasons there are few information about presence and distribution of Vibrio. Positivities to Vibrio shown by our results are not numerous, however, we confirm that it is necessary acquire more knowledge about V. parahaemolyticus and V. vulnificus in Sardinia marine seawater. It is desiderable to include this microorganism in the surveillance systems for gastrointestinal diseases and in monitoring programs relative to mollusc collection areas.

REFERENCES:
Desmarchelier PM, 2000. Encyclopedia of food Microbiology, 2037-2042

Table 1 Determination of V. parahaemolyticus and V. vulnificus in samples of mollusks collected from ASL of Sardinia during 2013,

<table>
<thead>
<tr>
<th>ASL</th>
<th>n. total samples</th>
<th>n. surveillance samples</th>
<th>n. monitoring samples</th>
<th>n. positive samples V. parahaemolyticus</th>
<th>n. positive samples V. vulnificus</th>
<th>% positive samples V. parahaemolyticus</th>
<th>% positive samples V. vulnificus</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.1. SASSARI</td>
<td>64</td>
<td>63</td>
<td>0</td>
<td>2 (ner.)</td>
<td>0</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>N.2. OLbia</td>
<td>351</td>
<td>69</td>
<td>212</td>
<td>10 (monitor.)</td>
<td>7 (monitor.)</td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td>N.3. VIBIO</td>
<td>40</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>N.4. LANESE</td>
<td>70</td>
<td>12</td>
<td>58</td>
<td>2 (monitor.)</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>N.5. ORISTANO</td>
<td>185</td>
<td>43</td>
<td>142</td>
<td>12 (monitor.)</td>
<td>5 (monitor.)</td>
<td>6%</td>
<td>3%</td>
</tr>
<tr>
<td>N.6. SANTU</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>N.7. CIBORINA</td>
<td>27</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>N.8. CAGLIARI</td>
<td>137</td>
<td>81</td>
<td>254</td>
<td>12 (monitor.)</td>
<td>8 (monitor.)</td>
<td>4%</td>
<td>2%</td>
</tr>
<tr>
<td>Tot. samples</td>
<td>1079</td>
<td>425</td>
<td>554</td>
<td>68</td>
<td>20</td>
<td>18%</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 2 Determination of V. parahaemolyticus and V. vulnificus in oyster’s samples collected from ASL of Sardinia during 2013, number of samples analyzed in Monitoring and Sanitary Control, their positivity samples and the percentage of positivity

<table>
<thead>
<tr>
<th>Sample type</th>
<th>n. sample</th>
<th>n. positive samples</th>
<th>n. positive samples V. parahaemolyticus</th>
<th>n. positive samples V. vulnificus</th>
<th>% positive samples V. parahaemolyticus</th>
<th>% positive samples V. vulnificus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surveillance samples</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>n. monitoring samples</td>
<td>24</td>
<td>1 (monitoring)</td>
<td>2</td>
<td>5%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>n. total samples</td>
<td>40</td>
<td>3 (75%)</td>
<td>2</td>
<td>5%</td>
<td>2%</td>
<td></td>
</tr>
</tbody>
</table>
DETECTION AND CHARACTERIZATION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) ISOLATED IN RAW MILK FROM APULIA REGION (ITALY)


Keywords: methicillin-resistant Staphylococcus aureus, raw milk, food safety

INTRODUCTION:
Methicillin-resistant Staphylococcus aureus (MRSA) infections are a serious public health concern. MRSA infections are localised mainly in the hospital environment (hospital acquired, HA-MRSA) and in the community (community acquired, CA-MRSA). More recently, a number of livestock associated (LA-MRSA) clones, that have a zoonotic potential, has emerged. The contact with food producing animals and the handling/consumption of foods of animal origin could provide a route of exposure for the general population. S. aureus is an important cause of mastitis in cattle and the emergence of MRSA strains in cows could represent a source of human infection (7). The aim of this report was to establish the prevalence and the characteristics of MRSA in bulk tank milk produced in Apulia Region (Italy).

MATERIALS AND METHODS:
Bulk tank milk samples (BTM) were collected during 2012 and 2013 from 398 dairy farms of Apulia Region, South Italy. One ml of milk was added to Mueller-Hinton broth supplemented with 6.5% (W/v) NaCl and after incubation each culture was spread onto a MRSA-SELECT® plate (Bio-Rad, France). Suspected MRSA colonies were confirmed as S. aureus using a specific PCR (6). PCR for the detection of the mecA gene and SCCmec elements were carried out as previously described (8). MRSA isolates were typed using spa-typing and Multi-Locus Sequence Typing as described elsewhere (3; 4).

RESULTS:
MRSA were isolated in 8 (2.0%) of the 398 tested milk samples. The mecA gene was detected in all the suspect isolates. The results of typing are summarized below: ST1-t127-SSCmec IVa n=2; ST1-t174-SSCmec IVa n=1; ST5-t688-SSCmec V n=2; ST398-t011-SSCmec V n=1; ST398-t899-SSCmec IVa n=1; ST45-t015-SSCmec IVa n=1.

DISCUSSION AND CONCLUSIONS:
It is ascertained the MRSA strains of zoonotic origin could cause serious human infections. Further, the handling or consumption of foods contaminated with MRSA can act as potential vehicle of infections for humans (2). In our survey we detected eight MRSA belonged to a wide number of genotypes. Among our isolates, the most representative ST was ST1 (t127 or t174). The spa-type t127 (ST1), known as a human MRSA genotype, has been associated with serious human infections in the United States and in Germany (1). Two out the eight isolates belonged to ST398 (SCCmec types IVa or V), a sequence type associated to cases of severe pneumonia in Europe and isolated in Italy from one case of invasive disease in a cattle farmer (5). Our results found a low prevalence of MRSA in raw milk produced in Apulia Region. However, the findings of recognized zoonotic genotypes confirm the potential risk of transmission of MRSA to humans through this source.

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MONITORING OF ZOONOTIC PATHOGENS IN FERAL PIGEONS IN A RURAL AREA OF NORTHERN ITALY

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IZSLER ~ Lodi ~ Italy, IZSLER ~ Pavia ~ Italy

Keywords: pigeons, campylobacter, VTEC, chlamydia

INTRODUCTION:
Pigeons are naturally infected with several microorganism that are pathogenic to humans, such as Campylobacter spp., Salmonella spp. and verocytotoxin-producing E. coli (VTEC) and Chlamidia psittaci. These birds are widespread in Italy and frequently they live in permanent colonies within the herds, representing a possible vehicle of microorganism to food animals. Aim of this work was to determine the prevalence of four bacterial pathogens in pigeons captured in dairy cattle herds of the Lodi province in Northern Italy.

MATERIALS AND METHODS:
A total of 149 pigeons, 13 to 20 per farm, were captured in 8 dairy farms of the Lodi province. Cloacal swabs were collected and one per pigeon was inoculated in 10 ml of Buffered Peptone Water (BPW) and incubated at 37°C for 18h then DNA was extracted (Blood and Tissue Kit, QIAgen; the presence of VTEC (vtx1, vtx2, eae) and Salmonella spp. was detected by real-time PCR. VTEC isolation from the positive BPW was conducted according to ISO/TS 13136:2012. A second swab was inoculated into 10 ml of Bolton enrichment broth, incubated 48h at 37°C and plated onto mCCD agar and Skirrow agar (Oxoid) after filtration through a 0.45-µm membrane. Colonies with typical morphology after incubation at 42°C for 48h in microaerophilic atmosphere, were identified with a multiplex PCR for six Campylobacter spp.. The third swab was put in 3 ml of SPG transport medium and stored at 4°C until the analysis. Swabs were vortexed for 1 min and then 0.2 ml of SPG medium were used for DNA extraction. A real-time PCR targeting the 23S-rDNA of Chlamydia spp was performed. Positive samples were further analysed by specie-specific real-time PCRs for C. psittaci and C. avium sp. nov.

RESULTS:
None of the birds examined showed signs of disease. Ten pigeons (6,7%) tested positive for VTEC and the virulence gene combination found was: vtx2 (4 samples), eae-vtx1-vtx2 (2), eae-vtx2 (2), vtx1-vtx2 (1), eae-vtx1 (1). In three cases it was possible to isolate the strain (2 positive for vtx2 and one positive for eae-vtx1, serogroup O103). No samples tested positive for Salmonella spp., while Campylobacter spp. were found in 22 birds (12,1%). Eighteen strains were identified as C. jejuni by specific PCR and the remaining 4 as unidentifiable Campylobacter spp. Out of 140 samples tested for Chlamydia, 17 were C. psittaci positive (12,2%), but no one resulted positive for the new specie C. avium.

DISCUSSION AND CONCLUSIONS:
Our study showed that pigeons living in contact with cattle in the considered areas are frequently carrier of zoonotic pathogens such as VTEC, C. jejuni and C. psittaci. The presence of VTEC in pigeons has already been demonstrated in several countries especially in urban areas, but it is interesting to compare these strains with those of cattle of the same farms where the pigeons were captured. Concerning C. jejuni, we previously evidenced that pigeons usually harbor a specific clone, different from those found in livestock or humans. This should be confirmed for the strains of the present study. The prevalence of Chlamydia psittaci in pigeons is similar to that reported in previous studies. As the investigated microorganisms could represent an hazard for humans and livestock, further genotyping of the strains is necessary to assess the health risk caused by feral pigeons in rural areas.

REFERENCES:
PRESNCE OF NOROVIRUS AND HEPATITIS A VIRUS IN UNPROCESSED AND MINIMALLY PROCESSED VEGETABLES PRODUCED IN THE REGION OF SARDINIA FROM 2011 TO 2013

Salza S., Mudadu A., Marras A., Bulla M., Mara L., Canu A., Pisanu M.

Istituto Zooprofilattico Sperimentale ~ Sassari ~ Italy

Keywords: vegetables, PCR Real Time, PCR Real Time

INTRODUCTION:
Vegetable products are recommended for every lunch, in consideration of their fiber's abundance, mineral, antioxidant characteristic and low calories. In Italy, the vegetable's consumption in the last 20 years is considerably increased (1). We have considered two categories of vegetables: 1) Unprocessed vegetables. 2) Minimally processed vegetables, fresh and exposed at very light transformation. These are considered "ready to eat" and are really appreciated by the consumers (2). They have growth with annual rate of 18.6% (3). Respecting the storage temperature, the minimally vegetable's shelf life can vary between 5 to 8 days. Nowadays we haven't pandemic diseases, but different outbreaks, due to animal products ingestion and also with vegetable foods (4), contaminated by waters, soil, fertilizer and workers (5).

Considering all foodborne diseases, 25% are represented of vegetables consumption (6), 50% of them have bacterial origin, 7% viral origin, 6% parasitical origin and 35% unknown origin (5). Among five Noroviruses's genogroups, GI and GII have been detected in humans. Outbreaks caused by HAV and Norwalk-like viruses have been associated with frozen strawberries, lettuce, melons, salads, diced tomatoes, and fresh-cut fruit (7). The aim of this work was to obtain knowledge of this viruses in vegetables marketed in Sardinia.

MATERIALS AND METHODS:
Between 2011 and 2013, 250 unprocessed and 195 minimally processed vegetables were purchased from supermarkets and street markets of Olbia and Sassari. For the analysis's preparation of all samples we used the Norovirus's method detection in vegetables using real-time RT-PCR. Part 2: Method for qualitative detection in use in the ISS. The virus extraction in vegetable samples added with process control virus material, is implemented by elution with agitation followed by precipitation with PEG/NaCl. The RNA extraction method based on virus capsid disruption with chaotropic reagents followed by adsorption of RNA to silica particles. The 7900HT Fast Real Time PCR System (Applied Biosystems). The area selected for NoV detection is the well-conserved region at the 5' end of ORF2. In order to control the presence of inhibitory substance, external control (EC) RNA is added to a sample's aliquot and tested using the RT-PCR method. We used 7900HT Fast Real Time PCR System (Applied Biosystems). After the primary reverse transcription to 55°C for 60 min, the cycling protocol was 95°C/5 min (initial denaturation), followed by 45 cycles of 95°C/15 s (denaturation), 60°C/60 s (annealing) and 65°C/60 s (extension). Positive results for each target virus are determined by Cq value and expressed as "virus genome detected".

RESULTS:
All samples tested were negative for NoV GI, GII and Hepatitis A virus.

REFERENCES:

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4- Marotta V. et al . “Frutta e verdure: quali rischi per il consumatore?” Inserto Ce.I.R.SA
6- Lessons learned from the EHEC outbreak in Germany, Prof Bernd Appel, BFR, Germany, Meeting del progetto Europeo AnioBioThreat, Goteborg 19 – Ottobre 2011.
7- “Outbreaks Associated with Fresh and Fresh-Cut Produce. Incidence, Growth, and Survival of Pathogens in Fresh and Fresh cut Produce”. Chapter IV (www.fda.gov).

Table 1. Type of samples collected

<table>
<thead>
<tr>
<th>Type of samples collected</th>
<th>n. of samples</th>
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<tbody>
<tr>
<td>Unprocessed Vegetables</td>
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</tr>
<tr>
<td>Lettuce</td>
<td>38</td>
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<tr>
<td>Iceberg lettuce</td>
<td>32</td>
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<tr>
<td>Belgian endive</td>
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<tr>
<td>Prickly lettuce</td>
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<tr>
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<tr>
<td>Arugula</td>
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<tr>
<td>Tomatoes</td>
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<td>Artichoke</td>
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<td>Fennel</td>
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<tr>
<td>Total</td>
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<tr>
<td>Minimally processed Vegetables</td>
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<td>Iceberg lettuce</td>
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<tr>
<td>Carrots</td>
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<tr>
<td>Arugula</td>
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</tr>
<tr>
<td>Valerian</td>
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</tr>
<tr>
<td>Lettuce</td>
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</tr>
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<td>Prickly lettuce</td>
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</tr>
<tr>
<td>Cabbage</td>
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<td>Sugar leaf</td>
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<td>Mix</td>
<td>97</td>
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<tr>
<td>Total</td>
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</tbody>
</table>
RAPID DETECTION OF HEPATITIS A IN SOFT FRUIT PERFORMED WITH THE PATHATRIX® AUTO SYSTEM: IMMUNO MAGNETIC PRECIPITATION AND DETECTION BY REVERSE TRANSCRIPTION REAL TIME PCR

Savoldi Boles M, [1], Agnelli E,[1] Viola F,[2], Ruffini C,[1], Vailati Nodari M, [3]

Keywords: Hepatitis A, real time PCR, Pathatrix® Auto

INTRODUCTION:
Hepatitis A (HAV) is responsible for acute infectious hepatitis. Last year in Italy a big food borne outbreaks of HAV has been associated with soft fruit. The main obstacles concerning routine detection of HAV in food include the presence of inhibitory substances in the samples and the low concentration of virus recovered. The efficiency of virus recovery and presence of potential inhibitors must be monitored with a process control (Mengovirus MV). The aims of this project were to develop a novel methodology for the rapid and sensitive detection of HAV from soft fruit, testing the Pathatrix® Autosystem. The MV isolate was used as a surrogate for the initial testing and development of the methodology. The Pathatrix method was also compared with PEG precipitation method.

MATERIALS AND METHODS:
The samples (25g soft fruit) were artificially contaminated by pipetting viruses HAV or MV. Viruses were eluted according with the method described in the ISO/TS 15216. Supernatants were collected and pH was subsequently adjusted in to 7.2 with HCl. Pathatrix (Life Technologies) is a commercialized recirculating affinity magnetic separation system. The system was tested together with positively charged paramagnetic beads that attract the negatively charged virus capsid.

Supernatants were incubated with 150 µl of paramagnetic beads and mixed by vortexing. The samples were incubated for 20 min and then placed into the Pathatrix. The magnetic beads were resuspended in PBS. RNA was extracted using thermal lysis (80°C 10 min).

Viruses were also concentrated by adding 5x PEG solution as indicated in the ISO/TS 15216.

Potential inhibitors of the PCR reactions were removed using OneStep PCR Inhibitor Removal Kit (Zymo Research).

