Typing of *Chlamydia psittaci* to monitor epidemiology of psittacosis and aid disease control in the Netherlands, 2008 to 2013

E R Heddema (e.heddema@orbisconcern.nl)[1], E J van Hannen[2], M Bongaerts[1], F Dijkstra[3], R J ten Hove[4], B de Wever[5], D Vanrompay[6]

1. Department of Medical Microbiology and Infection Control, Orbis Medical Centre, Sittard-Geleen, the Netherlands
2. Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, the Netherlands
3. Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
4. Department of Medical Microbiology, Rijnstate, Velp, the Netherlands
5. Department of Medical Microbiology, Academic Medical Centre, Amsterdam, the Netherlands
6. Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

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A simple genotyping method was developed and validated for all known *Chlamydia psittaci* reference genotypes. *C. psittaci* is currently divided into nine genotypes (A-F, E/B, M56 and WC), all more or less associated with the preferred avian host. This method targeting variable domain 4 of the *ompA* gene has a lowest detection limit of 1 to 10 copies per PCR and was applied to 69 human samples collected in nine hospitals in the Netherlands from September 2008 until the end of October 2013. Genotype A was the most prevalent genotype. In addition, genotypes B, C, a new genotype, and *C. abortus* were found. A *C. caviae* infection was detected as a result of extension of this surveillance study to the national level. The sensitivity of this method compared with our real-time diagnostic PCR was 0.98 (66/67 typable samples). Specificity was 1.0 based on 33 commonly encountered bacterial and yeast species and 20 human respiratory samples. This typing method could help monitor *C. psittaci* infections in humans and provides insight into the relationships between notified human 'psittacosis' cases and the probable avian and other animal sources. When needed, a result can be obtained within 24 hours.

Introduction

Psittacosis, a notifiable disease in many countries, is caused by *Chlamydia psittaci* (also known as *Chlamydophila psittaci*). Clinical signs of psittacosis range from none to life-threatening disease requiring admission to intensive care. In 1999, Everett et al. proposed splitting the single genus *Chlamydia* into two genera, *Chlamydia* and *Chlamydophila*, based on clustering analyses of the 16S rRNA and 23S rRNA genes [1]. However, taxonomic separation of the genus based on ribosomal sequences is not consistent with the natural history of the organism as revealed by recent genome comparisons. Consequently, the proposal was made to reunite the *Chlamydia* in a single genus [2]. The single genus nomenclature was published in the latest edition of the Bergey's Manual of Systematic Bacteriology [3]. *C. psittaci* is currently divided into nine genotypes (A-F, E/B, M56 and WC), all more or less associated with the preferred avian host in each case.

The genotypes WC and M56 have been found in cattle and a muskrat, respectively, and are probably not associated with birds [4]. Genotype A is associated with *Psittaciformes* (cockatoos, parrots, parakeets, lories), B with *Columbiformes* (doves and pigeons), C with *Anseriformes* (mainly ducks and geese) and D with turkeys. Genotype E is the most diverse; ca 20% of strains were isolated from pigeons, but genotype E has also been found in ratites. Genotype E/B is mainly associated with ducks. Genotype F is encountered rarely in *Psittaciformes* and Turkeys [5]. *C. psittaci*, together with the closely related *C. abortus*, has also been found in cattle [6]. The role of these mammals as vectors of zoonotic *Chlamydia* spp. infections still needs to be established.

For epidemiologic purposes, characterisation of *C. psittaci* in human samples provides knowledge on the most prevalent genotypes in human infections, infers probable avian sources and aids in the process of notification, surveillance and outbreak management. For decades, the diagnosis has been based on serological tests. In the past decade, diagnostic *C. psittaci* PCR assays were developed and introduced in the clinical setting. In the Netherlands this aided the diagnostic process for suspected psittacosis cases [7,8]. One of the advantages of the PCR approach over serological testing is the presence of *C. psittaci* DNA in these clinical samples. These samples are therefore suitable for further genotyping assays. Genotyping

can be done by real-time PCR with competitor probes [9], melting curve analysis [10], MLVA [11], MLST [4], microarray or other sequence analysis [12]. Previously, a sequencing-based approach aimed at the outer membrane protein gene (ompA) was successful on human clinical samples, but the method proved laborious and was not very sensitive, mainly owing to the relatively long PCR product and abundant side products [13].

