Diagnosis of tularemia using biochemical, immunochemical and molecular methods: a review

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ABSTRACT: Tularemia, an infection caused by the intracellular gram-negative bacterium *Francisella tularensis*, is accompanied by high mortality and occurs throughout the Northern Hemisphere. The causative agent is also considered one of the most important biological warfare agents. As well as its taxonomy and epidemiology, the basic immunochemical, biochemical, and molecular approaches for disease diagnosis are outlined in this review. Aspects of immune responses during tularemia and damage to specific organs are discussed with regards to the predictive value of standard biomarkers. Bacterial burden is also considered as a limitation for polymerase-chain-reaction-based diagnosis.

Keywords: *Francisella tularensis*; tularaemia; zoonosis; pathogenesis; assay

List of abbreviations

ALT = alanine aminotransferase, **AST** = aspartate aminotransferase, **CFU** = colony forming unit, **GLU** = glucose, **IFN** = interferon, **iNOS** = inducible nitric oxide synthase, **LD** = lactate dehydrogenase, **LVS** = live vaccine strain, **PCR** = polymerase chain reaction, **TNF** = tumour necrosis factor

Contents

1. Introduction

Tularemia (or tularaemia in some sources) is a zoonotic infectious disease caused by the gram-negative bacterium *Francisella tularensis*. The causative agent is transmittable to humans and can cause serious disease (Meric et al., 2010; Snowden and Stovall, 2010). Despite the relatively high number of tularemia-positive animals in the environment (Zhang et al., 2006; Treml et al., 2007), the total incidence in humans is on the decrease (Tarnvik and Berglunci, 2003). Game animals, and lagomorphs in particular (such as the European brown hare (*Lepus europaeus*)), are the most important sources of human infection (Hauri et al., 2010; Bandouchova et al., 2011). The clinical manifestation of tularemia is not uniform, and ulceroglandular (approximately 60% incidence), typhoidal (18%), glandular (15%),

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Figure 1. Mouse (BALB/c) suffering from tularemia. Profound apathy is typical for the phase of acute clinical manifestation. However, the symptoms disappear rapidly within approximately two days of the acute phase in surviving animals. The photograph was taken three days after subcutaneous application of the *F. tularensis* live vaccine strain (LVS)

oropharyngeal (7%) and oculoglandular (1%) forms are the most common (Rohrbach et al., 1991). Tularemia-related symptoms are not specific and the disease can be misdiagnosed. The most common symptoms include high fever, dry cough, aching body and ulcers (Ploudre et al., 1992; Haristoy et al., 2003). Lymphadenopathy is also a symptom that should be investigated in suspected cases (Dlugaiczyk et al., 2010). A mouse suffering from tularemia is shown in Figure 1.

Given its high virulence, transmission, mortality and simplicity of cultivation, *F. tularensis* could be used as a biological warfare agent or in terrorist attacks (Kman and Nelson, 2008). Proper treatment procedures are necessary for patients suffering from tularemia. Misinterpretation in the diagnosis is a complication with possible fatal consequences. This may arise owing to the fact that some aspects of tularemia pathogenesis are not well understood. The instrumental diagnosis of tularemia is reviewed in this paper. Special attention is paid to pathological aspects of tularemia from the diagnostic point of view. The scope and limitations of these methods of diagnosis are discussed.

2. Taxonomy

The causative agent of tularemia was first found and isolated in Tulare (California) in 1911 during a local epidemic. The microorganism was thus named *Bacterium tularensis* (McCoy and Chapin, 1912). In the following decades, the taxonomy of *F. tularensis* remained uncertain and it was considered to belong to the *Pasteurella* or *Brucella* genus. A separate genus was proposed at the end of the 1940s (Dorofeev, 1947) and the name *F. tularensis* was established, although the genus name *Tularecella* was also seriously considered (Philip and Owen, 1961). The *Francisella* genus was chosen in honour of Edward Francis, who first recognized the human disease (Gurcan, 2007). Finally, fatty acid and DNA investigation confirmed that the genus *Francisella* has only one species (*tularensis*) and that all subspecies are quite similar despite their different pathogenesis (Broekhuijsen et al., 2003).

