

Current and emerging assays for *Francisella tularensis* detection: a review

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ABSTRACT: This paper presents an overview of methods for detection and identification of the pathogenic bacterium *Francisella tularensis* such as cultivation tests, enzyme-linked immunosorbent assays, flow cytometry, polymerase chain reaction, immunosensor, microarray, mass spectrometry, and chromatography. Included references are chosen according to their practical importance or perspectives for the future.

Keywords: tularaemia; zoonosis; diagnostic techniques; identification

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1. Introduction

Francisella tularensis, the causative agent of tularaemia, represents not only a severe human zoonotic pathogen but also a potential biological warfare agent. *F. tularensis* is a relatively small, non-motile and non-spore forming, gram-negative coccobacillus. Small mammalian species represent the natural reservoir of tularaemia. Natural foci of tularaemia in Central Europe were thoroughly investigated (Pikula et al., 2002, 2003). Infection may be spread by ticks, flies, mosquitoes, and con-

taminated aerosol particles; however other ways, such as presented in one case report describing tularaemia spreading by means of infected dog's fur (Siret et al., 2006), and contacts with infected individuals could be risky. *F. tularensis* growth in protozoa was also reported (Abd et al., 2003).

F. tularensis was formerly divided into two subtypes: A, and B. Currently, the division into four subspecies has a wide consensus. The subspecies *tularensis* (also known as subtype A) naturally occurring only in North America but one isolate in the continental Europe (Gurycova, 1998) is the

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most virulent one. This subspecies was described historically as metabolizing glycerol and L-citrulline in comparison with the subspecies *holarctica* (Olsufjev et al., 1959). At present, the analysis of 16S rRNA (Forsman et al., 1990) seems to be more relevant to distinguish the above subspecies. The well-known and very virulent strain SCHU belongs to the subspecies *tularensis*. The subspecies *holarctica* (formerly referred to as subtype B) is the second one in the virulence scale having ten times lower virulence than the subspecies *tularensis* (Olsufjev and Meshcheryakova, 1983). It occurs frequently under favourable conditions of endemic areas (Pikula et al., 2003, 2004a) over the Northern Hemisphere. Reservoir hosts of *F. tularensis* subsp. *holarctica* are rodents and lagomorphs (Pikula et al., 2002, 2004b; Zhang et al., 2006; Treml et al., 2007) and ticks play a role as vectors of infection (Hubalek et al., 1998). The subspecies *mediaasiatica* shows only low virulence, despite its great similarity to the SCHU strain of *tularensis* subspecies recently recognized (Broekhuijsen et al., 2003). The last subspecies *novicida* was added to *F. tularensis* species as the last one in the late 1980s (Hollis et al., 1989). The subspecies *novicida* is of minimum importance in comparison with the others due to low ability of spread together with its low virulence.

Some reliable methods and approaches for *F. tularensis* detection have already been reviewed (Spletstoesser et al., 2005) and compared (Porsch-Ozcurumez et al., 2004). However, there still remains an interest in the construction of advanced devices. Routinely available or promising methods for future *F. tularensis* assays are presented in this paper, including, for example, detection based on whole cells and methods convenient for serological diagnosis. Many other analytical approaches for a broad group of analytes exist. However, their use in *F. tularensis* assays has not been reported yet. Therefore, these methods are not included in this review.

2. Cultivation tests

Cultivation tests belong to traditional procedures for the identification of microorganisms and *F. tularensis* is not an exception. However, unambiguous identification only by cultivation is not possible. Glutaminase, asparaginase, and citrulline ureidase activities are typical markers of the activity of pathogens including *F. tularensis*. Therefore, cultivation

media for *F. tularensis* should contain these supplements (Fleming and Foshay, 1953). Adenylic acid, adenosine diphosphate or triphosphate could be considered as the critical growth factors (Mager et al., 1954). Many cultivation protocols are based on blood enrichment agars such as presented by Gaspar et al. (1961). Another work found liquid media supplemented with sodium thioglycolate, blood, and glucose useful (Lukas, 1962). Good results can also be obtained using blood-free media based on, for example, glucose, thiamine, cysteine and histidine effectively supporting the growth of *F. tularensis* SCHU-S4 strain (Tresselt and Ward, 1964). Chocolate agar was developed by McLeod in 1927. It is based on yeast extracts and peptones and was demonstrated useful for a large group of fastidious microorganisms including *F. tularensis* as reported in some cultivation protocols (Berdal and Soderlund, 1977). Estimation of the metabolic activity of *F. tularensis* is an important way for subspecies differentiation. Citrulline ureidase activity can be estimated by supplementation of L-citrulline to the cultivation medium; in a similar way, glycerol fermentation can be estimated as presented by Sandstrom et al. (1992). Skilled laboratory personnel can recognize *F. tularensis* forming colonies after one or two days of cultivation in humid atmosphere and at a temperature adjusted to 37°C. *F. tularensis* colonies are characteristically opalescent in white light.