Culturing of *C. psittaci* could provide sufficient DNA for more in-depth typing methods such as MLVA or MLST. However, culturing is hampered by limited sensitivity, previous antibiotic use and the necessarily strict biosafety regulations. When culturing *C. psittaci*, biosafety level 3 precautions are needed. Nowadays, *C. psittaci* is only cultured in a few specialised laboratories.

None of the above typing methods has been evaluated on more than a handful of human samples. Even in a recently described outbreak of psittacosis in Sweden, only four of 12 available human samples could be typed [14]. In this study, we describe a new simple typing method for *C. psittaci* based on variable domain 4 (VD4) of the *ompA* gene. It does not require any specialised equipment other than a real-time PCR cycler and a (remote) sequencing facility. This method was applied directly on human samples positive for *C. psittaci*.

Methods

Bacterial strains and control DNA

C. psittaci genotypes A-F, E/B, M56 and WC were used as positive controls. The following strains were used: Genotype A: Orni (human), Genotype B: CP3 (pigeon), Genotype C: GR9 (German duck), Genotype D: NJ1 (New Jersey turkey), Genotype E: CPMN (human), Genotype F: VS225 (parakeet), Genotype E/B: WS/RT/E30 (German duck), Genotype M56 (muskrat), Genotype WC (*Bos taurus*). A quantified (15,000 copies per µl) commercially available *C. psittaci* DNA (Amplirun *C. psittaci* genotype A control, Vircell, Granada, Spain) control was used for determination of the lower limit of detection. The new assay was evaluated with the Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) External Quality Assessment (EQA) pilot panel for *C. psittaci* 2013 (CPS13).

The following strains and samples were used for specificity testing: 27 ATCC (American type culture collection) strains, one NCTC (National Collection of Type Cultures, Public Health England) strain and three Dutch quality control (SKML; Dutch Foundation for Quality Assessment in Medical Laboratories) strains, as well as QCMD EQA *Mycoplasma pneumoniae*/*Chlamydia pneumoniae* panel CP.MP13 samples 09 and 05 containing *C. pneumoniae* and *M. pneumoniae* DNA (Table 1).

Clinical samples and DNA extraction

For specificity testing, 20 respiratory samples negative for *C. psittaci* DNA were tested with the newly developed typing method.

Clinical samples (sputa, bronchoalveolar lavage fluid, (naso)pharyngeal swabs and serum) positive in diagnostic *C. psittaci* PCRs were obtained from nine hospital laboratories from the Netherlands. Most of these laboratories use real-time PCRs that detect, but do not differentiate, at least *C. psittaci* and *C. abortus* and sometimes also *C. caviae* and *C. felis* [7,8]. This means that clinical samples could contain these very closely related species as well. Nucleic acid purification was performed at the nine Dutch laboratories with the Magnapure (Roche Diagnostics), EasyMag (BioMérieux) or Versant kPCR Molecular system (Siemens Healthcare Diagnostics). Two clinical samples were obtained from Scotland and related to a previously reported outbreak [15]. Clinical samples and/or eluates were sent to the Orbis Medical Centre in the Netherlands for further analysis. Archived samples were collected from 2008 to 2012. Since September 2012, the typing method has been implemented nationally and samples have prospectively been typed and reported to submitting laboratories and public health authorities.

For validation experiments, nucleic acids were purified with the Versant kPCR Molecular system using Sample Preparation (SP) Kit 1.0 with SP protocol 250 µl sample input and 100 µl eluate output (Siemens Healthcare Diagnostics). All positive control strains were spiked with a background of pooled *C. psittaci*-negative sputum samples to simulate the diagnostic setting as close as possible. QCMD samples were processed according to the accompanying instructions.

This research was submitted for consideration to our local accredited medical ethical research committee METC Atrium-Orbis-Zuyd. According to this committee, this research does not fall under the scope of the Medical Research Involving Human Subjects Act. All prospective samples were obtained for diagnostic use and handled accordingly. Retrospective samples were analysed anonymously to the extent reasonably possible.