Two subtypes of tularemia are known: the most virulent A (also known as nearctica) and the less virulent B (palaeartica). At present, four subspecies have been confirmed, i.e., *tularensis*, *holarctica*, *mediaasiatica* and *novicida* (Mitchell et al., 2010). Pathogenic microorganisms such as *F. philomiragia* and *F. hispaniensis* are considered to be independent of the *F. tularensis* group (Hollis et al., 1989; Huber et al., 2010). Interestingly, a new fish pathogen named *F. noatunensis* was recognized recently (Brevik et al., 2011). The above-mentioned *F. tularensis* subspecies differ in their ability to induce host mortality. The highest mortality (4–24%) is caused by *F. tularensis* subsp. *tularensis*. *F. tularensis* subsp. *holarctica* causes mortality in approximately 7% of cases according to the Centers for Disease Control and Prevention in Atlanta, Georgia (Kugeler et al., 2009). The *F. tularensis* subsp. *holarctica* can be divided into three biovars: erythromycin-sensitive biovar I, erythromycin-resistant biovar II and biovar japonica. All these biovars have an almost four times lower case fatality rate than the *tularensis* subspecies (Olsufjev and Meshcheryakova, 1983; Petersen and Molins, 2010). The remaining two subspecies of *F. tularensis*, i.e., *mediaasiatica* and *novicida*, are less virulent than the other two (Pavlovich et al., 1991). The subspecies *mediaasiatica* can be found in Central Asia, and exhibits low virulence. However, it metabolizes L-citrulline and glycerol like the *tularensis* subspecies and exhibits high genomic similarity to the *tularensis* subtype. The genetic similarity between the *F. tularensis* sub-

Table 1. Summary of data on *F. tularensis* subspecies

F. tularensis subsp.	Virulence	Expected mortality in humans* (%)	Distribution
tularensis	high	up to 24	North America
holarctica	high	7	North America, Europe, Asia
mediaasiatica	medium	not available	Central Asia
novicida	low	not established	North America

*according to Kugeler et al. (2009)

species *mediaasiatica* and *tularensis* was recently investigated using the microarray technique and significant genetic similarities were found between *mediaasiatica* and the *tularensis* strain Schu S4 (Broekhuijsen et al., 2003). The other subspecies, *F. tularensis* subsp. *novicida*, was isolated from water in Utah in the 1950s. It is not fully virulent and proliferates only in immuno-compromised humans and mice (Anwar and Hunt, 2009). Investigation of the *Francisella* genus resulted in the recommendation to consider the subspecies *novicida* as a third species of the Francisella family, in addition to *tularensis* and *philomiragia* (Ellis et al., 2002). The differences between the subspecies are summarized in Table 1.

As seen in the taxonomy of the *F. tularensis* subspecies, tularemia can be caused by an incongruous group of subspecies that differ in their virulence. Fortunately, the application of antibiotics such as streptomycin and gentamicin is commonly effective against all the subspecies. Tetracycline and chloramphenicol can be used as an alternative to the above-mentioned drugs (Enderlin et al., 1994; Urich and Petersen, 2008).

3. Epidemiology of tularemia

The most common natural reservoirs of tularemia are lagomorphs and rodents (Pikula et al., 2002, 2003, 2004). Ticks and mosquitoes are the most important vectors of tularemia (Salinas et al., 2010; Triebenbach et al., 2010). In the South Moravian region of the Czech Republic, for example, the ticks *Dermacentor reticulatus* and *Ixodes ricinus* are positive for *F. tularensis* with an incidence rate of 2.6% and 0.2%, respectively (Hubalek et al., 1996). Similar results were reported from selected regions of the Czech Republic and Austria (Hubalek et al., 1997).