3. Classical immuno-assays

Immuno-assays represent a widely available approach for *F. tularensis* bacterial cell detection as well as serological diagnosis. Polyclonal or monoclonal antibodies are very useful for the recognition of whole cells or their parts using the same preparation protocols (Fulop et al., 1991; Hotta et al., 2007). Antigenically related *Brucella* sp. may cause false positive reactions. Therefore, the antibodies should be examined for cross-reactivity (Behan and Klein, 1982; Nielsen et al., 2004).

Chromogens such as fluorescein or enzymes such as horseradish peroxidase or alkaline phosphatase are the most frequently used labels since labelling by radioisotopes is limited due to health risks resulting in legislative obstacles. Agglutination tests and the enzyme-linked immunosorbent assay (ELISA), in particular, are probably the most frequently employed diagnostic tests in clinical laboratories.

Several publications describe assays performed with fluorescein labelled antibodies. The earliest one was presented in the 1940s (Coons et al., 1941). The presence of *F. tularensis* in rabbit's tissues was detected by fluorescein labelled antibodies (McCahan et al., 1962). An assay based on fluorescent antibodies was also performed to examine hare samples; 109 tularaemia cases were positively diagnosed from a total of 1 500 *post mortem* examined hares (Morner et al., 1988b). Another assay employing antibodies covalently bound with fluorescein was designed for detection of airborne *F. tularensis*. Living cells were captured on slides in a cascade of impactors and incubated further with antibodies resulting in the limit of detection as low as 10 cells per glass slide (Jaeger et al., 1961). Surface lipopolysaccharide antigens of SCHU and LVS strains were examined using fluorescein labelled IgM monoclonal antibodies in the slide agglutination test (Narayanan et al., 1993). Fluorescent antibodies were also used for detection of antibodies against *F. tularensis* in 91 privately owned cats (Magnarelli et al., 2007). Volunteers in one study were infected by *F. tularensis* and production of antibodies was assayed by outer membrane lipopolysaccharide based ELISA during the time frame of up to 2.5 years. Results were compared with a tube agglutination test and approximately ten times higher sensitivity of ELISA was proved (Carlsson et al., 1979). *F. tularensis* cells in tissues of infected animals or in liquid media were determined by ELISA with the limit of detection slightly above 10^4 CFU/ml (Meshcheriakova et al., 1988). Microagglutination and ELISA were used for diagnosis during a tularaemia outbreak in central Norway in 1984 (Bevanger et al., 1988). The presence of antibodies against *F. tularensis* outer membrane protein and especially 43 kDa protein was studied in 57 patients suffering from tularaemia. Competition between human antibodies in the patient serum and the rabbit serum for 43 kDa antigen from the outer membrane was examined by ELISA and all 23 patients were positive in the presence of antibodies against 43 kDa protein (Bevanger et al., 1989). High agreement between titres obtained from sera and lung extracts was found in a study with experimentally infected beavers and goshawks, nevertheless, the titres obtained from sera were approximately twice higher (Morner et al., 1988a). Slide agglutination was compared with ELISA; a total of 119 rabbit serum samples were assayed both by agglutination and by ELISA

resulting in seven positives by agglutination and 19 by ELISA, confirming ELISA to be more sensitive (Lepitzki et al., 1990). Similar results were obtained by Bevanger et al. (1994). They compared individuals eight years after tularaemia infection by microagglutination and ELISA. The best results were obtained by ELISA recognizing 95% of the individuals as anti *F. tularensis* antibodies positive in contrast to microagglutination that proved only in 64% as positive. In one epidemiological study, partially purified lipopolysaccharide from *F. tularensis* was successfully used for the serological diagnosis of human serum samples. A total of 1 253 serum samples were subsequently examined with 104 out of them being found tularaemia positive (Schmitt et al., 2005). A large testing of 6 632 serum samples was performed to compare ELISA, western blot, flow cytometry and immunofluorescence in which western blot and flow cytometry provided slightly better results. However, some other technical parameters such as the amount of samples necessary for tests confirmed ELISA as a favourable choice (Porsch-Ozcurumez et al., 2004).