PCR based on variable domain 4 of the *ompA* gene

With the aid of Primer3Plus, a new primer set was developed to include a variable part of the *ompA*, the VD4 domain [16]. The amplified VD4 sequence permits differentiation of at least nine *C. psittaci* genotypes (A-F and E/B) and the closely related *C. abortus*. The primer set was verified with in-silico amplification [17] and revealed positive results for available *C. psittaci* genomes and *C. abortus* S26/3 only (setting: 'maximum two mismatches allowed'). The primer set consisted of CPVDF 5'–GTC AAG AGC AAC TTT TGA TGC–3' and CPVDR 5'–ATT TTG TTG ATC TGA ATC GAA GC–3' (nucleotide positions CPVDF 897-917 and CPVDR

1,057-1,079 of the *ompA* gene of the *C. psittaci* VS1 strain, GenBank accession number AY762608). A fragment between 174 and 183 base pairs, depending on the genotype, is amplified. *C. caviae* primers were constructed by substituting five nucleotides in the above primer pair to obtain complete homology with the *C. caviae* VD4 sequence. CCVDF 5'–GTC CAG AGC TAC ATT TGA TGC–3' and CCVDR 5'–ATT TTG TTG ATT TGA AGC GAA GC–3'. *C. caviae* species confirmation was done by PCR high-resolution melting (HRM) curve analysis as described by Robertson et al., using DNA of the *C. caviae* reference strain (GPIC) as positive control [18].

Reactions for the VD4 PCR were performed in the Stratagene MX3005P QPCR system (incorporated in the Siemens Versant kPCR system). The uracil-N-glycosylase system (UNG) was used to prevent false-positive reactions due to amplicon carry over. After optimisation, the final reaction volume (25 µl) included 5 µl eluate, 12.5 µl (2x) Greenmaster qPCR mix with ROX reference dye, uracil-N-glycosylase (Jena biosciences, Jena, Germany) and 0.5 µM of each primer. The real-time PCR steps were as follows: 1) 50 °C for 2 min, 2) 95 °C for 3 min, 3) 40 cycles of 95 °C for 5 sec, and 60 °C for 60 sec. Fluorescence was detected in the FAM channel and normalised on the ROX signal. Subsequently, a dissociation curve was generated by continuous fluorescence acquisition from 60 to 95 °C to observe possible additional PCR products and establish the formation of the expected PCR amplicon by determining the melting temperature (Tm). At first, all reference genotypes were used as positive controls in each run. For ease of application, we decided later to use only three genotypes (A, C and D) as controls.

Validation of the VD4 PCR

Analytical sensitivity was determined by testing 10-fold dilutions of the commercially available Amplirun *C. psittaci* genomic DNA control starting from 10^4 genome equivalents per PCR reaction. Serial dilutions were prepared in Tris/EDTA buffer, pH 8.0, supplemented with 20 ng/µl salmon sperm DNA). Reactions were performed in triplicate. Limiting dilutions were tested with and without previous nucleic acid extraction. When applying nucleic acid extraction, a matrix of *C. psittaci* DNA-negative, pooled and liquefied sputum samples was used. Sequence analysis was performed only on the lowest positive dilution series to confirm the identity of the positive control strain. For comparison, all dilutions were also tested with the previously described full-length *ompA* PCR and the diagnostic PCR [7,13].

Specificity was determined on a panel of bacterial and yeast species commonly encountered in human (respiratory) specimens (Table 1). Strains were diluted to a 0.5 McFarland standard turbidity equivalent to ca 10^8 colony-forming units (CFU)/ml, and 250 µl of this suspension was subsequently subjected to nucleic acids purification. Diagnostic specificity was tested using 20 human respiratory samples, previously tested negative for *C. psittaci* DNA by our previously described *C. psittaci* PCR [7]. Clinical sensitivity was determined on all *C. psittaci* PCR-positive clinical samples sent to our laboratory with a request for genotyping.

Table 1

Strains used for specificity testing of the *Chlamydia psittaci* VD4 PCR (n=33)