Tularemia is found throughout the Northern Hemisphere, including North America, Europe, and Asia (plus Japan). *F. tularensis* subsp. *tularensis* occurs only in North America. In contrast, the *holarctica* subspecies is found throughout the Northern Hemisphere (Foley and Nieto, 2010). A similar epidemiological situation has been reported in countries with well-developed public health systems. The highest incidence rate of more than one case per 100 000 habitants was reported in regions with dry weather conditions and large forest areas suitable for ticks and rodents in North America (Eisen et al., 2008). Similar data were reported from European areas with deciduous forests (Hubalek et al., 1997). An outbreak of tularemia was confirmed in post-war Kosovo in 1999 and 2000 probably due to contamination of water, food and poor health conditions in general. The misuse of tularemia in Kosovo as a biological warfare agent was also considered. However, this was eventually ruled out (Grunow and Finke, 2002). The presence of tularemia under natural conditions is probably more frequent than estimated based on the epidemiological situation in humans. Approximately 5.0% of rodents in China were positive for tularemia (Zhan et al., 2009). Importantly, ecological conditions and climate can significantly influence the rate of tularemia infection. For example, the rates of tularemia in wild rodents in six Chinese regions ranged from 0 to almost 12% (Zhang et al., 2006).

4. Immunology

F. tularensis is an intracellular pathogen that proliferates in macrophages. After being phagocytosed by macrophages, it is able to escape from the phagosome into the cytosol, in which it proliferates (Akimana et al., 2010). Cytosolic bacteria activate caspase-1 within the inflammasome complex and the infected cell dies in a process called pyroptosis (Henry and Monack, 2007). Restriction of *F. tularensis* growth is based on the activation

of macrophages, which are then able to resolve the infection. Elevated interferon (IFN) g induces NADPH-oxidase and inducible nitric oxide synthase (iNOS), which potentiates the ability of macrophages to kill intracellular pathogens (Edwards et al., 2010). The tumour necrosis factor (TNF) is another cytokine of innate immunity that is required to resolve the disease. TNF-knock-out mice are highly sensitive to tularemia and infection can be fatal even at low bacterial doses (Cowley et al., 2008). T cells are also irreplaceable for the control and resolution of tularemia in the host organism. Moreover, memory T cells are able to enhance the suppression of tularemia when it next invades the host (Salerno-Goncalves et al., 2009). The role of B cells in protection against tularemia is of lower importance. However, B cells are also activated during infection. The role of the lipopolysaccharide on the outer membrane of *F. tularensis* is still under debate (Rahhal et al., 2007). Some authors have suggested that B cells have a role other than the production of antibodies during infection (Elkins et al., 1999).

The expected shifts in cytokines levels are shown in Table 2. IFN g was assessed by Ray et al. (2009). They infected BALB/c mice with the *F. tularensis* live vaccine strain (LVS) at a dose of 10^4 and 10^5 CFU (colony-forming units). After 14 days, IFN g levels in the spleen had increased by 3.7 and 13 times, respectively, or to approximately 0.3 and 0.75 ng/ml, respectively, in absolute values. Kim et al. (2008) investigated the immune response after BALB/c mice exposure to either a fully virulent isolate of *F. tularensis* subsp. *holarctica* or a *F. tularensis* LVS. The applied dose was 2×10^4 CFU, administered intradermally. Among other parameters, they assayed RNA for IFN g and TNF a in the liver and IFN g in the blood. IFN g and TNF a were expressed in the liver throughout the experiment, i.e., seven days, with maximal expression after five days. IFN g in the blood was elevated five days after exposure. However, it decreased rapidly after reaching its peak level. The limitation to the use of cytokines in assays for the assessment of tularemia is the short period during which they are detected at increased levels. Moreover, the time at which they reach their peak corresponds to the clinical manifestation of the disease and onset of antibody production. Antibodies against surface *F. tularensis* antigens appear a few days after the beginning of infection (Pohanka and Skladal, 2007; Pohanka et al., 2007a). The overall level of immunoglobulins is also elevated. The IgM and IgG isotypes reach their maximum after five and 12 days, respectively. On the 12^{th} day of infection, the overall level of antibodies in BALB/c mice is elevated from the initial 6 mg/ml to 15 mg/ml after exposure to 10^4 CFU of *F. tularensis* LVS (Pohanka, 2007). Specific antibodies are elevated to a similar degree to the overall level of antibodies. It should be emphasized that murine animals contain some non-specific antibodies that interact with the *F. tularensis* cell homogenate, and a positive reaction can occur in the absence of tularemia infection. However, specific antibodies are significantly elevated in response to tularemia infection (Pohanka et al., 2007a), and relatively high levels of antibodies may persist. This has also been demonstrated in plasma samples of European brown hares (*Lepus europaeus*) from South Moravia examined for the presence of anti-*F. tularensis* antibodies