Real time PCR reactions for the detection of MV and HAV were performed with CEERAM kit.

RESULTS:
Various volumes of cationic beads and different buffers were evaluated. Highest recovery was obtained with TGBE, and if 150 µl of cationic beads were added.

A slight enhancement in recovery was also achieved when a 20 min incubation step was added. Different time and temperature were tested for the lysis step and the best performance was obtained with 10 min 80°C.

Application of a commercial PCR inhibitor removal kit resulted in less inhibitory substances in undiluted samples.

The Pathatrix method was compared with PEG precipitation for concentrating viruses. The efficiency of recovery was measured with real time PCR.

Pathatrix method was slight more efficient method for concentrating viruses and displayed a lower degree of inhibition in PCR. MV and HAV were detected for PEG precipitation in PCR with recoveries of 6.7±3.4% and 9.2±4.8 %. The analysis of the diluted samples indicates that there is a lot of inhibition in PCR.

The Pathatrix method displayed recoveries of 7.1±1.2% and 9.8±0.8%.

DISCUSSION AND CONCLUSIONS:
Several approaches for virus concentration have been developed, but many of them are inefficient, laborious, or time-consuming. Cationic beads in combination with the Pathatrix is an easy and relatively quick method for virus concentration from large sample sizes.

In order to further evaluate the methodology, the Pathatrix was compared with PEG precipitation, which is currently the standard method, and is used routinely in our laboratory. However, a major drawback is that the method is time-consuming. The Pathatrix method gave slight higher efficiency than PEG concentration and displayed a low degree of inhibition in undiluted samples with less time consuming, but it needs to be further optimized before it can be used for concentrating viruses from soft fruit in routine diagnosis.

REFERENCES:
REFINING THE LPS-ANTIGEN IN SALMONELLA ANTIBODY ELISA FOR POULTRY ENHANCED SPECIFICITY WITHOUT IMPAIRING SENSITIVITY

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National Veterinary Institute, DTU ~ Frederiksberg ~ Denmark

Keywords: Salmonella, ELISA, Poultry

INTRODUCTION:
In the Danish serological surveillance for Salmonella in poultry (serum and egg yolk) a mix-ELISA is used, based on S. typhimurium and S. enteritidis antigens (Feld et al., 2000). When we evaluated results of the test retrospectively, over the years an unacceptably large fraction of seropositive findings could not be confirmed by the subsequent confirmatory bacteriological sampling in the herd. Therefore we tried to enhance specificity of the ELISA, without losing sensitivity, by refining the antigens used.

MATERIALS AND METHODS:
New LPS antigen preparations were made based on the antigens currently used in the ELISA (Feld et al. 2000). Lyophilized antigen was resuspended in running buffer with 0,25% deoxycholate and gel filtered on a HiPrep 26/60 Sephacryl S-100 HR column (Sigma Aldrich) (Klausen et al. 2007, Wiuff et al. 2002). The product was collected in fractions and characterized by SDS-PAGE (Fig.1). Fractions containing high molecular LPS were selected for the new antigen preparation (Fig.1). They were pooled, dialyzed and then lyophilized. This antigen was resuspended in MilliQ water for validation in the ELISA. Coating concentrations of the new antigens were adjusted, for the two serotypes separately, to give the same values for the internal control sera in the test as did the old antigens. For the validation a total of 365 samples (serum and egg yolk) were collected in the laboratory during 2010-2012. The samples originated from the Danish Salmonella surveillance and represented submissions where one or more samples had been classified as non-herd confirmed reactors i.e. serologically positive samples that could not be confirmed at the following bacteriological sampling in the herd. Both ELISA positive and ELISA negative samples were represented in this material. As positive gold standards for the validation we used samples from well described Salmonella seropositive poultry (Fig.2).

RESULTS:
When testing the 365 samples from non-herd confirmed reactors 58 samples were found positive with both antigens and 220 samples were found negative with both antigens. No samples that were negative with the old antigen were found positive with the new antigen. 87 samples that were positive with the old antigen were found negative with the new antigen indicating that specificity was improved. Sensitivity was unaltered when testing the gold standard samples (Fig.2). Finally we tested 240 of our daily diagnostic samples, which primarily consist of negative samples, with the old and new antigen in parallel and got the same results with both antigens.

DISCUSSION AND CONCLUSIONS:
This new method for preparing Salmonella antigen for our mix-ELISA does with benefit replace the antigen preparation that we have used until 2013. Without decreasing the sensitivity of the test, many positive reagents, that could not be verified bacteriologically, have been removed.

REFERENCES:
HUMAN LEPTOSPIROSIS IN SARDINIA FROM 2005 TO 2014

Piredda I., Palmas B., Noworol M., Falchi A., Ponti M.N.

Istituto Zooprofilattico Sperimentale della Sardegna ~ Sassari ~ Italy

Keywords: Leptospirosis, Human, MAT

INTRODUCTION:
Leptospirosis is a disease caused by the genus Leptospira that affects animals and humans worldwide. Infection occurs by contact with the urine of sick animals or through contaminated water or soil (1,2)(Fig.1). The clinical symptoms can range from an asymptomatic, subclinical anicteric leptospirosis manifesting as an influenza-like presentation of fever and myalgia to a fatal hepatorenal syndrome, well known as Weil’s disease (3).

The aim of this report is to resume the actually epidemiological status of human leptospirosis in Sardinia in the last ten years.

Fig. 1 The cycle of leptospiral infection of human

MATERIALS AND METHODS:
In the period from January 2005 to April 2014 were tested 395 samples of human sera, with microscopic agglutination test (MAT), 27 urine samples with bacteriological culture, and 46 specimens (whole blood and urine) with molecular analysis (Tab.1). A panel of 16 live reference strains were used as antigens for MAT. It was assigned serum reactivity for MAT titer >1:20 and was defined positive the titer >1:160 in agreement with KIT Biomedical Research Laboratory in Amsterdam. Whole blood and urine samples were tested with the bacteriological culture in semisolid EMJH medium. Incubation of cultures took place in aerobic conditions at 28-30 °C in the dark for 90 days. The same specimens were used for the direct molecular diagnosis by polymerase chain reaction (PCR), using a pair of primers (LigA and LigB) amplify a partial sequence of the 16S rRNA.

Tab. 1 Samples analyzed

<table>
<thead>
<tr>
<th></th>
<th>MAT</th>
<th>COLTURE</th>
<th>PCR</th>
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<tbody>
<tr>
<td>Tot samples</td>
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<td>27</td>
<td>46</td>
</tr>
<tr>
<td>Reactive samples</td>
<td>34</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>% Reactive</td>
<td>8.60%</td>
<td>0%</td>
<td>8.70%</td>
</tr>
</tbody>
</table>

RESULTS:
The presence of antibodies was confirmed in 34 (8.6%) out of 395 patients by MAT (Tab.1). In the early of this study antibodies (Ab) against the serovar L. Icterohaemorrhagiae were found in 10 cases (29%), Ab against serovar L. Copenhageni and L. Bratislava in 6 cases (18%). Number of analyzed samples increased after 2011. In the last three years L. Bratislava has increased its seroprevalence with 7 cases (39%) whose titers were from 1:320, 1:640 to 1:2560. The 2% were confirmed in patients hospitalized with diagnosis of leptospirosis. The 8.7% of 46 whole blood and urine samples positive for qualitative PCR. No isolation of Leptospira was obtained in 27 samples examined with bacteriological culture. The data analyzed in this study highlight the spread of L. Bratislava in humans also in according to the official data (4).

DISCUSSION AND CONCLUSIONS:
The MAT and PCR are the two techniques allowing to confirm the diagnosis of leptospirosis. The culture requires three months incubation and does not allow to make a quick diagnosis. The MAT detects late anti-leptospires antibodies and it is necessary a second blood sampling to confirm leptospirosis. PCR on a blood samples is possible only for a few days during the first week of the disease. Thus, there is an urgent need to develop new techniques for an easy to use and quick detection of antibodies or antigens at the acute stage of the disease.

There are no human vaccines licensed for use in Europe or North America, however the protection is relatively short, and boosting at regular intervals is necessary to maintain protective titers of antibodies. Therefore, prevention remains the only usable tool.

REFERENCES:
INTRODUCTION:
Environmental chemical contaminants are a concern for food safety assessment. Exceptions to the general food production hygiene were described in the Regulation 852/2004/EC for “traditional foods”; the “Caso conzato”, a luscious cheese that has ancient origins and a very intense flavour, was classified as a traditional food by the Italian law, that introduced some measures to preserve its typical production process, ensuring at the same time consumer’s protection. It is produced using sheep, goat or cow’s milk, in variable proportions, then adding natural goat rennet. A study was performed to evaluate contamination of “caso conzato” by heavy metals (lead, cadmium, mercury, arsenic, chromium, copper, zinc, manganese, iron) and aflatoxins, analyzing meadow grass for feeding, raw milk and dairy intermediates as well as the final product.

MATERIALS AND METHODS:
All analyses were carried out by quantitative test methods validated and accredited according to the Regulation 882/2004/EC.
Heavy metals analysis – Dairy intermediates and cheese were homogenized, 0.50 ± 0.01 g were added with nitric acid, hydrogen peroxide and mineralized in a microwave oven; the samples were diluted by MilliQ water and analyzed by atomic absorption spectrophotometry (AAS). Hg was determined by cold vapour technique, while Cu, Zn, Fe and Mn by flame AAS; Pb, As, Cr and Cd were determined by grafite furnace with Zeemann correction technique.
Aflatoxin M1 was analyzed in milk and cheese by both ELISA and quantitative HPLC methods; the limit of quantification (LOQ) of the method is 0.002 μg/kg in milk and 0.025 μg/kg in cheese. Aflatoxins B1, B2, G1 and G2 were analyzed in grass by a quantitative HPLC method; the LOQ is 0.00025 mg/kg.

RESULTS:
Very low levels of AF B1, B2, G1 and G2 were determined in the meadow grass (in the range 0.0003-0.0007 mg/kg), below the maximum limit at 0.005 mg/kg set by EU regulations. Aflatoxin M1 (AFM1) is a metabolite of AFB1 and AFG1 contaminating feeds, and can be detected in milk; because of its carcinogenic effect, a maximum limit has been set for milk, but not in cheese. Regarding “caso conzato”, AFM1 was not detected in both raw milk and the final product. About heavy metals, “Caso conzato” is not contaminated significantly by environmental contaminants; only zinc was determined in the concentration range 5.55 - 10.59 mg/kg, because it derives from milk and represent a relevant nutritional element.

DISCUSSION AND CONCLUSIONS:
All the steps to produce “Caso conzato” were considered to evaluate the possible presence of the main chemical contaminants that is aflatoxins, for their carcinogenicity, and heavy metals. Meadow grass, raw milk , dairy intermediates and the final products were analyzed.
No significant levels of AF B/G were determined, as a consequence no AFM1 was detected in both raw milk and the final product. About heavy metals, “Caso conzato” is not contaminated significantly by environmental contaminants; only zinc was determined in the concentration range 5.55 - 10.59 mg/kg, because it derives from milk and represent a relevant nutritional element.
THE EUROPEAN UNION (EU) REFERENCE LABORATORY FOR ESCHERICHIA COLI: 8 YEARS EXPERIENCE IN THE ORGANIZATION OF PROFICIENCY TESTING FOR THE DETECTION AND TYPING OF VEROCYTOTOXIN-PRODUCING E. COLI (VTEC)


Istituto Superiore di Sanità ~ Roma ~ Italy

Keywords: Proficiency test, VTEC, Escherichia coli

INTRODUCTION:
The EU-RL for VTEC was established in 2006 by the EC Directorate General for Health and Consumers (DG Sanco), according to the Regulation (EC) No. 882/2004 on official controls. It coordinates a network of 35 EU National Reference Laboratories (NRLs) and the main objective of its mandate is to ensure that the methods used by the NRLs for the identification and typing of pathogenic E. coli strains and their detection in food and animal samples are standardized.

MATERIALS AND METHODS:
The EU-RL accomplishes its mandate by developing and evaluating methods, distributing reference materials, organizing proficiency tests (PT) and hosting scientists from NRLs for training stages. The EU-RL VTEC also collaborates with other EU structures (EFSA, ECDC) in establishing monitoring and surveillance programs for VTEC.

RESULTS:
Since 2006, the EU-RL has developed and evaluated standard operating procedures for the identification and typing of VTEC and for their detection in food, mainly based on PCR detection of virulence genes. In particular, it coordinated the development of the ISO Technical Specification: CEN/ISO/TS 13136:2012 (1) on the detection of VTEC in food and animal feed, based on the Real Time PCR (RT-PCR) screening of food enrichment cultures.

To evaluate both the methods and the performance of the NRL network in their application, the EU-RL organized 13 rounds of PT. Seven PTs were dedicated to bacterial typing and involved the detection of VTEC virulence genes by PCR and the identification of the serogroups most involved in human disease in Europe both by serological and molecular methods. The last three PTs also included molecular (PFGE) typing of VTEC strains, with the quality of PFGE images being evaluated according to criteria of the PulseNet International Protocol.

Six PTs were dedicated to the detection of VTEC in different matrices, including carcass swabs, milk, spinach, water, seeds and sprouts, by using the RT-PCR-based CEN/ISO/TS 13136. A positive trend was observed in both the number of participating laboratories (Figure 1) and their performance (2). From 2010, the PT results are submitted directly by the NRLs through an on-line system, using a “Restricted Area” in the PT Section of the EU-RL website. This section could be also used by the participants to print out their own individual reports at the end of every study.

DISCUSSION AND CONCLUSIONS:
The control of pathogenic VTEC in food and animals represents a challenge for the development of specific detection methods and requires a network of skilled and trained laboratories throughout the EU for their detection in the vehicles of infection. The EU-RL is working to consolidate such a network, in order: i) to contribute to the knowledge of the epidemiology of VTEC infections in Europe; ii) to gather harmonized data on the prevalence of these pathogens in the food samples finalized to the definition of microbiological criteria for VTEC; iii) to provide the EC with more standardized operative structures and tools to face possible emergencies in this field of food safety, as happened during the large outbreak sustained by the mosaic VTEC-EAggEC O104:H4 strain, which occurred in Germany in 2011 (3).

REFERENCES:
1) Guidance on the technical specifications for the monitoring and reporting of VTEC on animals and food, EFSA Journal 2009; 7:1366
NEW TEST METHODS FOR DETERMINATION OF MYCOTOXINS AND HEAVY METALS IN VEGETABLES

Gallo P., Salini M., Rossini C., Urbani V., Serpe L.

*Istituto Zooprofilattico Sperimentale del Mezzogiorno ~ Portici (NA) ~ Italy*

Keywords: vegetables, metals, mycotoxins

INTRODUCTION:
The new competence of the Istituti Zooprofilattici Sperimentali for the official control of vegetables required to develop test methods to assess food safety. The development, validation and accreditation of methods for determination of mycotoxins and heavy metals in cereals, fruit and vegetables are described. These methods must allow to detect the analytes below the maximum limits set by the Regulation 1881/2006/EC, and be compliant with the analytical performance criteria in the EU Regulations 882/2004/EC, 401/2006/EC and 333/2007/EC.

Actually, several normalized methods are published, but they are not completely adequate for official control, because of limited applicability, detection limits higher than the maximum limits set by law, validation criteria non compliant with the EU regulations. Thus, quantitative test methods were developed and validated for determining:
- cadmium and lead by atomic absorption spectrophotometry using grafite furnace with Zeemann correction technique (ZETA-AAS) in cereals, fruit and vegetables
- aflatoxins B and G (AF B/G), ochratoxin A (OTA) zearalenone (ZEA), deoxynivalenol (DON) by HPLC after immunoaffinity chromatography purification (IACs)

MATERIALS AND METHODS:
For each method, samples representative of fruit, vegetables and cereals were selected, according to the maximum limits set by EU regulations. Heavy metals analysis – Fruit, nuts, legumes, lettuce, cereals, mushrooms were analyzed. Samples were ground and homogenized, then 0.50 ± 0.01 g were added with nitric acid, hydrogen peroxide and mineralized in a microwave oven; the samples were diluted by 25 ml MilliQ water and analyzed by ZETA-AAS.