Species	Strain[a]	VD4 PCR
Klebsiella pneumoniae	ATCC 700603	negative
Klebsiella pneumoniae	ATCC 13883	negative
Streptococcus pyogenes	ATCC 19615	negative
Streptococcus pneumoniae	ATCC 6303	negative
Streptococcus agalactiae	SKML 1905	negative
Staphylococcus aureus	ATCC 25923	negative
Staphylococcus aureus	ATCC 29213	negative
Staphylococcus epidermidis	ATCC 12228	negative
Staphylococcus aureus (meticillin-resistant)	ATCC 43300	negative
Enterococcus faecalis	ATCC 29212	negative
Enterococcus faecium	ATCC 35667	negative
Moraxella catarrhalis	SKML 967	negative
Haemophilus parainfluenzae	ATCC 7901	negative
Haemophilus influenzae	ATCC 35056	negative
Neisseria meningitidis	ATCC 13090	negative
Legionella pneumophila	SKML 2013	negative
Bacillus cereus	ATCC 11778	negative
Enterobacter aerogenes	ATCC 35028	negative
Proteus vulgaris	ATCC 13315	negative
Proteus mirabilis	NCTC 10975	negative
Escherichia coli	ATCC 25922	negative
Pseudomonas aeruginosa	ATCC 27853	negative
Burkholderia cepacia	ATCC 25416	negative
Bacteroides fragilis	ATCC 25285	negative
Peptostreptococcus anaerobius	ATCC 27337	negative
Prevotella melaninogenica	ATCC 25845	negative
Bacteroides thetaiotaomicron	ATCC 29741	negative
Candida albicans	ATCC 90028	negative
Candida tropicalis	ATCC 13803	negative
Candida krusei	ATCC 6258	negative
Candida glabrata	ATCC 90030	negative
Mycoplasma pneumoniae	QCMD CPMP13–09	negative
Chlamydia pneumoniae	QCMD CPMP13–05	negative

[a] ATCC: American Type Culture Collection; NCTC: national collection of type cultures; QCMD: external quality assessment samples; SKML: Dutch quality control assessment strains.

Sequence analysis

Sequence analysis was performed by an external Sanger sequencing facility (Baseclear BV, Leiden, the Netherlands). A 1:10 dilution of the amplification product in PCR-grade water was added to the forward or reverse primer with a final primer concentration

Table 2

Lower detection limit of the *Chlamydia psittaci* VD4 PCR and comparison with the diagnostic PCR and the previously described full-length *ompA* PCR

Copies/PCR	Diagnostic PCR	VD4 PCR	Full-length ompA PCR	Diagnostic PCR	VD4 PCR	Full-length ompA PCR
	without nucleic acid extraction			with nucleic acid extraction[a]		
10,000	3/3	3/3	3/3	Not done	Not done	Not done
1,000	3/3	3/3	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3[b]	3/3	3/3	3/3[c]
10	3/3	3/3	0/3	1/3	2/3	0/3
1	3/3	2/3	0/3	0/3	0/3	0/3
0.1	0/3	0/3	Not done	0/3	0/3	Not done
Negative control	Negative	Negative	Negative	Negative	Negative	Negative

Results shown as number of positive samples vs number of samples tested.

[a] Nucleic acid extraction of the dilution series in a background of *C. psittaci* DNA-negative pooled and liquefied sputum.
[b] All three weak reactions.
[c] One of three was a weak reaction.

of 1 pmol/µl. Sequences were delivered by email as original peak plots. The forward and reverse overlapping sequences were edited to obtain the complete sequence. Alignment and calculation of a similarity index p-distance was done with MEGA 5.1 [19]. The newly discovered VD4 genotype was subjected to complete *ompA* sequencing as previously described [13]. A phylogenetic tree was constructed using the neighbor-joining method. Reference *ompA* genotype sequences A-F, E/B and *C. abortus* (strain S26/3) available in the GenBank database (accession numbers AY762608–12 and AF269261) were included in this analysis.

Data acquisition and descriptive statistics

Systematically collected data concerning notified human cases from September 2008 until the end of October 2013 were obtained anonymously from the national database for notifiable diseases at the Dutch Centre for Infectious Disease Control as far as possible. Age, sex, hospital admission, mortality, probable country of acquisition and suspected source of infection were noted. Occasionally, additional information was provided on submitted laboratory forms or provided via personal communication.

Results

Validation of the VD4 PCR

The newly designed primer set allowed for amplification of *ompA* VD4 regions of all nine *C. psittaci* reference strains. The lowest detection limit was 1 to 10 copies per PCR (Table 2). In a background of sputum, sensitivity was slightly lower, but still 10 to 100 copies per PCR. Dissociation curves showed only one PCR amplification product for each genotype. The genotypes were visible as dissociation peaks with Tm's ranging from 79 to 83 °C. Several replicates of the reference genotypes showed overlap between the Tm of the different genotypes. It was not possible to separate each genotype solely by Tm. The CPS13 panel was tested during validation and all truly positive samples were detected, and all negative samples were correctly identified as well. Specificity was 100% based on 33 commonly encountered bacterial and yeast species and 20 human respiratory samples. Sixty-nine *C. psittaci* real-time PCR-positive samples from 66 human individuals were available for typing. The expected amplification product was obtained in 66 of 69 available human samples. The Ct values (quantification cycle) of these clinical samples ranged from 22 to 38 cycles. Three of 69 samples were negative in the VD4-PCR. Two of them contained *C. caviae* (see below), leaving only one of the remaining 67 samples untypable. Overall, the sensitivity of this method compared with our real-time diagnostic PCR was 98% (66 genotypes from 67 typable samples).