Table 2. Selected immunochemical markers of tularemia in BALB/c mice

Marker	Sample	Microorganism and dose	Time post-infection (days)	Marker change	Reference
IFN γ	spleen	*F. tularensis* LVS, *p.o.* 10^4 CFU *F. tularensis* LVS, *p.o.* 10^5 CFU	14	4× 13×	Ray et al. (2009)
RNA for IFN γ	liver	*F. tularensis* subsp. *holarctica*, *i.d.* 2×10^4 CFU	5 (3)	45× (> 5×)	Kim et al. (2008)
RNA for TNF α				55× (15×)	
IFN γ	blood		5	from non significant level by ELISA to 4.5 ng/ml	
Total immunoglobulins	plasma	*F. tualarensis* LVS, *s.c.* 10^4 CFU	12	2.5×	Pohanka (2007)
Anti *F. tularensis* LVS homogenate antibodies			5	2.8	Pohanka et al. (2007a)

LVS = live vaccine strain; *i.d.* = intradermal; *s.c.* = subcutaneously; *p.o.* = per oral

(Pohanka et al., 2007b). The main disadvantage of the immunodiagnosis of tularemia is the possibility of false-positive cases of tularemia in individuals suffering from other bacterial diseases such as brucellosis (Russell et al., 1978). Cross-reactivity can be suppressed using dithiothreitol (Behan and Klein, 1982). Cross-reactivity is also a problem in analytical tools using antibodies to identify *F. tularensis*, and novel assays should be tested for the false-positive assessment of *Brucella* sp. (Pohanka et al., 2008). However, an optimized immunoassay based on immunoglobulin G shows almost 100% sensitivity and specificity for tularemia in human as well as animal sera (Splettstoesser et al., 2010).

5. Biochemical aspects of disease

After invading the body, *F. tularensis* proliferates in macrophages, and can reach multiple organs. Park et al. (2009) illustrated the ability of *F. tularensis* to invade organs. They immunohistochemically proved the presence of *F. tularensis* subsp. *holarctica* in an infected hare (*Lepus brachyurus angustidens*) and confirmed the presence of *F. tularensis* in the skin, spleen, lymph nodes, lungs, adrenal glands, brain and bone marrow with acute necrotizing splenitis, lymphadenitis, hepatitis, pneumonia, myelitis, adrenalitis and encephalitis. The expected biochemical markers corresponded with the damaged organs.

Bandouchova et al. (2009a) extensively investigated biochemical markers in BALB/c mice and European common voles (*Microtus arvalis*). Since common voles are more resistant than mice, the biochemical markers assayed were altered accordingly. It was shown that markers of liver function such as alanine aminotranferase and aspartate amino transferase were significantly elevated in mice from the third day after experimental infection with *F. tularensis* subsp. *holarctica*. Markers indicating nephropathy (urea and creatinine) and muscle disorders (creatinine kinase) had lower sensitivity to tularemia progression. Lactate dehydrogenase, which is commonly considered to be a less specific marker, was found to be the most sensitive marker in tularaemia-infected mice. Tularemia is also accompanied by other metabolic imbalances such as a four-fold decrease in glucose within five days after infection.