AFB1, AFB2, AFG1, AFG2 , OTA, ZEA were analyzed by HPLC with fluorescence detection (HPLC-FLD), DON by HPLC with UV-visible detection (HPLC-DAD). For sample clean up IAC was employed, using specific column for each mycotoxin and AF B/G group. HPLC separation was attained by a reversed phase stainless steel column Synergi Polar-RP 80 (Phenomenex).

After the development step, the methods were validated evaluating the following analytical parameters:
- Specificity: no matrix interference from at least 20 blank samples representative of the food and feeds (for mycotoxins) to be analysed
- Trueness: the mean recoveries of analytes were calculated spiking blank samples at 3 concentrations, below and above the maximum limits set for different food and feeds
- Precision: repeatability and within-laboratory reproducibility were calculated over at least 2 working sessions, analyzing six replicates
- Ruggedness, varying the lot of solvents, IAC columns, chromatohgraph

RESULTS:
The following test methods have been developed and validated:
- Determination of AF B/G in feeds and cereals by HPLC-FLD
- Determination of DON in feeds and cereals by HPLC-DAD
- Determination of OTA in feeds, cereals and raisin by HPLC-FLD
- Determination of ZEA in feeds and cereals by HPLC-FLD
- Determination of lead in fruit, cereals and vegetables by ZETA-AAS
- Determination of cadmium in fruit, cereals and vegetables by ZETA-AAS

For each method, the LOQ is below the maximum limits set by the EU, ensuring applicability for the scope of official control.

All the requirements for the analytical parameters set for the determination of both heavy metals and mycotoxins were satisfied, in terms of precision, trueness, specificity and LOQ.

All the test methods were accredited according to the International standard UNI CEI EN ISO/IEC 17025:2005.

DISCUSSION AND CONCLUSIONS:
Six methods were accredited for the official control of mycotoxins, lead and cadmium in vegetables and cereals. A novel validation scheme was applied, to ensure the methods are compliant with EU regulations and can be used for a large variety of vegetables and cereals, spanning over a wide range of concentrations. This way, the limitations of several standardized methods were overcome; moreover, the methods to analyze mycotoxins can be applied also to all kind of feeds, allowing to reduce analysis cost and time.

REFERENCES:
IMPACT OF DIFFERENT DIET REGIMES ON THE PRESENCE OF ANTIBIOTIC RESISTANCE GENES IN HUMAN GUT MICROBIOME

Losasso C.[1], Cibin V.[1], Di Cesare A.[2], Lettini A.A.[1], Gerolimetto E.[1], Corno G.[2], Ricci A.[1]

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Keywords: Antibiotic Resistance, Human gut microbiome, Diet regimes

INTRODUCTION:
The human intestine is a complex biological network that accounts for a great variability of microorganisms of significant magnitude. Human gut microbiota dynamically interact with environmental micro ecological niches (both animals and vegetables related) in a complex way: from animals and humans, through manure and faeces to water and soil and return to humans and animals, by food and feed [1]. This via food connection between human and environmental microflora increases the possibility of genetic exchange between their associated microbiomes, allowing for the transfer and selection of potentially novel genes to human gut, including those carrying antibiotic resistance (AR) determinants. Even though it is well known that the use and misuse of antibiotics in animal husbandry and agriculture select for AR bacteria and genes that can be directly transferred to human associated microbioma by food ingestion [2], the extent of AR determinants transfer from food to human gut has been poorly documented and it is not yet estimated whether diet composition could influence the AR profile of human gut. Aim of the present study is to investigate the relations between three different diet regimes and AR profiles in human gut microbiota focusing on tetracyclines resistance genes.

MATERIALS AND METHODS:
The human experimental sample was recruited among people attending the regional service for “food hygiene and nutrition prevention” and via associations involved in veganism dissemination. The sampling scheme was designed on the basis of statistical criteria in order to have a sufficient and comparable number of participants for each selected categories (vegans, vegetarians, omnivores). Participants were enrolled using the following inclusion criteria: being strictly vegan, vegetarian or omnivore; being adults (more than 18 years old); being not under antibiotics use or having assumed antibiotics in the last twelve months. Each participant was asked to record a food frequency diary for two weeks and to provide a fresh faecal sample. Total DNA was extracted from faecal samples by commercial kit. For each sample the extracted DNA was quantified and checked for purity by a plate reader spectrophotometer and then a fragment of 142 bp of the 16SrDNA gene was amplified by PCR (3,4) in order to confirm the success of DNA extraction with respect of prokaryotic cells. The same procedure was applied for the measurement of presence/absence of the ARG resistance genes for tetracyclines (5).

RESULTS:
116 participants were recruited, 51 omnivora, 28 vegan and 37 vegetarian, of age spanned between 18 and 65 years. The 16SrDNA overall copy number was similar for all DNA samples (104-105 copies). This gene was used to normalize the Antibiotic Resistant Genes ARG copy number. The PCR screening for the presence of the tetracyclines resistance genes showed a significant difference between the three sub-population groups especially in the case of the tetM gene that was detected in 19.61% omnivores, 35% vegetarians, and 50% vegans.

DISCUSSION AND CONCLUSIONS:
These results strongly suggest that foods of animal and vegetal origin can have a different role into AR determinants transfer to human gut and, consequently, that different diet regimes could be characterized by different AR loads. This observation is of paramount importance if we consider that worldwide dietetic recommendations reflect only the nutritional point of view and essentially lack of any concern regarding the exposition to hazards potentially present in food, including AR determinants, which could strongly affect the success of pharmacological treatments.

REFERENCES:
SURVEY OF PATIENTS IMMUNOSUPPRESSED AFTER KIDNEY TRANSPLANTATION FOR EVIDENCE OF ZOONOTIC INFECTION BY NON-PRIMATE FOAMY VIRUSES

Materniak M.[1], Serwacka A.[2], Rydzewski A.[3], Rudzki S.[3], Bocian L.[4], Kehl T.[5], Loechelt M.[5], Kuzmak J.[1]

[1]Department of Biochemistry, National Veterinary Research Institute ~ Pulawy ~ Poland, [2]Clinical Department of Internal Medicine, Nephrology and Transplantology of Central Clinical Hospital Ministry of Interior in Warsaw ~ Warsaw, Poland ~ Poland, [3]First Chair and Department of General, Transplant Surgery and Clinical Nutrition, University of Medical Sciences ~ Lublin ~ Poland, [4]Department of Epidemiology and Risk Assessment, National Veterinary Research Institute ~ Pulawy ~ Poland, [5]Department of Genome Modifications and Carcinogenesis, German Cancer Research Center ~ Heidelberg ~ Germany

Keywords: foamy virus, zoonotic potential, immunosuppression

INTRODUCTION:
The zoonotic introduction of an animal pathogen into the human population followed by extension or alteration of its host range pose a serious risk for world-wide health care. Since it may result in new transmissible diseases with unpredictable pathogenic potential. Therefore it is very important to identify suitable animal populations from which transmission to humans may occur and recognize human populations which are at risk to be exposed to zoonotic agents or are particularly vulnerable, such as immunocompromised individuals. Many recent reports clearly demonstrate that foamy viruses can be zoonotically transmitted as was shown for men in Africa and Asia, exposed to contact with monkeys infected with simian foamy virus. But even though the exposure to foamy viruses to non-human primates is restricted to limited population of people, we can not exclude that exposure to FVs from companion and life-stock animals is much more likely and possibly affects very large population. Therefore in our study we aimed to determine seroreactivity of plasma samples collected from immunosuppressed patients using recombinant antigens specific for non-primate foamy viruses: bovine foamy virus (BFV), feline foamy virus (FFV) and equine foamy virus (EFV).

MATERIALS AND METHODS:
117 blood samples were collected from immunosuppressed patients in the Clinic of Nephrology and Transplantology of Central Clinical Hospital in Warsaw. Control group consisted of 44 blood samples collected from people donating blood for routine blood testing in Pulawy medical center. All people involved in the study were asked to fill in the questionnaire regarding their gender, age, place of residence, frequency and type of their contact with cows, horses and cats. Plasma of collected blood samples was tested by GST ELISA with antigens specific for BFV, FFV and EFV, while peripheral blood leukocytes were used for DNA extraction. Statistical analysis was performed using Mann-Whitney test and Spearman rank correlation test.

RESULTS:
Plasma samples were tested for the presence of seroreactivity to EFV Gag, BFV Gag and Bet as well as BFV Gag and Bet antigens. In the group of immunosuppressed patients 2 samples showed increased seroreactivity to BFV antigens, 6 samples to FFV antigens and 8 to EFV antigen, while in control group 1 samples showed higher reactivity to BFV antigen, 2 to FFV antigens and 3 to EFV antigen. DNA samples were tested by virus specific PCR tests to confirm ELISA results. Investigation of differences in net-OD values between immunosuppressed and control patients, according to the frequency of contacts with animals showed no statistically significant differences in patients having contact with animals (p>0.05), however, the values close to significance were seen for BFV and FFV antigens. Furthermore, weak statistically significant correlation was found for BFV Gag antigen (r=0.206, p<0.05) in immunosuppressed group, when correlation between the ELISA net-OD values and the frequency of the contacts with animals in both groups was investigated.

DISCUSSION AND CONCLUSIONS:
This study presents the first report showing the investigation of seroreactivity to non-primate foamy viruses in patients immunosuppressed after organ transplantation. Although only single plasma samples showed overreactivity to particular antigens, statistical analysis showed a weak positive correlation between net-OD values and frequency of contacts for BFV Gag in the group of immunosuppressed patients. Obtained results show that zoonotic potential of those viruses can not be excluded, however more samples should be tested to verify presented data. This study was supported by grant no. 2011/01/B/NZ7/04282 from National Science Center.

REFERENCES:
A THREE-YEARS (2011-2014) SURVEILLANCE ACTIVITY ON SALMONELLA SPREAD IN DOMESTIC AND WILD ANIMALS IN SICILY


Istituto Zooprofilattico Sperimentale della Sicilia ~ Palermo ~ Italy

Keywords: typing of Salmonella spp., diversity, spread

INTRODUCTION:
Salmonella species are important zoonotic enteropathogens that affect humans, livestock, companion and wildlife animals. Foodborne illness due to Salmonella has been associated with increasing of human death worldwide. Some serovar caused outbreaks in different part of the world. In Europe, bacteria of the genus Salmonella cause 31% of cases of food-borne bacterial enteritis in humans1. However, despite Salmonella decreases in several countries, Italy reported more human Salmonella cases in 2009 than in 2008, which account for 39.5% of the confirmed cases in Europe1. Furthermore, the number of recorded animals with Salmonella is increasing, and many of isolated strains showed antimicrobial resistance. The aims of this survey is to describe the salmonella distribution in the different animal species and to identify strains widespread in Sicily.

MATERIALS AND METHODS:
Specimens (swabs, feces and organs) collected from equids, ruminants, suids, canids, felids, rabbits, hedgehogs, birds, reptiles, dolphins and fishes were evaluated for the research of Salmonella spp. at the Istituto Zooprofilattico Sperimentale della Sicilia according to the ISO standard 6579:2002 (ISO, 2002) from 2011 to 20142. The presumptive positive Salmonella isolates were characterized through biochemical analysis using the API 20E identification system, macroscopic assays, rapid sera agglutination and antimicrobial susceptibility tests. All isolates were serotyped at the National Reference Center for Salmonellosis.

RESULTS:
Out of 180 Salmonella spp. isolated from different animals 162 were typed. Salmonella enterica diarizonae is the main species found in this survey (n=17), followed by S. manhattan (n=16) and S. muenster (n=10) as reported in table 1. Less common Salmonella isolates included the following: S. blockley, S. brandenburg, S. derby, S. elomrane, S. enteritidis, S. hermannswerder, S. nashua, S. santpaul with two cases each and S. abony, S. alachua, S. bovismorbificans, S. coeln, S. goldcoast, S. hessarek, S. kambole, S. kenya, S. kimbabwa, S. kottbus, S. larochelle, S. mbandaka, S. muenchen, S. napoli, S. panama, S. rissen, S. richmond, S. paratyphi, S. heidelberg, S. virchow, S. vleuten and S. worthington with one case each. Ten strains are under typing. Foxes were the species more affected (n=49) by Salmonella spp. followed by sheep (n=26), dogs (n=25) and birds (n=24).

Antimicrobic susceptibility tests revealed that most of the isolated strains were sensitive to all antimicrobial tested.

DISCUSSION AND CONCLUSIONS:
Salmonella spp. is the main agent of gastroenteritis in human due to consumption of contaminated food and water. The importance of companion animals and wildlife as Salmonella spp. carriers has been highlighted and well-established previously by several studies on a variety of species. This survey revealed that Sicilian animals are carriers of Salmonella spp. but all the isolated strains were susceptible to all antimicrobial molecules tested.

REFERENCES:

Table 1 - Typing of isolated Salmonella from different animal species

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Group</th>
<th>Number of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. diarizonae</td>
<td>P, Y, Z</td>
<td>17 (10.5)</td>
</tr>
<tr>
<td>S. manhattan</td>
<td>C2</td>
<td>16 (9.8)</td>
</tr>
<tr>
<td>S. muenster</td>
<td>B1</td>
<td>10 (6.2)</td>
</tr>
<tr>
<td>S. enterica</td>
<td>B, C1, C9, F, S, W</td>
<td>9 (5.5)</td>
</tr>
<tr>
<td>S. seftenico</td>
<td>T</td>
<td>8 (4.9)</td>
</tr>
<tr>
<td>S. salmonae</td>
<td>S, T</td>
<td>7 (4.3)</td>
</tr>
<tr>
<td>S. ichtinovivium</td>
<td>B</td>
<td>6 (3.7)</td>
</tr>
<tr>
<td>S. homology</td>
<td>T</td>
<td>5 (3.1)</td>
</tr>
<tr>
<td>S. monterilico</td>
<td>C1</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>S. serp</td>
<td>G2</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>S. dublin</td>
<td>D1</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>S. hampelmann</td>
<td>P, U</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>S. mantroucoque</td>
<td>G2</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>S. beauger(V)</td>
<td>Y</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. brolingy</td>
<td>B</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. hadar</td>
<td>C2</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. ladina</td>
<td>B1</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. neugert</td>
<td>C2</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. tevergord</td>
<td>C1</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>S. vennidiana</td>
<td>F</td>
<td>3 (1.8)</td>
</tr>
</tbody>
</table>
POSTER PRESENTATIONS

QUALITY ASSURANCE AND AUTOMATION
A NEW LEVEL OF STANDARDIZATION IN REAL-TIME PCR USING A MODULAR SYSTEM


Keywords: BVDV, PCR, Modular

INTRODUCTION:
Real-time PCR has revolutionized diagnostic testing over the past years. While real-time PCR is continuously developing, commercial assays often deliver a set of reagents designed for testing a precise number of samples for a specific target(s). The IDEXX RealPCR real-time PCR tests aim to provide a new level of standardization to PCR diagnostics by using reagents in a modular system. The components of this modular system are shared over the entire test platform.

MATERIALS AND METHODS:
The RealPCR BVDV RNA Test was evaluated using characterized samples and synthetic oligonucleotides. To ensure reliable results, the test employs a multiplexed internal sample control (ISC) to detect endogenous bovine RNA. For maximum flexibility, different sample types, extraction methods and pooling possibilities were tested.

RESULTS:
The test shows an analytical sensitivity of ≤ 15 copies/reaction for Type I, Type II and HoBi BVDV with efficiencies of > 95% over at least a 7-log range. The test displays no cross-reactivity with many common bovine viral pathogens either by in silico analysis or with diagnostic specificity testing. In addition to individual whole blood, serum, plasma and ear notch claims, the test detects BVDV in sample pools of up to 50 for blood fractions or 25 for ear notches. A rapid lysis protocol for ear notches has been validated, eliminating the need for full RNA extraction which greatly reduces sample handling and processing time.