In our hands, the method (excluding technician costs) costs ca EUR 22 per sample. This includes four PCR controls per run, nucleic acid extraction, PCR reagent and two sequence reactions. When needed, a typing result can be obtained within 24 hours.

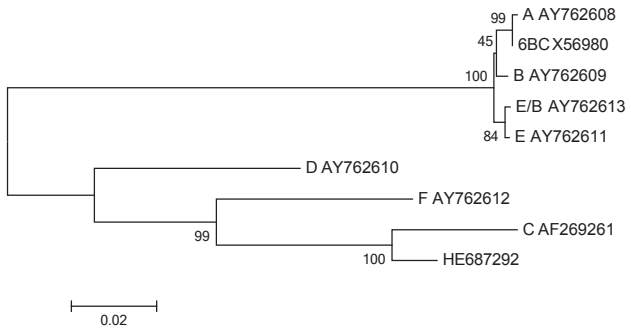
Sequence analysis and distribution of genotypes

During validation, the Amplirun *C. psittaci* genomic DNA control (only on the lowest positive dilution series), all reference strains and the CPS13 panel were successfully sequenced. All 66 VD4 PCR-positive clinical samples were sequenced revealing *C. psittaci* genotype A in 42 samples. Genotype B was found in 14 samples, E/B was found in two samples and genotype C in one sample.

Four samples revealed a new identical sequence type. Sequence analysis showed this strain to be a unique

Figure

Phylogenetic tree displaying the *Chlamydia psittaci ompA* sequence HE687292 in relation to reference genotypes A–F and E/B



Neighbor-joining method, Jukes-Cantor model, using 1,000 bootstraps created with MEGA.

ompA genotype with homology to the *ompA* of both genotype C and D. In the similarity calculation the sequence was most similar to reference genotype C strain (95% and 86% similarity to the *ompA* VD4 of genotype C and D respectively). These four samples were subjected to full-length *ompA* sequence analysis including all four variable domains. Full-length *ompA* could only be obtained for two of the four samples. These two full-length *ompA* sequences revealed an identical but new genotype in both samples. The sequence was submitted to the EMBL nucleotide sequence database and assigned reference number HE687292. The Figure shows the phylogentic relationships between the new genotype and genotypes A–F and E/B. BLAST search did not reveal an identical sequence. Phylogenetic analysis showed the *ompA* gene of this strain to be most similar to that of the *C. psittaci* genotype C (96% homologous to genotype C) but still discordant in 37 nucleotides resulting in nine different amino acids.

Chlamydia caviae and *Chlamydia abortus*

One sample contained *C. abortus*. Two VD4 PCR-negative samples from another patient were suspected to contain *C. caviae* DNA rather than *C. psittaci* DNA. This assumption was based on information from public health officials, who told us that the patient showed clinical symptoms after purchasing guinea pigs. *C. caviae* was indeed detected by use of *C. caviae*-specific primers and subsequent sequence analysis of the amplified *ompA* VD4 gene region. Both samples were also analysed by a PCR-HRM and confirmed positive for *C. caviae*.

Descriptive epidemiology

For 54 of the 66 PCR-positive patients, data were available in the national notification database.

Table 3

Characteristics of notified patients harbouring *Chlamydia psittaci* genotype A and B, the Netherlands, 2008 to 2013 (n = 50)

Genotype	A (n = 37)	B (n = 13)
Age in years: median (IQR)	63 (51 – 72)	70 (62 – 73)
Sex (male)	27	11
Deaths	1	0
Probable source of infection (location)[a]		
Home	21	10
Bird show	4	0
Bird dealer/bird dealing company	3	0
Pet shop	2	1
Public resort	1	0
Abroad (farm)	0	1
Unknown/not reported	10	2
Probable source of infection (type of bird)[a]		
Columbiformes	0	10
Captive	0	8
Wild	0	3
Psittaciformes[b]	8	0
Passeriformes[c]	5	0
Anseriformes (duck)	0	1
Galliformes (pheasant)	1	0
Unofficial bird groups	0	0
Poultry	3	2
Wild, free ranging birds	3	0
Aviary birds	6	0
Unknown/not reported	15	1
Date of disease onset		
First quarter	17	3
Second quarter	14	2
Third quarter	4	6
Fourth quarter	2	2

[a] More than one source could be noted.