The changes in selected markers over the course of infection are shown in Table 3. Levels of inflammatory markers (such as TNF a) change at the same time as those of biochemical markers in BALB/c mice (Kim et al., 2008; Bandouchova et al., 2009a). Measurement of biochemical markers could provide an alternative to immunochemical diagnosis for the acute phase of infection when antibodies have not yet been produced.

6. Perspectives for molecular diagnosis

Bacterial burden can vary significantly according to the seriousness of infection. The highest levels of *F. tularensis* colony forming units can be expected in the blood, spleen and liver. Bandouchova et al. (2009b) followed the *F. tularensis* subsp. *holarctica* burden in common voles and BALB/c laboratory mice, and found up to 10^8 CFU/g in the blood, spleen and liver of mice that did not survive the infection. The lungs and kidney had a significantly lower bacterial burden. A similar situation was presented by Troyer et al. (2009) for BALB/c mice and *F. tularensis* LVS. However, in this case mice had a higher level of *F. tularensis* cells in the lungs. The bacterial burden in organs peaks approximately five days post-infection. After that, it decreases and sur-

Table 3. Relative level (%) of biochemical markers in plasma of tularemia-infected BALB/c mice compared to healthy individuals*

Days after infection	1	2	3	4	5
ALT	115	138	1340	1370	1725
AST	59	93	593	550	1051
LD	97	194	430	459	965
GLU	48	64	46	33	25

Mice were infected with 160 CFU

*data recalculated from Bandouchova et al. (2009a)

Table 4. Sequence of primers for the fopA gene*

Sequence	Nucleotide position
5´- GGCAAATCTAGCAGGTCA-3´	824–841
5´-GCTGTAGTCGCACCATTATC-3	1052–1073

*according to Fujita et al. (2006)

viving animals become *F. tularensis*-free 20 days post-infection at the latest (Ray et al., 2009).

The presence of high bacterial levels in infected individuals is a good prerequisite for direct assessment. The polymerase chain reaction (PCR) is one of the most exact and reliable methods for identifying the pathogen. The genes *tul4* and *fopA*, coding for the outer membrane 17 and 43 kDa proteins, are common markers for *F. tularensis* (Ellis et al., 2002; Hepburn and Simpson, 2008). The sequence of primers for the *fopA* gene according to Fujita et al. (2006) is shown in Table 4. Amplification of genes for the chaperones *cpn10* and *cpn60* and their respective 16S rRNA can also be used to identify *F. tularensis* (Ericsson et al., 1997; Maurin et al., 2010). Diagnosis of tularemia by PCR is not limited to serum or blood, as other matrices such as lymph nodes are also suitable. Lubbert et al. (2009) diagnosed tularemia in a 20-year-old woman five months after she was bitten by an infected tick. The woman suffered from lymphadenopathy and had increased levels of protein C. Surprisingly, PCR analysis of the serum and lymph nodes was negative. However, serological tests confirmed tularemia. This case report demonstrates the limitation of PCR as the pathogens are eliminated. On the other hand, PCR is uniquely able to identify isolates and could be used to confirm tularemia in hard-to-diagnose oculoglandular cases (Kantardjiev et al., 2007) or environmental samples (Ozdemir et al., 2007). It can also be used successfully for tularemia diagnosis during the acute phase of the disease (Chitadze et al., 2009). PCR is also suitable for precise identification of the *F. tularensis* subspecies in infected individuals, which is not possible by standard serological diagnosis (Tarnvik and Chu, 2007).

7. Conclusions

A quick and precise diagnosis of tularemia is necessary for appropriate treatment. Apart from standard serological diagnosis, biochemical and molecular tests are suitable for diagnostic purposes. The present review has summarized the basic markers that can be examined in humans or animals with suspected tularemia. Each method has its advantages and limitations and should be selected according to the condition of the patient and the overall anamnesis.

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