DISCUSSION AND CONCLUSIONS:
The shared components make it possible to run any pathogen-specific detection mix with a standard master mix and positive control. Moreover, the IDEXX RealPCR modular system maintains a single cycling protocol for all tests and shared quality controls and guidelines across the entire platform. This new approach to PCR testing will increase standardization in PCR laboratories without compromising the performance of the test.

REFERENCES:
INTRODUCTION:
The Office Acceptance at the headquarters of IZSM in Portici receives approx. 20,000 samples a year directly from its jurisdiction area and approx. 10,000 samples from the diagnostic departments of the provinces of Campania and Calabria. During its activity the Operating Acceptance Unit deals as well with rejections of samples due to problems connected with mistakes in the accompanying documents and/or the noncompliance of the samples. In order to optimize this activity, it has become necessary to switch from the ordinary paper records of the rejections to a computerized procedure.

MATERIALS AND METHODS:
The creation of the computer version has followed the following steps:

a. Rejections’ input in software of the years 2012 and 2013;
b. Classification of reasons for refusal by codes. In this way it was possible to create a first division into two main groups, on the causes of rejection;
   1) Technical reasons (non-conformity of the samples submitted or their wrappers),
   2) Administrative reasons (compile errors of the reports, delay in delivery, improper requests generically,
   c. Further and more detailed classification of the two initial groups in different sub-groups, each identified with a specific code;
   d. Adoption ministerial codes of rejections used in the 2014 NRP;
   e. Processing of the data stored;
   f. Integration of the data contained in the old register paper with additional data.

RESULTS:
The computerization of rejections has allowed the achievement of several objectives, including:

1) Much faster consultation of the input data;
2) Analysis of the flow of rejections;
3) Processing and analysis of the major causes of rejection;
4) Preparation of internal and external reporting on the deducted conclusions;
5) Allocation of responsibilities among the involved parties;
6) Ability to take corrective actions that can reduce this circumstance;
7) Assessment of possible training activities for the different professionals involved;
8) Consulting of the rejections database by the different U.U.O.O. (e.g. observatories and laboratories).

DISCUSSION AND CONCLUSIONS:
Between 2013 and 2012 there was a decline in total rejections (607 vs. 974) and, more importantly, a reduction of samples eliminated as unadjusted (359 vs. 442).

The analysis of the data stored by means of the new procedure has allowed to put in evidence different aspects of the problem rejections and it is possible to implement appropriate corrective and/or preventive measures, in order to further reduce the number of rejections in 2014. This reduction results in a significant cost savings by the user who performs the sampling, and optimization of the activities within the office acceptance.

REFERENCES:
Nappo C., 2012 Use and evolution of a computer system for the management of laboratory analysis: Acts XIV National Congress S.I.Di.L.V.
DEVELOPMENT OF A VALIDATION PROCEDURE TO DETECT BOHV1 IN RUMINANT BIOLOGICAL SAMPLES BY USING REAL TIME AND END POINT PCR

Messana E., Gobbi E., De Marco L., Caruso C., Maddalena R., Palmegiano P., Andrà M., Masoero L.

Keywords: Validation procedure, Alphaherpesviruses, PCR

INTRODUCTION:
Bovine herpesvirus 1 (BoHV1) is a member of the family Herpesviridae known to cause several diseases worldwide in cattle. As BoHV1 is antigenically related to others alphaherpesviruses of ruminants, namely BoHV5, caprine herpesvirus 1 (CpHV1), cervine herpesvirus 1 and 2 (CvHV1-2) and Bubaline herpesvirus (BuHV1), diagnostic tests able to discriminate BoHV1 from these related viruses are needed to avoid misdiagnosis. Aim of this work was to validate a sensitive and specific molecular diagnostic procedure for detection of BoHV1 in ruminant biological samples by using a rt-PCR described in OIE Manual (1) followed by End Point PCR (2). The parameters included in validation procedure were accuracy, limit of detection (LOD), precision, sensitivity, specificity and robustness according with internal quality procedures of “IZSTO” (3).

MATERIALS AND METHODS:
A panel of 36 samples, 20 positive and 16 negative tested in cell isolation, inter-laboratory analysis and immunofluorescence were selected. Samples included tissue from lung, lymph nodes, biological fluids and cell lysates. BoHV1 was produced on MDBK cells and used as positive control. Viral DNA was extracted with PureLink Genomic DNA Kit (Qiagen). gB and gD gene region were targeted for rt-PCR and End Point PCR, respectively. Both reactions were performed using the qPCR ProbeMaster with UNG (Bioscience). Mix was composed by 12,5µL Master Mix (2X), 1µL of each primer (5µM), 5µL of DNA; for rt-PCR mix, 1µL of TaqMan probe (3µM) was added. Thermal profile for both PCR was as follows: 50°C for 2min, 95°C for 2min, 45 cycles of 95°C for 15sec, and 60°C for 45sec. The thermocyclers were Stratagene Mx3005 and Applied Biosystem 2720.

RESULTS:
Accuracy, consisting of diagnostic sensitivity and specificity, for both PCR was 100%, resulted in 20 positive and 16 negative confirmed samples. LOD was determined with serial dilutions of BoHV1 DNA (from 6 to 5x10-6ng); the estimated LOD was 6x10-5ng for rt-PCR and 3x10-4ng for End Point PCR, respectively. As expected, rt-PCR resulted to be more sensitive than End Point PCR. Precision was evaluated according two parameters: intra-assay repeatability, performed by the same operator, confirming the same results on 2 replicates of 5 samples; intra-assay reproducibility, performed by two operators analyzing all the samples panel, with a Cohen’s kappa coefficient of 1±1.96 x 0.167. Primers specificity used in both PCR was verified with the software NCBI tool BLAST; furthermore 8 different alphaherpesviruses, included BoHV1, antigenically correlated viruses and no target viruses, were tested. Results showed that gB primers used in rt-PCR amplified also for BuHV1, CvHV1, CpHV1 (Fig.1), while primers designed on gD gene region amplify only for BoHV1 (Fig.2). Robustness was determined using two different amplification kits (Bioscience; Applied Biosystem) to detect the lowest concentration of DNA found in LOD analysis, confirming no different results for both kits.

DISCUSSION AND CONCLUSIONS:
For implementation of a test procedure for laboratory routine use, it is essential to set up a validation methodology. In this work, validation parameters to detect BoHV1 with two different PCR methods were examined. Rt-PCR targeting gB gene region, results more sensitive than End Point PCR targeting gD gene region; on the other hand the latter method is strongly specific for detection and discrimination between BoHV1 and others alphaherpesviruses. Since domestic and wild ruminant are susceptible to heterologous infection, it could be useful to set up both methods to increase analytical sensibility and specificity.

REFERENCES:
-3 UNI CEI EN ISO/IEC 17025. 2005
VALIDATION OF A BIOMOLECULAR METHOD FOR VETERINARY PATHOGEN IDENTIFICATION

Corrado F., Cecere B., Criscuolo D., De Roma A., Cerrone A., Paradiso R., Riccone N., Galiero G.

Istituto Zooprofilattico Sperimentale del Mezzogiorno ~ PORTICI ~ Italy

Keywords: MicroSeq Microbial Identification System, 16S ribosomal DNA, Bacterial identification

INTRODUCTION:
Identification of bacteria in microbiology laboratories is traditionally performed by isolation of the organisms and study of their phenotypic characteristics. The goal of this work is the development and the validation of an alternative method for veterinary pathogen identification based on the MicroSeq Microbial Identification System. Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new “gold standard” for identification of bacteria to the species level. In this system, the first 527-bp fragment of the 16S rRNA gene of the bacterial strain is amplified, sequenced, and analyzed using the database of the system.

MATERIALS AND METHODS:
The MicroSeq 500 16S ribosomal DNA (rDNA)-based bacterial identification system (Applied Biosystems Division) has been designed for rapid and accurate identification of bacterial pathogens. The analytic procedure is divided in the following steps:
- Extraction of bacterial DNA from cellular lysates.
- PCR and purification of amplified products.
- Sequencing PCR and purification of sequencing products.
- Identification with MicroSeq Microbial Identification Software.

RESULTS:
The method validation was performed through the identification of 50 clinically significant bacterial strains. The bacterial lysate derived from swab of animal organs. The characterization was performed with an identification to species and genus level.

DISCUSSION AND CONCLUSIONS:
We used DNA sequencing of the complete 16S rRNA gene to analyze 50 veterinary bacterial strains. We compared the data obtained with this system with the results obtained through microbiological characterization. We are able to identify also samples that showed ambiguous biochemical profiles, such as Pasteurella spp, Campylobacter spp and Mycoplasma spp. Moreover we detected some pathogens of specific veterinary interest non available in the MicroSeq Identification Library, such as the Mycoplasma agalactiae e Taylorella equigenitalis. Therefore, in accordance with the MicroSeq equip, we would like to update the MicroSeq library in order to supply an alternative and valid analytical protocol for the veterinary diagnostics.

REFERENCES:
- UNI EN ISO 22174:2005 Microbiology of food and animal feeding stuff – Polymerase chain reaction (PCR) for the detection of food-borne pathogens – General requirements and definitions.
- Usefulness of the MicroSeq 500 16S Ribosomal DNA-Based Bacterial Identification System for Identification of Clinically Significant Bacterial Isolates with Ambiguous Biochemical Profiles

QUALITY CERTIFICATION FOR THE SERVICE PROCESSES IN VETERINARY HEALTHCARE PUBLIC SYSTEM


[1]Istituto Zooprofilattico del Mezzogiorno ~ PORTICI ~ Italy, [2] REGIONE CAMPANIA ~ NAPOLI ~ Italy

Keywords: ISO 9001, Quality Assurance System, Public Health System

INTRODUCTION:
The present abstract reports what achieved from “Istituto Zooprofilattico del Mezzogiorno” to allow the issuance of UNI EN ISO 9001:2008 Certification for the service processes disbursement in Veterinary Public Healthcare field and for Food Safety of Campania area. Such activity involved the Veterinary Health Departments of Campania area.
The working group was spurred by the need to build a Quality Assurance System in healthcare because, on one hand, the Country will to improve the proficiency and efficacy of its health organization and, on the other hand, a precise customer demand to receive optimal services. For development of Quality system in a healthcare setting are necessary implement same ISO 9000 which afford the necessary guidelines.

MATERIALS AND METHODS:
The ISO 90012008 Certification program had been conducted in 2012/2013 through different steps:
- First of all, creation of a web platform called “CE.SE.SA.RE” (Regional Sanitary Certification Service), activated on the Regional Food Assurance Observatory. This interesting enterprise had the intent to optimize the information’s flow to share with different staffs dislocated on a large area.
- Recognitions of the healthcare structures that required a certification, data processing of Quality System documentation (Manual, procedures, etc.).
- Training activity for the company staff. Audits to evaluate the implementation level reached.
- Starting and final audit by public certification authority for reach the purposes of setting certification.

RESULTS:
For each settings certifiable has been nominated same representatives of Quality. Precisely same meeting have been sighted 4 Representatives in Veterinary field, 1 for prevention field, healthcare and 5 vet for each ASL: 1 vet for area A, 1 for area B e 1 for area C and also 2 SIAN doctors in all local ASL.
The results of work which has done and coordinated by Representatives of Quality are:
- Procedures for the quality management system (ISO 9001);
- Standard operating procedures for the process to be certified;
- Quality Manual;
- Internal audits.
The documentation and the results of audits conducted were shared among the various structures through the web platform, with a view to ensure communication and sharing results achieved by each working group with the coordination of Istituto Zooprofilattico. Results of this effort has been achieved the target of the project and therefore the certification of veterinary services and SIAN of the Campania region and the ASL.

DISCUSSION AND CONCLUSIONS:
Realise the ever customer urgent contemplations enjoins to add the complex assurance quality disciplines, the organization and existents resource.
So we ask for to the company to operate in effective and proficient way. For such intent aiding the ISO 9000 criteria who have the target to provide a product/service according to customer contemplations. This target is obtained define responsibility, planning, treatment, transcription, constant improvement and checking the final created.
For the maintenance of the obtained targets, certification related, in the next years through specifics, programs, will begin a constant improvement of the quality system such as reference criteria required.

REFERENCES:
ISO 9000 - Quality management
VALIDATION OF THE ENDOPEP-MS METHOD FOR QUALITATIVE DETECTION OF ACTIVE BOTULINUM NEUROTOXINS IN HUMAN AND CHICKEN SERUM

Björnstad K.[1], Tevell Åberg A.[1], Kalb S.[2], Wang D.[2], Barr J.[1], Bondesson U.[1], Hedeland M.[1]


Keywords: botulinum neurotoxin, botulism, Endopep-MS, MALDI-Q-TOF MS, mass spectrometry

INTRODUCTION:
Botulinum neurotoxins (BoNTs) are highly toxic proteases produced by anaerobic bacteria. Traditionally, a mouse bioassay has been used for detection of BoNTs, but for a long time, laboratories have worked with alternative methods for their detection. One of the most promising in vitro methods is a combination of an enzymatic and mass spectrometric assay called Endopep-MS. However, no comprehensive validation of the method has previously been presented.

MATERIALS AND METHODS:
The main purpose of this work was to perform a validation for the qualitative analysis of BoNT-A, B, C, D, D/C, E, and F in serum. BoNTs A, B, C, D/C, E, and F were purchased as complexes from Metabiologics (Madison, WI, USA). Recombinant BoNT-D and C/D (purified, not as complexes) produced in E. coli were purchased from Toxogen GmbH (Hannover, Germany). Monoclonal antibodies targeting the different BoNTs were purchased from Dr. James Marks at the University of California San Francisco (San Francisco, CA, USA) as 1 mg/mL solutions in different buffers (e.g. mixtures of glycine, tris, and phosphate buffered saline). The BoNTs were captured on antibody-coated magnetic beads. Target peptides for the Endopep-MS reaction were custom synthesized by by Xaia Custom Peptides (Gothenburg, Sweden). The mass spectrometric analysis of the was performed on a Synapt G2 instrument (Waters Corporation, MA, USA) with a MALDI interface operated in positive potential and MS resolution mode.

RESULTS:
The limit of detection (LOD), selectivity, precision, stability in matrix and solution, and correlation with the mouse bioassay were evaluated. The LOD was equal to or even better than that of the mouse bioassay for BoNT-A, B, D/C, E, and F. Furthermore, Endopep-MS was for the first time successfully used to differentiate between BoNT-C, D and their mosaics C/D and D/C by different combinations of antibodies and target peptides. In addition, sequential antibody capture was presented as a new way to multiplex the method when only a small sample volume is available. In the comparison with the mouse bioassay, all the samples analyzed were positive for BoNT-C/D with both methods.

DISCUSSION AND CONCLUSIONS:
The Endopep-MS method has for the first time been validated for qualitative analysis of active BoNT-A, B, C, D, D/C, E, and F in serum. The parameters LOD, selectivity, precision, stability in matrix and solution, and correlation with the mouse bioassay were evaluated with successful results. The Endopep-MS can challenge the mouse bioassay as the gold standard for the diagnosis of botulism, as the sensitivity is in the same order of magnitude or better than that of the mouse bioassay for most of the toxin serotypes, it measures toxin activity, it is faster and it does not require any experimental animals. Furthermore, it has for the first time been demonstrated that Endopep-MS can differentiate between BoNT-C, D and their mosaics C/D and D/C. Additionally, a new way of multiplexing has been presented, based on sequential capture with antibodies directed against the different serotypes.