[b] Four parakeets, one parrot, one cockatiel, one budgerigar, one unidentified parrot-like bird.

[c] Two zebra finches, one canary, one jay, one siskin.

Characteristics associated with infection with either genotype A or B are presented in Table 3.

Among all 54 cases, men predominated, one death was reported and all were admitted to hospital. One person probably acquired the infection outside of the Netherlands. Exposure to *Psittaciformes* and *Passeriformes* was reported for patients harbouring genotype A, while exposure to *Columbiformes* predominated among patients harbouring genotype B. Remarkable is the large proportion of genotype A cases in the first half of the year: 31 cases vs six in the second half (Table 3).

C. abortus was found in one patient. This patient, suffering from severe pneumonia requiring admission to an intensive care unit, was living on a farm raising sheep and goats [20]. The *C. caviae*-positive patient was only diagnosed because an extensive diagnostic investigation took place for sepsis of unknown origin, revealing psittacosis as the most likely diagnosis. The patient had recently purchased two young guinea pigs before becoming ill [21]. The new unique *ompA* sequence was found in four cases. In none of the four could a direct link to specific birds be found, although one of the infected people was a volunteer working with and exposed to many different kinds of birds. One case with a genotype C infection was detected. This case was related to a bird hospital.

Discussion

In this study, we present a simple, sensitive and cheap genotyping method to detect *C. psittaci* genotypes (A-F, E/B,WC,M56) and the closely related species *C. abortus*. The sensitivity of 98% compared with our real-time diagnostic PCR and a specificity of 100% were satisfactory. A result can be obtained in ca 24 hours. The lower detection limit of the VD4 PCR (at least 10 copies per reaction) is very sensitive and comparable to previously used *C. psittaci ompA* typing methods [9]. Geens et al. required separate PCR reactions for each genotype while our method is a singleplex format [9]. Although the VD4 PCR was validated in only one laboratory, we were still able to detect the genotype in 66 of 67 typable samples sent from across the Netherlands.

Although the dissociation curve analyses could roughly separate the *C. psittaci* reference strains used in this study, this method was not accurate enough on its own. In particular, overlap occurred in the Tm for genotypes A, B, E and E/B. Mitchell et al., using dedicated equipment for high-resolution melting curve analysis, also found 21% of their tested positive samples to be untypable owing to inconclusive melting curve data [10].

To the best of our knowledge, the current study characterises the largest series of human-derived psittacosis strains described to date. In the past we had analysed a limited number of 10 human strains originating from psittacosis outbreaks and sporadic cases. As in the present study, genotype A was the most prevalent strain [13]. Recently, an outbreak of psittacosis was described in Sweden. Twelve samples were available for *ompA* genotyping but sequencing of the *ompA* was successful in only four of them [14]. It should be noted that many *C. psittaci* typing methods described previously included hardly any human clinical samples [4,9-12]. They were validated mainly on bird samples or cultured strains. Validation on human samples is needed because the clinical matrix (for example sputum and bronchoalveolar lavage fluids) and the bacterial load can differ substantially between birds and humans.

Many of the genotype A-positive samples were obtained in spring 2011, during a period of increased psittacosis notifications. It seems that this temporary increase was in part due to these genotype A strains. A similar observation was described recently by Rehn et al. who reported a threefold increase in notified psittacosis cases from January to April 2013 [14]. A matched case–control study showed that cases were more likely than controls to have been cleaning bird feeders or were exposed to bird droppings in other ways.