Flow cytometry is a very sophisticated instrumental method that can provide unique information especially in the way of markers on activated lymphocytes and some other immunological studies. This type of analysis was performed in some clinical studies (Sumida et al., 1992; Porsch-Ozcurumez et al., 2004) and it was used for microbiological and immunological research purposes (Abd et al., 2003; Chen et al., 2005). However, the detection of *F. tularensis* or a valid diagnostic system based on flow cytometry still remain a challenge.

4. Polymerase chain reaction

Polymerase chain reaction (PCR) is the most commonly used nucleic acid amplification technique for the detection, identification, and typing of microorganisms. The PCR usually amplifies DNA; however, RNA could be amplified in the case of reverse transcription (RT) – PCR. Higuchi et al. (1992, 1993) improved the PCR by a system of real-time PCR that detects labelled amplification products as they accumulate. Multiplex PCR uses one or more primer sets to potentially amplify multiple templates within a single reaction (Chamberlain et al., 1988; Elnifro et al., 2000).

The *tul4* and *fopA* genes encoding 17 and/or 43 kDa protein from the *F. tularensis* outer membrane are

typical amplification targets (Ellis et al., 2002). New primers were designed for PCR detection purposes and a proper assay was introduced (Sjostedt et al., 1990; Fulop et al., 1996). Heat shock protein chaperones 10 and 60 are specific markers for the *F. tularensis* assay, so the amplification of *cpn10* and *cpn60* genes can be useful for the identification of *F. tularensis* (Ericsson et al., 1997).

Not only DNA studies but also the analysis of 16S rRNA is an important tool in identification techniques. It was proved to be useful for the determination of evolutionary relationships among different bacteria (Woese, 1987; Posthaus et al., 1998; Sacchi et al., 2002; Bavykin et al., 2004; Gee et al., 2004) and, consequently, for the discrimination of *tularensis* and *holarctica* subspecies (Forsman et al., 1990, 1994; Garcia Del Blanco et al., 2002).

PCR based on recognizing *tul4* gene in spiked blood samples and experimentally infected mice was introduced in the early 1990s (Long et al., 1993). It was found feasible for diagnosis of tularaemia in a murine model even one day after inoculation with 15 CFU of *F. tularensis* (Junhui et al., 1996). Multiplex PCR targeting *tul4* gene and 16S rRNA was successfully used in human cases of tularaemia (Sjostedt et al., 1997). PCR was also tested under field conditions and approved of as useful (Berdal et al., 2000).

Fujita et al. (2006) designed real-time PCR for a highly sensitive and specific assay of *F. tularensis* through the *fopA* gene. A linear response was observed using *F. tularensis* genomic DNAs from 20 fg to 2 ng. Emanuel et al. (2003) compared two PCR thermocyclers for *tul4* and *fopA* genes. The samples were analyzed using the laboratory-based Applied Biosystems International 7900 (ABI 7900) and the field device Smiths Detection-Edgewood BioSeeq. The limit of detection for both the *tul4* assay and the *fopA* assay using ABI 7900 was 50 fg, which is approximately 25 genome equivalents of *F. tularensis*. In further testing, the limit of detection was found higher for the BioSeeq platform. The *tul4* assay provided the limit of detection 200 fg in comparison with 300 fg when *fopA* was employed. In another study, *F. tularensis* was simultaneously detected together with *Bacillus anthracis* and *Yersinia pestis*. All the above-mentioned agents were identified and no cross-reactivity from 39 negatives used as controls was observed. Approximately 10 fg of DNA was sufficient for positive detection of any of the above agents (Skottman et al., 2007).

PCR and/or real-time PCR were employed in epidemiological studies as recently reported by several investigators (Kantardjiev et al., 2007; Matz-Rensing et al., 2007; Muller et al., 2007).