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POSTER PRESENTATIONS
MOLECULAR EPIDEMIOLOGY
USING RFLP ANALYSIS FOR IDENTIFICATION AND DIFFERENTIATION OF FIELD AND VACCINE STRAIN OF MYXOMA VIRUS

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Keywords: myxoma virus, B-82 vaccine strain, RFLP analysis

INTRODUCTION:
Myxoma virus (MYXV; family Poxviridae, subfamily Chordopoxvirinae, genus Leporipoxvirus) is a linear double stranded DNA virus that specifically infects rabbits and hares. Affected rabbits display conjunctivitis, anorexia, listlessness, and fever. Animals may die within 2 days after onset of symptoms. In a more prolonged disease course, depression and myxomas in the eyelids, nose, lips, vulva, or scrotum are observed. The mortality rate may reach up to 100% depending on the viral strain. The diagnosis of myxomatosis is based on clinical presentation of typical symptoms, histology and laboratory tests, including molecular studies on the basis of PCR. However, there is need for improvement of modern molecular techniques for identification and differentiation of myxoma virus in rabbits associated with the evolution of myxomatosis virus which causes atypical form of the disease. To detection differences between biological variants of the myxoma virus is using of RFLP analysis of PCR products.

MATERIALS AND METHODS:
For isolation DNA from different samples we used home kit. A set of primers was designed to amplify the sequence between the M129R and M131R genes of myxoma virus. PCR conditions were as follows: 94 °C 2 min, then 35 cycles of 94 °C 10s, 65°C 20 s and 72 °C 15s, with a final extension of 2 min at 72 °C.

PCR products were analyzed by agarose gel electrophoresis. PCR products were cut from gels and purified using the PCR clean-up system (Promega, USA). Purified PCR products were subjected to digestions using a BamHI (Fermentas, Lithuania) restriction enzyme (as per manufacturer’s instructions) and analyzed by agarose gel electrophoresis in order to look for RFLPs.

To assess the specificity of the method was used DNA isolated from vaccine strains “B-82” and “Mix-98”, a virulent strain MP, associated vaccine against myxomatosis and RHDV, field strains “Khabarovsk-2009”, “Smolensk-2011”, “Ivanovo 2012”. In addition, we used the DNA obtained from the matrix of bookmarks production of the vaccine strain B-82, lay down to museum in 1992 and 2010. As well as the series of vaccines which was produced when using them (issued 2004-2012). As heterogeneous samples used pathological material: RHDV and vaccinia virus.

RESULTS:
As a result of restriction analysis were confirmed the expected results for all samples. For vaccine strain “B-82”, “Mix 98”, and associated vaccine against myxomatosis and RHDV after restriction amplified genomic region showed two fragments of 170 bp and 394 bp, while DNA of virulent and field strains are not cleaved that allows to spend differentiation of the B-82 vaccine strain and field isolates.


DISCUSSION AND CONCLUSIONS:
We have found that the vaccine strain B-82 myxoma virus (used for vaccine production in Russia) on M130R region has site for specific cleavage recognizable with the BamHI restriction enzyme, whereas in the virulent strain, and in field strains circulating in Russia, this site is missing.

BamHI restriction site is located in the amplicon such a way that the endonuclease cleaves the amplified region of the genome of vaccine strain myxoma virus on 2 fragments of 170 bp and 394 bp, while DNA of virulent and field strains are not cleaved that allows to spend differentiation of the B-82 vaccine strain and field isolates.

The studies found that the developed assay can detect and differentiate the virus genome in samples of various organs and tissues of naturally and experimentally infected animals. The results of these studies led to the conclusion that the proposed assay has a good specificity and sensitivity and can be used for detection and differentiation of rabbit myxoma virus genome.

REFERENCES:
EVOLUTIONARY AND GENOMIC ANALYSES OF THE PANDEMIC H1N1 VIRUSES IN ITALIAN PIG FARMS

Moreno A. [1], Vaccari G. [2], Boni A. [1], Lelli D. [1], Lavazza A. [1], Zaccaria G. [2], Di Trani L. [2], Alborali G.L. [1], Cordioli P. [1]


Keywords: whole-genomic and evolutionary analyses, H1N1 pdm viruses, swine

INTRODUCTION:
In April 2009, a novel H1N1 (H1N1pdm) influenza A virus was identified as the cause of the present flu pandemic. Following the rapid spread of H1N1pdm virus, transmission from infected humans to swine has been detected worldwide. The first case was reported in a pig farm in Mexico in April 2009 [1] contemporary to the first isolation in humans. This virus was generated by a reassortment between Eurasian swine H1N1 influenza viruses and North American triple reassortant H1 viruses. Swine influenza surveillance performed in IZSLER from 1998 to 2012 revealed a continuous circulation of H1N1, H3N2 and H1N2 viruses and starting from 2009 the isolation of H1N1 pdm viruses in pigs. In 2009-2012, 15 H1N1pdm strains were isolated from 5 different farms located respectively in Milan, Palermo, Brescia, Rovigo and Benevento province. This study reports the phylogenetic analysis of the concatenated complete genome of the Italian pandemic strains and the comparison with reference human strains representatives of the eleven previously described clades [2] and other H1N1pdm strains originated from swine and turkeys. We performed phylogenetic and genome signature analyses based on the concatenated complete genome analysis and estimated both the rates of nucleotide substitution and time to the most recent common ancestor (tmrca).

MATERIALS AND METHODS:
Only strains for which whole-genome sequences were available and two human strains from the eleven described clades were included. The eight segments of 82 whole genomes were manually concatenated prior to phylogenetic tree construction. To infer the evolutionary relationships, we employed a Bayesian Markov chain Monte Carlo method using a strict molecular clock (GTR+G4 model), as implemented in the BEAST program.

RESULTS:
The bootstrap supported phylogenetic tree showed that swine and turkey strains clustered in five different clades starting from clade 6, with no isolates placed in the first five clades. Italian isolates were divided in five separate groups depending on the farm of origin. Two isolates from Rovigo and three from Milan belonged to clade 10, but the last ones formed a separate new sub-clade. Four isolates from Brescia and four from Benevento were related to other European swine strains deriving from a common ancestor, but they were divided in two different new clades. Of these, the most interesting was the one from Benevento that is characterized by an insertion of two amino acids (aa) in the receptor binding site of HA protein.

DISCUSSION AND CONCLUSIONS:
Our analysis showed a clear distinction of Italian strains depending on the farm of origin with no relation between them, probably due to an initial human-to-pig transmission. Subsequently H1N1pdm may undergo many pig-to-pig transmissions because of the continuous availability of susceptible pigs and circulate in the same farm for many months. These results indicated that H1N1pdm viruses have distinctively adapted and shared many substitutions. The last detected group from Benevento showed the highest number of aa substitutions including the quite rare double aa insertion in the HA protein.

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GENETIC TYPING OF RUMINANT PESTIVIRUS STRAINS FROM NORTHERN IRELAND COLLECTED BETWEEN 2008 AND 2011 AND FROM RUMINANT ANIMALS INTRODUCED TO NORTHERN IRELAND DURING 2010 AND 2011

Guelbenzu Gonzalo M.D.P.[1], Cooper L.[1], O’Neill R.[3], Welsh M.[1], Graham D.A.[2]


Keywords: Bovine Viral Diarrhoea, Phylogenetic analysis, 5’ untranslated region

INTRODUCTION:
The genus pestivirus within the family Flaviviridae includes bovine viral diarrhea virus (BVDV) types 1 and 2, border disease virus (BDV) and classical swine fever virus. The two recognised genotypes of BVDV are divided into subtypes based on phylogenetic analysis, namely a-r for BVDV-1 (Jackova et al., 2008; Vilcek, 2001; Vilcek et al., 2004; Yeşilbağ et al., 2014) and a-b for BVDV-2 (Flores et al., 2002). Ruminants introduced to Northern Ireland (NI) from other parts of the United Kingdom or continental Europe between January 2008 and December 2012 were routinely blood sampled for testing for Bluetongue virus (BTV; antibody and viral RNA). A study was conducted to investigate presence of pestiviral strains in the cattle population in NI and in ruminants introduced to this region during 2010 and 2011.

MATERIALS AND METHODS:
The genetic heterogeneity of pestiviruses from 91 BVDV samples collected between 2008 and 2011 was investigated. RNA from samples from 839 bovine and 4,437 ovine animals introduced to NI during 2010 and 2011 were tested for the presence of pestiviral RNA with an RT-PCR assay (Ambion BVDV Taqman AgPath, Life technologies). Positive samples were further sequenced. A 288 base pair portion of the 5’ untranslated (UTR) region was amplified by RT-PCR and the product sequenced. The sequences were aligned and compared with each other and with the corresponding sequences of a number of reference strains and with other previously reported sequences from NI and the Republic of Ireland. Nucleotide sequence data was aligned using MUSCLE software. Phylogenetic trees were calculated using the MEGA programme package version 5 based on the neighbour-joining Kimura two-parameter method. The robustness of the phylogenetic analysis and the significance of branch order were determined by bootstrapping method carried out on 1,000 replicates.

RESULTS:
The analysis indicated that the predominant subtype circulating in NI is BVDV-1a (86 samples out of 91, 94.5%). Five out of the 91 NI samples clustered close to reference strains in subtype BVDV-1b (5.5%). 18 out of the 839 samples from bovine animals introduced to Northern Ireland gave a positive result (2.14%, Ct<37) and 8 an inconclusive result (0.9%, Ct ≥37) comprising a total of 24 animals. Positive samples were sequenced and all of them (eight samples in total) clustered close to reference strains in subtype BVDV-1b.

DISCUSSION AND CONCLUSIONS:
Given the fact that only the BVDV-1a subtype was detected in samples collected between 1968 and 1999 (Graham et al., 2001), this study suggests that at least one new subtype has been introduced to NI between 1999 and 2010 and highlights the potential for importation of cattle to introduce new strains.

REFERENCES:
INTRODUCTION:
The Orf virus (ORFv) or Parapoxvirus (Poxviridae) is an epitheliotropic virus affecting domestic and wild ruminants (1). Due to the absence of a systemic virus spread, the infection remains usually localized in the skin of the lips, around the nostrils and in the oral mucosa (1). The mucocutaneous lesions generally heal during 1-2 months, even if fever and mortality can be observed in the acute forms. The virus can also affect humans in close contact with infected animals, causing painful nodules on hands and more rarely on the face (1). Aim of this survey was to study the genetic characterization of Parapoxviruses detected from episodes of Contagious Ecthyma (C.E.) occurred in Southern Italy.

MATERIALS AND METHODS:
Between 2011 and 2014, 12 outbreaks of suspected C.E. were observed in sheep in different areas of the Apulia and Basilicata regions. Twenty-three clinical samples (Tab.1), were collected from crusted scab lesions and sent to the Virology Department of IZSPB in Foggia for analyses. The first diagnosis was achieved by negative staining electron microscopy, in order to confirm or to exclude the presence of Parapoxvirus. The following step consisted in the molecular analysis of the samples by a PCR based on the amplification of the gene 045 of ORF virus, that encodes for the late transcription factor VLTF-1(1). All the amplified products were later purified and sequenced by Eurofins (Edersberg, Germany) in one direction using the forward primer 045F. Nucleotide sequences were submitted to GenBank and compared to the deposited sequences of the same gene. The sequences were also aligned by Phylogeny.fr software for the phylogenetic analysis.

RESULTS:
All the 23 analyzed samples by E.M. evidenced the presence of enveloped, ovoid virions, 220-300 nm long and 140-170 nm wide, with filaments on the surface membrane. On the basis of the morphological characteristics these particles were referable to Parapoxviruses. The investigated clinical specimens, successfully amplified by PCR a fragment of 392 bp. The results of the phylogenetic analysis based on the partial 045 ORF gene, demonstrated that all the ORFv strains were closely related and the most (20/23) were identical, while 3 strains, coming from 3 different farms, showed little nucleotide differences (Fig.1). The sequences of the samples 10278 PZ and 10279 PZ had 3 variable sites, while the strain 3472 FG showed 4 variable sites and 2 variable sites in comparison to the strains 10278 PZ and 10279 PZ. Furthermore, the nucleotide BLAST, revealed that 20 strains had higher similarity with the sequence of the late transcription factor of the ORFv strain B029 (KF837136.1), while the other 3 were more similar to the partial genome of the ORFv isolate Shiraze3. The ORFv strain B029 was isolated in a study carried out in Germany (2), while the ORFv Shiraze3 was isolated in Shiraz region (Iran)(3) (KC334486.1).

DISCUSSION AND CONCLUSIONS:
This study represents the first contribute on the molecular characterization of Parapoxviruses in Southern Italy. The phylogenetic analysis based on the 045 ORF gene revealed the high degree of identity, although there was a slight, but significant genetic diversity. On the basis of our preliminary data It can be hypothesized that some of the ORF viruses present in Italy, could also have been introduced from some foreign countries, for example Middle-East countries, as result of the animal trade. However, more genomic regions of the ORF virus should be investigated, for a better understanding of the virus circulation, epidemiology and evolution.

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INTRODUCTION:
BPVs are double-stranded DNA viruses causing diseases of considerable importance in cattle. Although Papillomaviruses are mostly species-specific, BPVs play a major role in the pathogenesis of equine sarcoid. 13 viral types have been characterized and classified in 3 genera: Xipapillomavirus, Deltapapillomavirus and Epsilonpapillomavirus. The Xi-papillomavirus genus includes BPV3, 4, 6, 9, 10, 11 and 12 and infects keratinocytes inducing epithelial papillomas. The genus Delta-papillomavirus, consisting of BPV1, 2 and 13, determines fibropapillomas involving epithelium and dermis of paragenital areas, skin, alimentary tract and urinary bladder. BPV5 and 8, included in the Epsilon-papillomavirus genus, cause papillomas and fibropapillomas mostly localized at the udders and teats level. BPV7 still remains unclassified [1].

Mainly spread BPVs are divided in 2 subgroups depending on genome similarity, biological activity and immunology. Particularly Subgroup A (BPV1,2 and 5) causes fibropapillomas, while Subgroup B (BPV3,4 and 6) induces epithelial squamous papillomas [2].

Aim of this work is to assess viral presence in Piedmont and to improve diagnostic procedures to characterize different BPVs.

MATERIALS AND METHODS:
59 samples (55 bovine, 3 donkeys, 1 horse), deriving from Piedmont farms and previously identified as “papillomas” by histological diagnosis were analyzed.

Two PCRs were designed to detect BPVs belonging to Subgroup A and B. Both PCR reactions were targeted to L1 gene, codifying viral capsid proteins and PCR mixtures and amplification protocols were performed as described by Maeda et al [3]. Reaction mixtures were electrophoresed on a 2% agarose gel containing Gel Red (Nucleic Acid Gel Stain 10.000x) and detected by UV transillumination.

Sequencing was performed on 15 positive cases, selected in reason of their proximity to farms breeding equids.

After PCR products purification (NucleoSpin Gel and PCR clean-up -Macherey-Nagel) sequencing was performed with forward and reverse primers by Cycle Sequencing Kit (Big Dye Terminator, version1.1, Applied Biosystems) and a 310DNA Analyzer (Applied Biosystems). Forward and reverse complementary sequences were aligned by BioEdit software (version 7.0.9.0, Hall, 1999) and results were analyzed by BLAST search on GenBank database.

RESULTS:
BPV was detected in 69% of cattle samples and in one donkey tissue. Particularly, 37 cattle samples resulted positive to Subgroup A and B. Both PCR reactions were targeted to L1 gene, codifying viral capsid proteins and PCR mixtures and amplification protocols were performed as described by Maeda et al [3]. Reaction mixtures were electrophoresed on a 2% agarose gel containing Gel Red (Nucleic Acid Gel Stain 10.000x) and detected by UV transillumination.

Sequencing was performed on 15 positive cases, selected in reason of their proximity to farms breeding equids. After PCR products purification (NucleoSpin Gel and PCR clean-up -Macherey-Nagel) sequencing was performed with forward and reverse primers by Cycle Sequencing Kit (Big Dye Terminator, version1.1, Applied Biosystems) and a 310DNA Analyzer (Applied Biosystems). Forward and reverse complementary sequences were aligned by BioEdit software (version 7.0.9.0, Hall, 1999) and results were analyzed by BLAST search on GenBank database.