Until now, a source for this temporary increase in the Netherlands of psittacosis notifications in Spring 2011 has not been found. This clearly emphasises the need for genotyping *C. psittaci* strains in human samples prospectively, as it could provide earlier information on probable avian sources, allowing for appropriate outbreak control measures. In the spring of 2013, the same effect was seen, albeit on a smaller scale. Within three months, eight people were infected with *C. psittaci* genotype A. Three of these eight were traced back to a bird show. Bird shows present a zoonotic risk. Visitors can be infected during their visit or afterwards as psittacine and passeriforme birds are often traded and disseminated at such events which are mainly held in the spring. In our dataset, genotype A strains were more prominently found in the first half of the year. Genotype A is most often associated with *Psittaciformes*. This genotype is highly virulent for these birds, which excrete the bacterium in large amounts for long periods of time [5]. This might be one of the reasons of the high virulence in humans. The high proportion of genotype A could possibly be related to more intensive exposure to the main bird source of these genotypes (*Psittaciformes*). These birds are frequently kept as pets inside the house, while birds harbouring the other genotypes more often live outside a person's home. Remarkable is the exposure to *Passeriformes* in patients harbouring genotype A strains, possibly also due to exposure to these birds as pets. Analysis of the fluctuations in genotypes and possible causes should be a subject of ongoing surveillance. Genotype B is mainly associated with *Columbiformes*. In this sample set, we found 14 such strains. Previous research also determined genotype B to be the second most prevalent genotype in humans [13].

Four patients were infected with a new *C. psittaci ompA* VD4 genotype, showing the highest *ompA* VD4 sequence homology with the genotype C GR9 strain. Full-length *ompA* could only be obtained for two of these four cases, underlining the lack of sensitivity of this typing method. The DNA and amino acid sequence of the full-length *ompA*/MOMP of this strain confirmed its unique sequence as we could not find a single match by BLAST. This raises the question of which birds or animals host these strains. Two of the samples were obtained in the context of a previously described outbreak in which a bird source could not be identified [15].

C. abortus was found in one patient. In the Netherlands, *C. abortus* is known to be endemic in sheep and goats [22]. Human *C. abortus* infections have been described. The infection can cause severe septic shock and fetal loss in pregnant women [23-25]. In most cases, testing for psittacosis is only performed when medical history reveals obvious bird contact. Therefore underestimation of these pulmonary *C. abortus* cases is quite likely. The same is true for the *C. caviae*-positive patient. It was only due to the sepsis of unknown origin that an extensive diagnostic investigation was done, revealing psittacosis as the most likely diagnosis based on a positive PCR of the conserved domain of *ompA* (which besides *C. psittaci*, also detects *C. felis*, *C. caviae*, and *C. abortus*). The discrepancy of this positive diagnostic PCR and the negative VD4 PCR led us to consider *C. caviae* as the cause of this infection, which was confirmed by molecular characterisation. Knowledge on the zoonotic potential of *C. caviae* is limited and until now, *C. caviae* has not been linked to fulminant sepsis in humans [26-28].

Human medicine should be aware of the zoonotic potential of *Chlamydia* as there is accumulating evidence that these species are more abundant in animals than previously assumed [6]. These cases also stress the need for close collaboration of physicians, medical microbiologists and public health officials involved in the notification process, as crucial information such as potential animal reservoirs with their associated *Chlamydia* can be missed. The distribution of genotypes in human hosts as found in this study should be carefully considered with respect to geographical location. The local fauna could be relevant when interpreting the results, and extrapolating them to other countries is probably premature. The interaction between human behaviour (urban vs rural) and the present wild bird species (tropical vs non-tropical) could influence local epidemiology. Accidental introduction of invasive, exotic pet bird species or invasion of foreign bird species could create a niche for certain genotypes, including genotypes not present in this study, and lead to unexpected increases in psittacosis cases [29].

In conclusion, this study shows that genotype A and B were the most prevalent causative strains of human psittacosis in the Netherlands. Psittacosis is a clinical syndrome caused by diverse *C. psittaci* genotypes, but typing results suggest that the clinical signs and symptoms are quite similar to closely related zoonotic *C. abortus* and *C. caviae* infections. The discovery of a unique *ompA* sequence points to currently unknown links between human cases and avian or other animal reservoirs.

Accession number

The EMBL accession number for the newly described *Chlamydia psittaci* genotype is HE687292.

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Conflict of interest

None declared.

Authors' contributions

Heddema: idea and design of the study, wrote the manuscript. Bongaerts: performed and analysed most of the typing results, revised the article draft and approved the final manuscript. Van Hannen, Ten Hove and De Wever: submitted archived samples, collected data, revised the article draft and approved the final manuscript. Dijkstra: revealed and analysed demographic and epidemiologic data, revised the article draft and approved the final manuscript. Vanrompay: Cultured and provided all control strains, analysed crucial samples (*C. caviae*), co-designed the study, revised the article draft substantially and approved the final manuscript.

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