MOLECULAR BIOLOGY RESEARCH REGARDING LYOPHILIZED
FRANCISIELLA TULARENSIS REVITALIZED AFTER 40 YEARS

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Summary

Francisella tularensis is a highly contagious Gram-negative bacteria that causes tularemia or “rabbit fever” and it is contagious to humans. There are four known subspecies of Francisella tularensis, two of them are the most studied: A Type strain that is the more virulent (found in North America) and B Type (subspecies holarctica, also referred to as palearctica strain, found in Europe) that is the less virulent. The two other non-virulent subspecies are: mediiasiatica, found in central Asia and novicida, of which is not know very much.

Our researches have been based on the revitalization of lyophilized strains of Francisella tularensis in order to obtain the positive controls required for the in house real time PCR kit for CCHFv and TBEv and Francisella tularensis and Borrelia burgdorferi s.l. agents transmitted by ticks.

Lyophilized strains were rehydrated in nutrient broth, cultured in Francisella tularensis specific medium (CHAB-PACCV) and passed on nutrient medium. Microbiological diagnosis (including optical microscopy) was confirmed by immunoassay (Tularemia biotreat Alert kit, Tetracore) and molecular tests: Real Time PCR with TaqMan Francisella tularensis detection kit, Applied Biosystems for two genes (fopA and tul4) and TicKitqPCR (in house kit) for one target insertion sequence-like element (ISFtu2).

The methods have confirmed the presence of Francisella tularensis strain in revitalized samples after 40 years of storage.

Key words: Francisella tularensis, identification, Real Time PCR, lyophilized, revitalized

Francisella tularensis (F. tularensis) is a highly contagious Gram-negative bacteria that causes tularemia or “rabbit fever” and it is contagious to humans. (1)

Among domestic animals, sheep are the most common host, but clinical infection has also been reported in cats, dogs, pigs, and horses. Tularemia is seen
more often in cats than dogs, and in young versus adult animals. A pet with a mild infection may show no symptoms or may suffer briefly from lack of appetite, lethargy, and a low-grade fever.

More serious infections can cause dehydration, high fever, swollen lymph nodes, eye infections, ulcers in or around the mouth, draining abscesses, jaundice, and an enlarged liver or spleen. (*)

Humans can become infected through several routes, including: tick and deer fly bites, skin contact with infected animals, ingestion of contaminated water, inhalation of contaminated aerosols or agricultural dusts, laboratory exposure.

Symptoms vary depending on the route of infection. Although tularemia can be life-threatening, most infections can be treated successfully with antibiotics.

Steps to prevent tularemia include: use of insect repellent, wearing gloves when handling sick or dead animals, avoiding mowing over dead animals. (**)

*F. tularensis* is classified as a Tier 1 Select Agent by the U.S. government due to its low infectious dose, ease of spread by aerosol, and high virulence.

*F. tularensis* is considered a monomorphic extracellular pathogen, molecular genotyping studies have shown that there are numerous differences between subspecies of *F. tularensis*.

There are four known subspecies of *F. tularensis*, two of them are the most studied: *A Type strain* that is the more virulent (found in North America) and *B Type* (subspecies *holarctica*, also referred to as *palearctica* strain, found in Europe) that is the less virulent. The two other non-virulent subspecies are: *mediasiatica*, found in central Asia and *novicida*, about this subspecies very little is known.

More recent studies showed that in within sub - type/species from genetical point of view there are significant differences particularly in the case of repetitive sequences (SNP-Single Nucleotide Polymorphism) of genome which affect the content of AT/GC of certain target genes involved in virulence. (3)

In addition, *F. tularensis* coexist on cellular and/or tissular lever in the bodies of vector (ticks) with other bacterial as Wolbachia persica, which has led to finding genetic targets very specific to pathogenic subspecies of Francisella. (2)

**Materials and methods**

For present experiments were used lyophilized samples in which *F. tularensis* was supposed to be still present and viable, lyophilized which are over 40 years old. Lyophilizates were rehydrated with liquid nutrient agar, 24 hours at 37°C. Cultivation of samples after rehydration was done on *F. tularensis* specific medium: cysteine heart agar with 9% chocolatized sheep blood, containing polymyxin B, amphotericin B, cyclohexamide, cefepime and vancomycin. (4)

The current methods for detection of *F. tularensis* are: microbiological diagnosis (including optical microscopy), immunoassay and molecular tests included molecular biology techniques based on Real Time PCR (usually in multiplex approach for specific gene targets as *IsFtu2, tul4* and *fopA*).
Immunoassay detection was made with Tularemia BioThreat Alert™ Test Strip who is a hand-held biological agent detection and identification device for *F. tularensis*, the causative agent for tularemia. The Tularemia BioThreat Alert™ Test Strip is a rapid qualitative test who utilizes a combination of monoclonal and polyclonal antibodies to selectively detect the presence of biological threat agents in aqueous samples.

DNA extraction from bacterial culture was performed with PureLink® Genomic DNA Mini Kit, Invitrogen which enables rapid and efficient purification of genomic DNA. Real Time PCR tests was made with TaqMan® *Francisella tularensis* detection kit, Applied Biosystems for two genes (*fopA* and *tul4*) and TicKitqPCR for one target insertion sequence-like element (ISFtu2).

**Results and discussions**

Our 12 lyophilized were analyzed using classical methods - bacterial culture on specific media and modern methods - rapid immunoassay and Real Time PCR.

After rehydration the samples were grown on specific medium, 48-72 h on 37°C (Fig. 1).

![Francisella tularensis colonies on CHAB-PACCV after 72 hours](image)

**Fig. 1.** *Francisella tularensis* colonies on CHAB-PACCV after 72 hours

Rapid immunoassay method revealed possible presence of *F. tularensis* which was confirmed by Real Time PCR.

The test is positive if two colored lines appear. One colored line will appear in the sample window and one in the control window (Fig.2).
Immunoassay test using Tularemia Biothreat Alert™ kit, Tetracore

DNA extraction from bacterial culture was performed with PureLink® Genomic DNA Mini Kit, Invitrogen. The total amount of DNA was quantified with NanoDrop One®, Thermo Scientific.

Gene amplification was performed using a Cepheid® SmartCycler Real Time PCR machine using a commercial kit with TaqMan® Francisella tularensis detection kit, Applied Biosystems for two genes (fopA and tul4) and TicKitqPCR for one target insertion sequence-like element (ISFlu2). As a positive control for genomic DNA we used Amplirun® Francisella tularensis DNA control (LVS strain, 1400copies/µl, CT = 17.05), Vircell (Fig.4).

The reaction outcome was visualized in real time using the software supplied with amplification system by monitoring the fluorescence after each amplification cycle and CT values were registred (Table 1).
Molecular methods had confirmed the presence of specific genes *tul4* (three positive samples), *fopA* (two positive samples) and *ISFtu2* (in all samples) in analysed vials.

We wondered why *ISFtu2* has so small values of all isolates. The possible answer is that *ISFtu2* is an insertion sequence-like element that is in a number of 16 copies per genom of *F. tularensis* (5) and thus is about 16x more sensitive than *fopA* and *tul4* who are one copy per genome.

**Conclusions**

Our research has shown, through microbiological methods, immunoassay and Real Time PCR, that the *Francisella tularensis* strain (classified as level 4 biological agents) can be revitalized after a period of 40 years from liophilized form.