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REPLACEMENT OF RABBIT HEMORRHAGIC DISEASE VIRUS (RHDV) GENOGROUP 1 BY NEW VARIANT RHDV2 IN PORTUGAL

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Keywords: Lagovirus, rabbit hemorrhagic disease virus (RHDV), genogroup 1, new variant RHDV2, Portugal

INTRODUCTION:
Rabbit hemorrhagic disease virus (RHDV) is a highly pathogenic virus from the genus Lagovirus, family Caliciviridae, that causes rabbit hemorrhagic disease (RHD) in European rabbits (Oryctolagus cuniculus). The disease is associated with hepatic necrosis and hemorrhages, and rabbits die within 1 to 3 days [1]. Six RHDV genogroups, G1-G6, are recognized [2, 3]; however, a new variant named RHDV2 recently emerged [4]. The new variant has a unique antigenic profile and causes mortality in young rabbits, that are not susceptible to G1-G6 strains [5]. Until 2012, only G1 was circulating in Portugal [6], but RHDV2 was recently reported [7]. Here we performed a study in rabbits found dead in the field in 2013 and 2014 to determine the prevalence of G1 and RHDV2 in Portuguese rabbit populations.

MATERIALS AND METHODS:
From 2013 to 2014, 57 rabbit liver samples from several Portuguese localities were received in our facilities. For seven young wild rabbits, carcasses were submitted to necropsy and samples of several organs were collected for histopathological characterization. Samples were screened for the presence of RHDV by PCR. Positive samples were sequenced and blasted against publicly available sequences. Genetic distances and a Maximum Likelihood (ML) tree were estimated.

RESULTS:
Necropsy of the seven rabbits revealed macroscopic changes, such as epistaxis, haemorrhagic tracheitis, sero-haemorrhagic pleurisy and pulmonary congestion. Observed livers were predominantly pale in appearance and in some cases were congested. Histopathology revealed hemorrhagic pneumonia and tracheitis, congestion of the liver and diffuse necrotizing hepatitis. Fifty samples were positive for RHDV2 (87.7%). The blast of the sequences revealed 98-100% of similarity with publicly available sequences of RHDV2 and an overall identity of ~78% with G1 strains.

DISCUSSION AND CONCLUSIONS:
During 2013 and 2014, wild rabbit populations in Portugal suffered severe outbreaks of RHD. Our results show that these outbreaks were caused by the new variant RHDV2 and no cases of G1 were detected. This confirms that RHDV2 is the main circulating variant and has replaced G1 in Portugal. A similar situation was already reported in France [8], suggesting a selective advantage of RHDV2 over other genogroups. Since the epidemiological outcome of this replacement is unpredictable, and RHDV2 is causing important ecological and economical losses in Portugal, continued monitoring is strongly recommended.

REFERENCES:
INTRODUCTION:
Avian influenza (AI) is caused by the Influenza A virus, member of the family of Orthomyxoviridae. Subtyping of AI is based on the surface proteins hemagglutinin (H) and neuraminidase (N). Most AI viruses cause only mild disease in poultry. However, AI viruses of subtypes H5 and H7 can evolve into high pathogenic avian influenza (HPAI) by only a few mutations. Since HPAI viruses cause severe disease and high mortality, AI caused by H5 and H7 subtypes is a notifiable disease for which immediate control action is compulsory.

In the Netherlands, monitoring of AI is, among others, based on serology, where each flock is tested with ELISA for the presence of antibodies against influenza nucleoprotein. All ELISA positive samples are subsequently tested in the hemagglutination inhibition test using H5 and H7 antigens. As this is an elaborate procedure requiring relatively large serum volumes, the current scheme does not allow for complete subtyping, thereby preventing full insight in the introduction of subtypes and their transmission. A bead-based suspension array with a comprehensive antigen panel would enable complete subtyping, beneficial for both veterinary and human medicine.

MATERIALS AND METHODS:
Recombinant H and N proteins (H1-16, N1-9; 1-9 variants per subtype) were coupled to paramagnetic Luminex beads using standard chemistry and protocols (1). With the resulting 54 bead sets (separated into six multiplex assays), a collection of sera from experimentally infected chickens was tested using the flow cytometry based Luminex 200-system, essentially as described (2), with phycoerythrin-labelled goat anti-chicken IgG as secondary antibody.

RESULTS:
The assay identified nearly all sera correctly. A H3N2 serum and a H16N3 serum did not give signals with any antigen, whereas a H5N1 serum did not give a signal with any of the N antigens. The results further show that some cross-reaction of certain sera with other H antigens can occur, e.g. H7N1 reacts with the H15 antigen and H12N5 reacts with a H9 antigen.

DISCUSSION AND CONCLUSIONS:
To enable comprehensive subtyping of AI virus infections in poultry, a serological multiplex assay was set-up in the format of a bead-based suspension array, using paramagnetic beads to enable automated washing. The 54-plex assay was able to identify most sera correctly. Two sera (H3N2 and H16N3) did not react with any antigen, which may suggest that the corresponding antigen variants are not present in this assay and/or that the protocols need optimization. Not all H5 antigens reacted with the H5 sera, confirming that within antigen types variation occurs that can affect serological results. Some (weak) cross-reactions were observed, which correspond to the genetic relationship of antigens, such as H7/H15 and H12/H9 (3).

In conclusion, the first results with a new bead-based suspension array suggest it is a viable approach for comprehensive subtyping of avian influenza infections in chickens. Currently, further optimization and evaluation with field samples is in progress.

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2 Van der Wal et al., 2013, Vet J 196:439-444
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INTRODUCTION:
Rotaviruses (RVs) are the major cause of acute gastroenteritis in infants and young animals of mammalian and avian species. RVs are classified in the genus Rotavirus of the family Reoviridae, with a genome consisting of 11 segments of double-stranded RNA. RVs are divided into eight groups (A-H) based on antigenicity and nucleotide sequence identities of the VP6 gene (1). In avian species, group A and D are represented with high frequency, while group F and G are sporadic. Enteric syndrome in young birds is a major concern to poultry industry, causing severe economic losses. Avian RV infections resulting in clinical disease are most frequent in turkeys, quite common in chickens, pheasants, guinea fowls, partridges, and quails, and are sporadically described in pigeons and ducks. Monitoring rotavirus distribution in different avian species is crucial to uncover diversity among strains and to better understand RV ecology in the field. Several studies on the epidemiology of RVs in different avian species were reported worldwide. However, very little is known about the characterization of avian RVs in Italy. The aim of this retrospective study (2006-2012) was to provide information on the prevalence of different RV groups and the genetic diversity of RVs in different avian species suffering enteritis in Italy.

MATERIALS AND METHODS:
We analysed a total of 36 intestinal contents and/or faecal samples collected during the period 2006-2012 from different avian species (chicken 14; turkey 7; pheasant 5; guinea fowl 5; partridge 5) suffering enteritis resulted positive for rotavirus by electron microscopy. All samples were processed as pooled specimens. Viral RNA was extracted from 200 μl of each suspension using QIAzol (Qiagen). Extracted viral RNA was subjected to RT-PCR assays with specific primer pairs for NSP4, VP6, VP4, VP7 of RV-A and RV-D groups and VP6 of RV-G and RV-F groups using the OneStep-RT-PCR kit (Qiagen). The PCR products were purified using NucleoSpin Gel and PCR clean-up kits (Macherey-Nagel), according to manufacturer’s instructions. Nucleotide sequences were performed by Big Dye Terminator (Applied Biosystems) using the same primers used for RT-PCR. The phylogenetic dendogram was constructed by comparing RV sequences from various groups, including human and animal group A RVs, group B-D RVs and group F-H RVs. Analysis was performed on the coding region of the four segments. The alignment was performed using the CLUSTAL W method in the MEGALIGN module of the DNASTAR software package and phylogenetic trees were constructed using neighbour-joining method as implemented in the MEGA V software package. Bootstrap analysis in phylogenetic trees was performed with 1000 replicates and 111 random seeds.

RESULTS:
On a total of 36 samples single infections were detected in 11.1% of cases (2.7% RV-A and 8.3% RV-D). Mixed samples containing RV-A and RV-D groups were detected in 16.6% of the analysed samples, while RV-F and/or RV-G together with RV-A and/or RV-D were detected in 72.2% of samples (2.7% RV-A+F; 8.3% RV-D+F; 5.5% RV-A+D+G; 25% RV-A+D+F; 19.4% RV-D+F+G; 11.1% RV-A+D+F+G). Up to now 115 complete sequences in the following gene segments were performed: 13 of NSP4A, 15 of VP6A, 28 of NSP4D, 29 of VP6D, 6 of VP4D, 15 of VP7D, 5 of VP6F, 3 of VP6G and 1 of VP4A. The phylogenetic analysis of these sequences showed for VP6 a clustering of groups A, C, D and F, separated from groups B, G and H; for VP4 and VP7 a clustering of groups A, C and D, separated from groups B and H; for NSP4 segments the group D forms a branch well separated from the other groups.

DISCUSSION AND CONCLUSIONS:
The results of this study provide novel data on RVs prevalence in avian species in Italy. In these set of samples, we observed a high presence of mixed infections of RV-A, D, F, G groups compared to single infections. In particular, RV-F and RV-G groups are highly present in Italian poultry farms (72.2%). Overall, our findings give the basis for further genomic and epidemiological studies aimed to better understand the characteristics of avian rotaviruses circulating in our country.

REFERENCES:
GENETIC DIVERSITY OF ALEUTIAN MINK DISEASE VIRUSES ISOLATED IN RUSSIA AND BELARUS

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Keywords: Aleutian mink disease virus, VP2 gene, phylogenetic analysis

INTRODUCTION:
Aleutian mink disease virus (AMDV) causes severe economic losses in domestic mink farms worldwide. The disease characterized by acute fatal pneumonia in kits and chronic immune disease and impaired reproduction in adult minks. AMDV is the only member of the genus Amdovirus of the Parvoviridae family. It has a single-stranded 5-kb length DNA genome that codes three nonstructural proteins (NS1, NS2, and the putative NS3) and two structural (VP1, VP2) proteins. The VP2 protein is the central capsid protein responsible for capsid assembly and DNA packaging and it also induces neutralizing antibodies [1]. The VP2 protein is expressed by the VP2 gene displayed a high degree of variability in different AMDV strains. Therefore, research of the full-length VP2 gene is very interesting in Aleutian mink disease diagnostics and viral identification. Previous phylogenetic analysis of AMDVs isolated in different mink farms of Russia from 2000 to 2004 was based on short VP2 gene fragment. To obtain a better understanding of the current genetic diversity of AMDVs in Russia and Belarus full-length VP2 genes of 7 strains isolated in 2005-2013 were sequenced and used for phylogenetic analysis.

MATERIALS AND METHODS:
Organ material submitted for the PCR detection of AMDV included blood, spleen and lymph nodes of minks from 5 Russian and 2 Belarus farms. DNA was extracted using DNA-sorb-B kit. The presence of AMDV was confirmed using AMDV-Eph PCR kit (AmpliSens, Russia). Three primer pairs were designed for amplification and sequencing of 300, 600 and 1200 bp overlapping fragments of the complete VP2 gene. Purified PCR products were sequenced in both directions. Complete VP2 gene sequences of Belarus and Russian AMDVs were aligned with full-length VP2 sequences available from GenBank and kindly provided by Dr.Zhang (China). The phylogenetic analysis of 29 sequences was performed using MEGA 4.0 software.

RESULTS:
A comparative analysis of 1941 bp VP2 nucleotide sequences showed that new sequenced strains shared 92-99% identity with AMDVs from GenBank and China [2] and were 95-99.6% identical to each other. The amino acid alignment showed 122 mutations among 647 amino acid residues of the VP2 protein from 29 AMDVs. The mutations occurred not only in the hypervariable region of VP2 but also in the immunoreactive region. The unique mutation of amino acid Glu to Lys was found in position 437 in isolate from Belarus. The highest mutation level among 29 AMDVs was found in Russian strain FarEast, isolated in 2003. We found that 2 Chinese (SD1 and DL1), and 3 Russian strains (FarEast, Rus-17 and P1) had unique deletion of amino acid residue at position 236 of the hypervariable region. A polyG deletion at the N-terminus of VP2 protein was not found in new AMDVs. At the amino acid level the most relative new sequenced strains were Rus-09 and Bel-01 (99.8% identity), the most diverged – Rus-17 and Bel-02 (94.7% identity with other new sequences).

DISCUSSION AND CONCLUSIONS:
Phylogenetic analysis of VP2 gene showed that 5 new AMDVs grouped with low-pathogenic European strain AMDV-SL3 and non-pathogenic strain AMDV-G from USA. One isolate from Belarus and one from Russia (Bel-02 and Rus-17) were placed in group with high-pathogenic strains FarEast and AMDV-Utah1. Phylogenetic analysis also showed that Russian and Belarus isolates had no relationship with Chinese endemic AMDVs [2]. In conclusion, we found that most of Russian and Belarus isolates genetically similar to AMDV strains circulating in Europe and probably could have evolved from strains imported with minks and feed from Denmark and Finland. Further studies of AMDV based on NS1 sequence data are still needed to reveal the population dynamics of AMDV strains and to realize effective control of Aleutian mink disease.

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POSTER PRESENTATIONS

NOVEL TECHNOLOGIES IN DIAGNOSTICS
RAPID DETECTION OF SALMONELLA SPECIES USING MODIFIED FLUORESCENCE IN SITU HYBRIDIZATION METHOD

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Keywords: FISH, PHMB, Bacteria

INTRODUCTION:
Fluorescence in situ hybridization (FISH) offers many advantages and involves the specific hybridization of complementary nucleic acid sequences within whole permeabilized bacterial cells using fluorescent labeled probes for detection. Effective permeabilization and delivery of Oligodeoxynucleotide (ODN) probes into bacteria remain a challenge for efficient hybridization and bacteria detection. Recently, the cell entry properties of polyhexamethylene biguanide (PHMB) as a cellular delivery agent of molecules were described in our laboratory (Chindera et al. 2013). We found that PHMB was capable of delivering a range of cargo types such as ODN, siRNA, proteins, plasmid RNAs and small molecule drugs. This study aimed to optimize intracellular delivery of ODN to viable Salmonella cells and improve on fluorescence in situ hybridization for pathogen detection using PHMB.

MATERIALS AND METHODS:
Sal3 ODN probe (Nordentoft et al 1997) with specificity for Salmonella enterica was used for PHMB-ODN delivery. For modified FISH studies, an additional designed mismatched probe (Mis-Sal3) was used as negative control. Target sequence matched and mismatched probes were labelled on the 5’end with 6’FAM fluorophore (Sigma Aldrich UK). PHMB was purchased from Arch UK Biocides under the trade name VantocilTM.

PHMB-ODN delivery and cell microscopy: An overnight culture of Salmonella in Muller Hinton broth was sub-cultured in fresh broth and incubated for 2 hours at 37ºC with continuous shaking. Cells at early logarithmic phase were harvested, washed and OD standardized (OD 600~0.2±0.050). Solutions of 100 µl consisting of PHMB and Sal3 mixtures were prepared at ratios of 7µg/ml: 1µM and 6 µg/ml: 1µM, added to bacteria, and incubated at 37ºC for 2 hours. The optimal complexation volume was found to be 40µl at a ratio of 7µg/ml PHMB: 1µM ODN, and was subsequently used for further delivery and PHMB-FISH assays. Then cells were pelleted and washed in 1×PBS by centrifugation and counterstained with DAPI and visualized under fluorescence microscope. PHMB-FITC was used as positive control.

Cell viability assay: Two assays were performed to determine if PHMB-ODN delivery required viable bacteria. This included testing ODN delivery into cells treated with sodium azide (NaN3) from concentrations 1-10mg/ml in a NaN3 killing assay, and temperature dependent assay by incubating cells at 4ºC, 25ºC and 37ºC.

Fluorescence in situ hybridization: After delivery of Sal3 and Mis-Sal3 as described above, cells were washed in 100µl wash buffer containing 0.7M NaCl, 20mM Tris/HC1 pH7.2 and 0.01% SDS preheated at 50ºC. Cell suspensions were incubated at 50ºC in a water bath for 30 mins. Cells were washed once with sterile distilled water by centrifugation and microscopy carried out as previously described.

RESULTS:

Cell viability assays:
Cell associated fluorescence signals were observed in NaN3 untreated cells and bacteria incubated at 37ºC, indicating cell viability was needed for PHMB-ODN delivery.

DISCUSSION AND CONCLUSIONS:
PHMB enhances the delivery of ODN probes for the specific detection of different Salmonella strains. Also, the use of PHMB enables fixation-free hybridization protocols, which may enable differential detection of live/dead bacteria. Further work will involve the detection of E. coli strains and further specificity studies.

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Chindera, K., (2013). Cellular uptake properties of polyhexamethylene biguanide (PHMB) and applications in intracellular delivery. PhD thesis submitted to the Royal Veterinary College, University of London.
A NOVEL LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) FOR A RAPID AND SIMPLE DETECTION OF COXIELLA BURNETII

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INTRODUCTION:
Q fever, caused by Coxiella burnetii, is a worldwide zoonosis with important consequences for human and animal health (1,3). Diagnosis from livestock using direct and indirect techniques is challenging (2). Nevertheless to tackle Coxiellosis in domesticated animals a rapid diagnosis is crucial. We carried out a novel LAMP molecular assay as an alternative to conventional diagnostic methods, based on DNA amplification under isothermal conditions for detection of C. burnetii. Our assay can rapidly and easily detect C. burnetii DNA in clinical samples of abortive material.

MATERIALS AND METHODS:
To develop the LAMP assay, 4 novel primers, FIP, BIP, F3, and B3 (Table 1) were designed using Primer Explorer Software (http://primerexplorer.jp/e/). The primers were based on the COM1 gene, which encodes a 27-kDa outer membrane protein of C. burnetii. This protein is the first outer membrane-associated immune reactive protein found in both acute and chronic Q fever disease. The LAMP reaction mixture (final volume, 25 µL) contained the following: 10 µL of isothermal amplification buffer 1X, 5.2 µL nuclease free water, 2.5 µL of dNTPs (1.5 mM), 1.6 µM each of the FIP and BIP primers, 0.3 µM each of the F3 and B3 primers, 2.5 µL of extracted DNA, and 1 µL (8 U) of Bst 2.0 WarmStart DNA Polymerase. The reaction mixture was incubated in a heating block at 65°C for 45 min and subsequently at 80°C for 5 min to terminate the reaction. The specificity of the C. burnetii LAMP assay was evaluated by testing 33 different bacterial DNAs. To test analytic sensitivity, 10-fold serial dilutions of C. burnetii DNA (Nine Mile strain) from 100 ng to 100 fg were subjected to LAMP. In order to evaluate the diagnostic efficiency of LAMP for C. burnetii, ten specimens (consisting of abortive products) were tested. Five of them resulted positive for C. burnetii and five were positive for both C. burnetii and Chlamydia abortus. The LAMP products were analyzed directly using UV illumination after the addition of propidium iodide to each tube (1:10 dilution of 10 mg/mL stock solution) (4).

RESULTS:
The LAMP amplification of all negative DNA controls did not produce an observable reaction on the agarose gel. The LAMP assay identified all of the positive clinical specimens and its products showed a characteristic ladder pattern on a 2.0% agarose gel (Fig. 1). The detection limit of our LAMP assay is 8 pg of C. burnetii DNA/µL. The total time required for the LAMP assay, which included amplification and detection, was about 45 min, whereas conventional PCR required 2 h. UV illumination of the reaction tubes containing the positive controls produced brilliant fluorescence, whereas the tubes containing the negative controls did not show a fluorescent signal (Fig. 1).

DISCUSSION AND CONCLUSIONS:
Our LAMP assay is useful because it can rapidly detect C. burnetii DNA in clinical samples of abortive material also when co-infections are present (i.e. C. burnetii and Ch. abortus). Moreover, LAMP does not require any specific or expensive equipment. These features enable testing in a large variety of laboratories and make LAMP a promising platform for the molecular detection of zoonotic infections in developing countries. In conclusion, our LAMP assay is a rapid, specific, economic, and simple method for the identification of C. burnetii DNA and has specificity comparable to a standard PCR assay used to detect C. burnetii DNA.

REFERENCES:
RAPID MOLECULAR DIAGNOSTICS IN THE BARN

Lorenzetti C.[1], Panara F.[1], Tabarrini F.[1], Carucci M.[1], Ragni A.[1], Biagetti M.[2], Pezzotti G.[2]


Keywords: NALF , LAMP, Respiratory diseases , Cattle, Novel Diagnostics

INTRODUCTION:

The complex of respiratory diseases is among the major causes of economic loss in cattle. Respiratory diseases usually have a multifactorial origin. Their onset is linked to the simultaneous presence of viral and bacterial agents. The main viruses are Herpesvirus Bovine type 1 (causative agent of infectious rhinotracheitis - IBR) and the Bovine Respiratory Syncytial Virus (BRSV) which may be associated with the Adenovirus cattle, the virus Parainfluenza 3 (P13) and the virus of Diarrhea virus (BVD). Among the major bacterial agents include instead Pasteurella multocida, Mannheimia haemolytica and Mycoplasma spp. which are also very common on farms. These can act as primary agents of disease, but, most often, they appear as secondary agents of preexisting viral infections. The purpose of this work is the development and validation of a new generation of diagnostic assay that has reduced costs and it can be used by farmers to identify respiratory diseases in cattle directly in the barn by nasal swab. Pathogens identified by the assay are IBR, Pasteurella multocida, Mannheimia haemolytica, and Mycoplasma bovis.

MATERIALS AND METHODS:

The swabs (two for animal) were processed one in laboratory by molecular routine methods and cultivation technique (1,2,3,4) and the other directly in the barn by new assay. Target DNA eventually present in the sample is amplified with an isothermal reaction (LAMP) at ~65°C. The isothermal reaction is specially designed in order to be highly sensitive and specific for the target, moreover a technology patented by RAPID Biotech is employed to conjugate some of the oligos with GNPs (Gold Nano Particles). The use of conjugated oligos allows the synthesis during the LAMP reaction, in the presence of the specific target, of a double labelled amplicons that can be visualized using a chromatographic device. Interpretation: after the amplification step the outcome is visualized on a specific chromatographic multi detection device which after max ten minutes will produce a well visible red line similar to a pregnancy test in the case of a positive sample. No line will be visible in the case of a negative sample.

RESULTS:

The NALF method reveals more positive respect to the classical PCR for IBR and Mycoplasma bovis, and also respect to the isolation for Pasteurella multocida, and Mannheimia haemolitica. The percentage of similarity of the results of analyzes carried out using traditional methods and innovative methods NALF out of a total of 139 samples is: IBR (93%), M. bovis (73%), Pasteurella (83%) e Mannheimia (90%).

DISCUSSION AND CONCLUSIONS:

From the results, the NALF method seems to be more sensitive than PCR and isolation. This may be due to the fact that molecular methods reveal the nucleic acids even if the pathogen is dead. In conclusion, the developed assay is rapid and low cost and can be performed directly on site for an early diagnosis and a prompt treatment.

REFERENCES:

OPTIMIZATION OF A REPORTER GENE ASSAY TO DISCLOSE ILLICIT TREATMENTS BY CORTICOSTEROIDS IN BOVINE

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Keywords: bovine urine, glucocorticoids, reporter gene assay

INTRODUCTION:
The illicit use of synthetic glucocorticoids (GCs) in livestock production has been widely described [1]. In Italy, screening methods for GCs have to demonstrate, at least, a detection capability (CCβ) of 2 ng mL⁻¹ toward each GC included in the validation study [2]. Recently, prednisolone traces have been found in several experimental and control bovines, posing doubt about the endogenous production of this hormone [3]. For this molecule the level of interest has been risen to 5 ng mL⁻¹. In the present study the applicability of GR CALUX as qualitative screening tool for the detection of synthetic GCs in calf urine samples was demonstrated. The validation study included only the GCs estimated by the Italian Residues Control Plan, and for which a confirmatory method is applied. The pro-hormone prednisone, which resulted as being not active, was not included [4].

MATERIALS AND METHODS:
1. Urine samples and sample preparation
Every urine sample applied in this study was obtained from experimental and control animals. The specimens were collected at the farm, after spontaneous micturition, and immediately stored at −20 °C. Samples were subjected to deconjugation and liquid/liquid extraction.

2. GR CALUX analyses
Cells were cultured in medium supplemented with foetal calf serum (FCS). For exposure, cells were plated in 96-well plates with phenol red-free medium supplemented with dextran-coated charcoal-stripped FCS. The next day, the medium was replaced by medium containing the extracts to be tested dissolved in DMSO. After 24 h, the medium was removed, the cells were lysed and luciferase activity was measured.

3. Validation study
The validation study was developed in accordance with the Commission Decision 657 of 2002, and included the following analyses:
- Cross-reactivity of analytes: standard solutions of flumethasone, dexamethasone, betamethasone, methylprednisolone, prednisolone and spiked urine samples.
- Mean response negative urine: 13 blank urine samples.
- Specificity: standard solutions of cortisone, cortisol, 20β-dihydrocortisol, 17β-estradiol, testosterone, progesterone, aldosterone and spiked samples.
- Decision Limit (CCα) and Detection Capability (CCβ): 24 blank urine samples and the corresponding 24 spiked specimens (prednisolone 5 ng mL⁻¹).
- Ruggedness: minor changes (+/- 10%) in standard conditions of sample preparation and analysis.
- Stability of analytes in matrix: different aliquots of a spiked specimen, within a period of a month.

RESULTS:
Physiological levels of endogenous corticosteroids did not appear to influence GR-CALUX performance, while sexual hormones resulted as being not active. The method showed a high sensitivity toward the synthetic molecules tested (Table 1). The validation study was carried out using prednisolone, molecule that showed the lowest GC activity on the method. All blank and spiked urine samples fulfilled the CCβ criteria (Figure 1), the method was specific and robust and the analytes in urine were stable for at least 30 days.

DISCUSSION AND CONCLUSIONS:
The analytical method employed in EU control programs for measuring steroid residues fail short of capturing the complexity of illicit practices. As demonstrated for estrogens and androgens, bioassay activity-based screening combined with chemical identification could provide an optimal way to detect the illegal use of these molecules [5]. The study shows that a reporter gene assay based on stably transfected human cells is suitable for the qualitative analysis of bovine urine samples.

REFERENCES:
RAPID DETECTION OF ANTIBIOTIC RESISTANCE GENES USING LUMINEX MULTIPLEX TECHNOLOGY

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Keywords: Antibiotic Resistance Genes, Luminex, Multiplex Technology

INTRODUCTION:
Traditional methods for antibiotic susceptibility testing of bacterial pathogens are cultivation based and require at least 24 hours of incubation after having the organism growing in pure culture. However, rapid and accurate determination of antibiotic resistance is essential for effective treatment and infection control. Since resistance genes are known to aggregate upon plasmids, horizontal gene transfer can readily confer multi-resistance between even distantly related bacteria. An implication of this is that it might not be possible to determine the complete resistance profile of bacterial pathogens by using conventional molecular methods for rapid detection, such as real time PCR, because of their limited multiplexing capacity. We here explore the possibility to use a novel PCR-based approach in combination with Luminex multiplex technology for the determination of antibiotic resistance profiles.

MATERIALS AND METHODS:
In brief, the method is based on the same basic principles of low primer concentrations as the rapid molecular pathotyping assays for avian influenza and Newcastle disease virus developed by Leijon et al. (1-2). However, it has been modified to allow simultaneous and specific detection of all PCR products by the Luminex xMAP system. Currently, the assay is able to detect 11 antibiotic resistance genes. These genes were selected based on the pUUH239.2 plasmid characterized by Sandegren et al. (4), which contains all of the resistance genes except NDM-1. This plasmid was also used for the optimization and validation of the primer constructs. Since there are two amplification steps, a first to increase the sensitivity and a second for specific detection, combinations of individual PCR products from the first step were used to assess cross- and/or nonspecific hybridization that can give rise to false positive signals in the second step. The optimized assay was then used to identify resistance genes in six samples of cultivated bacteria, five containing modified versions of the pUUH239.2 plasmid and one with a largely uncharacterized plasmid carrying the NDM-1 resistance gene. All experiments were performed in triplicate and the analyses of the bacterial samples were conducted as blind tests.

RESULTS:
All genes were readily detected after optimization of the assay. The positive signals, as measured in mean fluorescence intensity (MFI), were all larger than 1000, while the negative control is below 50. Luminex signals are generally regarded as positive if their values are four times higher than the background. The assessment of specificity, where one gene at the time was removed, demonstrated consistent results for all genes. The MFI of the missing genes were all on the same level as the negative controls. The analysis of the bacterial samples correctly revealed their antibiotic resistance profiles.

DISCUSSION AND CONCLUSIONS:
As our results demonstrate, we have developed a tool for culture-independent multiplex detection of antibiotic resistance genes. Similar to the pathotyping assays, it can easily be expanded or modified to fit a given set of genes. These abilities make it suitable for rapid determination of the antibiotic resistance of bacterial pathogens.

REFERENCES:
INTRODUCTION:
Recumbency in cow is caused by numerous different disorders and if the cattle are unable to rise for more than 24 hours after initial recumbency, they may develop a secondary recumbency from pressure damage to muscles and nerves, often termed “downer cow syndrome”. The most important pathophysiologic event that develops during prolonged recumbency is a pressure-induced ischemic necrosis of the thigh muscles that frequently affects both hindlegs. Downer cows have usually increased serum creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activity, as reported by other authors (Omnaz et al., 2011). Increased serum CK activity is a specific indicator of muscle damage, while AST is present in so many soft tissues that the enzyme is a sensitive but nonspecific marker of soft tissue damage and LDH is considered ubiquitous. In particular LDH molecule is a tetramer composed of four polypeptide chains that can be combined differently resulting in five isoenzymes which can be separated by electrophoresis. The prevailing type of LDH varies according to tissue type and serum LDH isoenzymes may be useful in differential diagnosis, since tissue damage releases isoenzymes contained therein. LDH2, LDH3 and LDH4 are found in various amounts in different organs while LDH5 is present mostly in skeletal muscle and LDH1 is found predominantly in the heart muscle (Kaneko, 2008). The objective of this study was to evaluate if in downer cows LDH isoenzymes differ from LDH isoenzyme in healthy cattle.

MATERIALS AND METHODS:
The study was carried out on 10 dairy cows (Holstein Frisian) belonging to 5 different farms of Northern Italy. In each farm a cow with downer syndrome and a clinically healthy cow (control) at the same time of lactation were selected. Blood samples were taken by tail veins into tubes without anticoagulant (Vacutainer®) and taken refrigerated to the laboratory. After clotting, the blood samples were centrifuged at 1,300 g for 10 min to separate the serum that was stored at -80°C until analysis. The serum AST, LDH and CK activities were determined by the biochemical automatic analyzer ILab Aries (Instrumentation Laboratory, Lexington, MA,USA) and its ready-to-use kits. Electrophoresys and staining of LDH isoenzymes were carried out with a LDH isoenzymes electrophoresis kit (Hydragel ISO-LDH, Sebia, Issy Les Moulineaux, France, PN 4130) using the instrument HYDRASYS and its PHORESIS software, after diluting serum to reach an activity of around 750 UI/l.

RESULTS:
The results of AST, CK, LDH activity and LDH isoenzymes distribution are reported in table 1.

<table>
<thead>
<tr>
<th>Cows</th>
<th>AST U/l</th>
<th>CK U/l</th>
<th>LDH1 %</th>
<th>LDH2 %</th>
<th>LDH3 %</th>
<th>LDH4 %</th>
<th>LDH5 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downer</td>
<td>498.5</td>
<td>27.0</td>
<td>15.8</td>
<td>12.3</td>
<td>26.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=5) SD</td>
<td>475.6</td>
<td>3651.1</td>
<td>2.8</td>
<td>3.2</td>
<td>8.2</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75.0</td>
<td>2162.0</td>
<td>41.8</td>
<td>27.8</td>
<td>18.3</td>
<td>6.8</td>
<td>5.3</td>
</tr>
<tr>
<td>(n=5) ND</td>
<td>18.8</td>
<td>575.7</td>
<td>3.5</td>
<td>1.9</td>
<td>0.7</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Means and standard deviation (SD) of AST, CK, LDH and LDH isoenzymes distribution (% of total LDH activity)

DISCUSSION AND CONCLUSIONS:
With respect to the range of normal reference values established in our laboratory for milking cows (Archetti et al, 2001, unpublished data) the AST and CK values of downer cows were beyond the 95th percentile of the distribution as opposed to control cows. In accordance to previous studies (Keller, 1974) there was also a trend to higher values of LDH5 in downer cows, which is consistent with its muscular origin. Nevertheless further studies are needed to validate this hypothesis.

This study was financed by Ministry of Health, PRC2011019

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EVALUATION OF MLVA AND NGS FOR TYPING OF SALMONELLA MBANDAKA: APPLICATION TO AN OUTBREAK IN CATTLE CAUSED BY CONTAMINATED FEED

Söderlund R. [2], Jinnerot T. [1], Frosth S. [1], Thelander M. [1], Aspan A. [1]


Keywords: Salmonella, next generation high-throughput sequencing (NGS), molecular epidemiology

INTRODUCTION:
Salmonella enterica subsp. enterica serovar Mbandaka (SMb) causes infection in domestic birds and cattle. Infected animals rarely develop symptoms, but SMb is a known human pathogen even though it is uncommon in patients in Sweden. Salmonella can survive long periods in low water-activity environments such as compound feed, feed raw materials, processing environments etc.

In April 2013, SMb was found in products from a Swedish feed factory. The contaminated feed was delivered to hundreds of farms, including chicken, sheep, pigs and cattle production farms, as well as horse stables. Of 168 farms sampled, ten cattle farms were found positive for SMb.

Pulsed-field gel electrophoresis has long been the gold standard for molecular typing of SMb. However, this method is time consuming, and difficult to interpret and reproduce. Thus, there was a need to evaluate alternative typing methods.

The project aim was to rapidly evaluate MLVA (Multiple Loci Variable-number tandem repeat Analysis) and next generation high-throughput sequencing (NGS) as typing tools for SMb, and to apply the methods to investigate the origins and course of the outbreak by comparing isolates from feed raw materials, the factory, compound feed, and infected animals.

MATERIALS AND METHODS:
MLVA typing
A small diverse panel of isolates was chosen to evaluate VNTR loci, from which a small set of variable loci could be selected and then applied on outbreak and references isolates. A total of 44 loci were investigated, of which 23 were queried by PCR followed by fragment analysis, while the rest were investigated in silico. The included loci were adopted from the literature or identified from NGS data.

Genome sequencing
Ten isolates of SMb were subjected to in-house MiSeq sequencing generating 2*250 bp paired-end reads. Sequenced isolates were from the feed factory 2010 (1) September 2012 (1), March 2013 (1) April 2013 (1); cattle faeces May 2013 (1 each from 2 farms); milk May 203; Imported soy 2011-2012 (4).

In silico analysis from whole genome sequencing data
From whole genome sequence data in silico MLVA, MLST (multi-locus sequence typing), and CRISPR (clustered regularly interspaced short palindromic repeat sequences) profiles were extracted using BLAST+. Single nucleotide polymorphism (SNP) typing was performed using an in-house analysis pipeline based on MUMmer.

RESULTS:
Of 44 VNTR loci investigated, only four were found to vary within the set of SMb isolates analysed. There was no combination of VNTR loci that could distinguish between the outbreak strain of SMb and unrelated SMb-isolates. Thus, MLVA was not considered an option for the outbreak investigation.

MLST profiles extracted from NGS-sequences revealed that all Swedish isolates belonged to ST-413.

DISCUSSION AND CONCLUSIONS:
Successful MLVA typing of Salmonella Mbandaka requires new and better markers, and further studies would need to be performed to identify these. NGS provided excellent typing information from species level down to within-outbreak micro-variation, and is particularly useful for highly clonal pathogens like SMb. Both MLST and CRISPR1-profiles were found to be consistent with patient isolates of SMb from the literature. SNP typing was the only method that could clearly separate the outbreak isolates from non-related findings of SMb.

Acknowledgements
This project was financed by the SVA research fund.
A NON INVASIVE BIOMOLECULAR METHOD FOR SEX DIFFERENTIATION IN SOME SPECIES OF TURDIDAE FAMILY

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Keywords: Turdus spp., molecular sexing, CHD gene

INTRODUCTION:
Sex identification in no-sexual dimorphism birds is an important goal but also a difficult parameter to determine. In order to define the birds sex for hunting purposes and so to collect only the male, the Fishing and Hunting Service of the Veneto Region supported this study focusing on sex discrimination in the Turdidae family. As in some Thrushes there is a lack of sex dimorphisms, bird sexing is not available and only invasive laparoscopic technique gives an immediate response. Because of the stress induced by this technique bloodless procedures should be implemented. The genotyping method is based on the PCR amplification of a conserved gene (CHD-chromobox-helicase-DNA-binding gene) present in both male and female sexual chromosomes (ZZ and ZW respectively).

Griffiths and collaborators [1] described a specific PCR with primers, P2 and P8, that amplify both a conserved exonic and an intron region in CHD-W and CHD-Z. The differentiation between male and female birds is easily evidenced through the electrophoresis of amplified PCR products, resulting in a single band for the male and two bands for the female.

Moreover, Fridolfsson and Ellegren [2] developed a method for molecular sexing based on the detection of a constant size gene region using highly conserved primers (2550F/2718R). PCR products show one or two fragments for females, whereas one fragment for males.

This work describes the use of a biomolecular method as a bloodless and non-invasive tool for sex-differentiation in birds belonging to Turdus spp. The technique is a specific PCR performed on the DNA extracted from pharyngeal swabs, blood and feathers of three species of Turdus (T. philomelos, T. pilaris and T. iliacus).

Two couple of primers P2/P8 and 2550F/2718R were checked at different thermal cycling conditions.

The P2 and P8 sexing-PCR was capable to discriminate between male and female birds in the three avian species investigated, whereas 2550F and 2718R couple of primers identify male birds, but not females.

MATERIALS AND METHODS:
DNA was isolated from blood, pharyngeal swabs and feathers of T. philomelos, T. pilaris and T. iliacus, using automatic extraction (Maxwell 16 Instrument - Promega*). Amplification of CHD gene was performed using two sets of primers: P8/P2 [1] and 2550F/2718R [2]. PCR products were run in 3% agarose gel, stained with SybrGold and visualized by BioRad® Chemidoc XRS. PCR thermal conditions have been modified to better discriminate DNA size variation.

RESULTS:
DNA extraction from feathers, pharyngeal swabs and blood with automatic technique gave good DNA samples
Swabs collected from the oral cavity gave results comparable to those obtained from blood and feather specimens.
For all the species of Turdus analyzed, the P2/P8 primer set allows clear and reproducible results (fig.1). However 2550F and 2718R couple of primers identify male birds, but not females.

DISCUSSION AND CONCLUSIONS:
Several trials described in literature were applied to get the easier way to get a clear sex differentiation.
Basing on our results the Griffiths’ PCR (with slight modification) represented a valuable tool for sexing in Thrushes (T. philomelos, T. pilaris and T. iliacus). Moreover the non-invasive sampling via pharyngeal swabs resulted a suitable and sensitive method for bird-sexing without compromising significantly the birds welfare. Basing on our results, we reached a goal technique for the differentiation of male and female birds belonging to Turdus spp.
This study has been supported by the Veneto Region, Fishing and Hunting Service “DGRV 2964/12”.

REFERENCES:

Figure 1: Sex identification in Turdus spp. with P2/P8 set of primers. Marker: DNA Ladder (100 bp) ♀: female; ♂: male.
INTRODUCTION:
The present case study concerns four flamingos that, unable to fly, were kept in a conservation oasis in Ferrara, Emilia Romagna Region. All birds have found dead, some of them headless. Although a fox (Vulpes vulpes) was initially considered responsible of the killing, the birds have been taken to the laboratory for further investigations. The present study aims at identifying the predator through analysis of its behaviour, the pathology investigation and the DNA analysis.

MATERIALS AND METHODS:
All four flamingos found dead on the morning of 14th January 2013 likely have been killed by a predator during the night before and therefore the bodies were still well preserved. The inspection of the area could not reveal some breakages in the fence, reinforcing the hypothesis that a predator had to climb the fence to enter the centre and reach the flamingos. After killing, only few parts of the birds have been eaten and two heads have been removed and not found. All these elements have been considered expression of behaviour of a fox. In the laboratory the carcasses of the birds have been classified and a pathology examination took place. Intercanine distance were measured. Swabs from the predator’s saliva have been collected from the edge of the lesions referable to bites. Six swabs have been collected for identification of fox (Vulpes vulpes) DNA. The analysis have been carried out at the National Reference Centre for Forensic Veterinary.

RESULTS:
The predator’s DNA has been successfully extracted from two out of the six salivary samples. Amplification and following sequencing of a portion of ND1 gene showed, through comparison with control sequences and international databases (GenBank), that the predator belonged to species Canis lupus instead of species Vulpes vulpes as it was initially thought based on first findings. The exam of mitochondrial DNA does not allow distinction between wolf and dog (both belonging to C. lupus species) and therefore an analysis of nuclear DNA through amplification of 20 STR loci has been required. The individual genotype of the predator has thus been obtained. The statistical analysis showed then that the salivary DNA belonged to a dog, excluding further the possibility that a wolf was the predator. (Lorenzini et al., 2014).

DISCUSSION AND CONCLUSIONS:
A literature research concerning the interdental distance of the lesions as described in the pathology, has been carried out. The intercanine distance is a species-specific element and therefore it can be used for identification of the predator (Ratz et al.,1999). With special reference to adult fox, the average intercanine distance is 24-27 mm (Hart et al., 1982), greater than those recorded through pathology. The local press claimed that a fox was the predator involved, but an accurate investigation brought full light on what had happened. It is worthwhile to highlight that genetic investigation, pathology and field observations have been effective in clarifying the whole episode.

REFERENCES:
INTRODUCTION:
Dolphins are widely distributed across the warm-temperate to tropical waters of the world. About this, Stenella is a cosmopolitan cetacean and the most abundant dolphin in the Mediterranean sea. In addition, because of their position at the top of the food chain, they are excellent indicators of the welfare of the waters in which they live. Based on mitochondrial DNA (mtDNA) we reported species identification regarding carcasses of cetacean stranded on the Sicilian coast from January 2014 till now. It has been shown as mtDNA genes sequencing analysis could be useful to identify the cetacean when the carcasses do not allow to recognize the species. In the present work we identified through DNA barcoding 12 cetacean species.

MATERIALS AND METHODS:
Samples were collected from 12 carcasses stranded in various regions of the Sicilian coast (Table 1). Genomic DNA was extracted using a specific commercial kit (EZNA Genomic DNA Isolation Kit) following the manufacturer’s instructions and spectrophotometrically quantized and employed in PCR test targeted to the mitochondrial region. The region considered for the genetic characterization is a stretch of the mitochondrial gene cytochrome b. This approach permits the use a couple of mammalian universal primers to amplify a species specific DNA sequence. Amplification of the partial Cyt B regions was performed by using universal primers Cyt B1 (5-CCAATGATATGAAAAACCATCGTT-3) and Cyt B2 5-GCCCCTCAGAATGATATTTGTCCTC-3 as previously described (Janczewski D.N. et al. 1995). Species was after revealed by application of cyclesquencing protocol on the purified PCR product. The purified amplicon were sequenced with the primers Cyt B1 and Cyt B2 in two different reactions using BigDye Terminator chemistry (Life Technologies). Sequencing was performed on an ABI Prism 3130 DNA sequencer; the obtained data were therefore first edited and then aligned against a reference database (GenBank), which provides us a result a percentage of identity. We considered as stringent parameter for strain identification a value of 97% (P<0.005).

RESULTS:
The sequencing data were checked on the electropherograms and analyzed by Wu Blast 2 sequence alignment software. Our sequence was aligned in GeneBank with the corresponding tract of the mitochondrial cytochrome B. Results showed that samples belong mainly to the striped dolphin species, Stenella coeruleolatba (n=9). The remaining species belong to the bottlenose Tursiops truncatus (n=3) (Table 1).

DISCUSSION AND CONCLUSIONS:
In this study, the authors identified by DNA barcoding 12 stranded cetacean. The use of molecular markers, such as the conserved region of the Cyt B provide a valid support both from genetic diversity and phylogeny as well as a valid support in characterization of species, especially if unusual conditions make it difficult to identify. In conclusion, the cytochrome b region, despite its conserved structure, it shows considerable variability which makes it highly informative for species identification and genetic population studies.

REFERENCES:
A NEW TAQMAN REAL TIME PCR TARGETING THE OMPA GENE FOR RICKETTSIA CONORII IDENTIFICATION

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Keywords: Rickettsia conorii, Real Time PCR, ompA

INTRODUCTION: The genus Rickettsia (family Rickettsiaceae, order Rickettsiales) is an important cause of emerging vector-borne diseases and includes 21 species pathogenic to humans classified into three groups: Spotted Fever Group (SFG), the typhus group and the R. bellii group. In addition, more than 20 Rickettsia isolated were described but not yet characterized [1]. The SFG Rickettsiae comprehend the Rocky Mountain Spotted Fever group (RMSF), which R. rickettsii belongs to, and the Mediterranean Spotted Fever group (MSF), including R. conorii [2]. This one is the main tick-borne rickettsial agent found in southern and eastern Europe. Currently used diagnostic tests have limitations. Serological tests are the easiest diagnostic method but data interpretation is complicated by the cross-reactivity among the Rickettsiae. Rickettsia species identification involves molecular methods based on PCR amplification and sequencing of various rickettsial genes, with a time consuming procedure. This work is aimed to develop a new test, based on a TaqMan Real Time PCR, to discriminate R. conorii infections without sequencing.

MATERIALS AND METHODS: R. conorii Malish 7 strain cultured in VERO cells was used as a standard material for Real Time PCR optimization. OmpA gene sequences from many different Rickettsia species were selected from GenBank and aligned using ClustalW in order to identify the appropriate region for primer and probe design using the Primer Express 3.0 software. For the assay optimization many annealing temperatures and primers concentrations were tested. Each reaction was performed in duplicate in a CFX96 Biorad Termocycler and in presence of at least a no-template control. Data were analyzed by the CFX Manager 1.6 software. Specificity was verified in silico using blast analysis on GenBank database and in vitro using a local collection panel of Rickettsia DNAs [3] and also in presence of DNA of Anaplasma [4], Ehrlichia [5], Babesia [6] and Theileria [6]. Sensitivity was determined by 10-fold serial dilutions of the standards.

RESULTS: The reaction was performed with TaqMan method and the customary values obtained for the standard curve (r =0.967 and a slope value of -3.185) indicated that the reaction was well optimised (Figure 1). The analysis performed with DNA from different Rickettsia species showed the presence of fluorescence only in presence of R.conorii DNA. All the other species of Rickettsia were negative as well as the samples positive for the other related pathogens. Reaction sensitivity was of 50 copies of pathogen DNA per μl.

DISCUSSION AND CONCLUSIONS: This study allowed developing a new method able to detect and quantify Rickettsia spp. pathogen DNA and also to discriminate R. conorii from other Rickettsia species. This opportunity is of great benefit since methods currently available for identification of rickettsial species are consuming and expensive. New assays are rapid, easy to perform and also sensitive.

Acknowledgements Research supported by the Italian Ministry of Health (IZSSI 07/08). Thanks to Rosa Filippi and Pippo Bono for their technical support.

REFERENCES:

Figure 1. Standard curve obtained in the ompA TaqMan Real Time PCR.
(R1: R.conorii; R2: R.aeschlimanni; R3: R.massiliae; R4: R.raoultii; R5: R.monacensis; R6: R.helvetica; R7: R.slovaca; R8: R.felis; R9: Rickettsia endosymbiont of Haemaphysalis sulcata and R10: Candidatus Rickettsia hoogstraali)
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