5. Proteomics

It is clear that not only (deoxy)ribonucleic acids harbour specific information that can be used for detection or typing purposes. The diversity of biological organisms is reflected also on the level of proteins/peptides and other biomolecules. However, the lack of methods comparable to polymerase chain reaction makes the analysis of such molecules more difficult. On the other hand, the recent progress in instrumentation has led to the development of high resolution separation techniques such as 2-dimensional gel electrophoresis and sensitive detectors such as mass spectrometers. These techniques can analyze proteins from bacterial samples with a possibility of using such results for discrimination between the organisms even on the subspecies level. Two-dimensional electrophoresis has the power to separate many bacterial proteins in one single map while the position of each spot is characteristic. The protein can then be identified by means of mass spectrometry. Such an approach was used for comparison between the three subspecies of *F. tularensis* and there were several protein spots characteristic of each subspecies (Hubalek et al., 2004). In combination with statistical methods such as principal component analysis, this method can be used for the identification of subspecies. These results are reliable; however, the drawback of such an approach is the speed of analysis. It takes at least a day of laboratory work to prepare the well resolved map.

The advantage of well-characterized protein spots on the gel led to the investigation of immunogenicity of *Francisella* proteins in the host (Hubalek et al. 2004). The resulting list of proteins points out to potential targets of immunoassays or subunit vaccines.

The mass spectrometry itself has been shown to be a powerful technique in the identification of bacteria (Fenselau and Demirev, 2001). The MALDI TOF measurement of surface biomolecules, mostly proteins, coming from whole bacterial cultures spotted onto the target is one of the most popular methods. The resulted fingerprints of biomolecules have been shown to be able to discriminate into the

subspecies of bacteria. There are variants of such an approach using the extraction of surface molecules instead of the analysis of whole bacteria that can result in a similar outcome. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry as a modification of the same principle of ionization as MALDI was designed for the discrimination of *F. tularensis* subspecies and differential protein profiles were obtained for each subspecies (Lundquist et al., 2005).

The method of MALDI measurement has the advantage of being very simple and robust; however, it requires quite pure cultures, since it is based on unidentified peaks. The alternative of this method is to use tandem mass spectrometry that leads to the fragmentation of selected peptide ions. Such information can be very specific and as it was demonstrated also sensitive. The discrimination of the subspecies *holarctica* vs. *tularensis* based on sequence specific peptides from each subspecies can be achieved on the level of 100 ng of proteins from the whole cell lysate (the authors' unpublished results).

6. Biosensors

Biosensors are analytical devices incorporating a biological sensing – biorecognition – element providing specificity together with a physical sensor – physicochemical transducer. Specific interactions in the biorecognition element are widely used for the detection of microorganisms including *F. tularensis*; the most frequently used are interactions between antigen and antibody, however, biosensors based on DNA are possible. The impact of biosensors for the biological warfare agent detection has recently been reviewed (Pohanka et al., 2007a).

Immunosensors were found useful in several studies. Piezoelectric biosensors could favourably be used in measuring systems where mass interaction between the biorecognition component and the analyte occurs. Piezoelectric immunosensors including *F. tularensis* disrupted cells as a biorecognition element were designed for the detection of *F. tularensis* in the form of immunoprecipitate with monoclonal IgM (Pohanka and Skladal, 2005) and in another way designed application, piezoelectric immunosensors with covalently immobilized antibodies seem to be approachable for the label-free detection of *F. tularensis* (Pohanka and

Skladal, 2007a). Piezoelectric immunosensors based on immobilized antigens were also employed for an assay of specific antibodies. The impact of tularaemia infection was summarized by this type biosensor in the murine BALB/c model; the progress of tularaemia caused by *F. tularensis* LVS infection was recognizable even one day after inoculation (Pohanka et al., 2007b); in a further study, a piezoelectric immunosensor was employed for the serological diagnosis of *F. tularensis holarctica* infected European brown hares and results were compared with the agglutination test (Pohanka et al., 2007c). A similar biosensor working in full flow-through arrangement was designed for the rapid characterization of monoclonal antibodies and allowed to estimate the kinetic rate constants and equilibrium constant of the interaction *F. tularensis* antigen – monoclonal antibody (Pohanka et al., 2007d). The last two studies are very interesting due to a label-free measuring order. An amperometric immunosensor including specific antibodies bound through protein A was used for the detection of *F. tularensis* viable cells; secondary antibodies covalently labelled with horseradish peroxidase enabled the sandwich complex formation resulting in a very low limit of detection, i.e. 10² CFU/ml (Skladal et al., 2005). Furthermore, the amperometric biosensor harbouring *F. tularensis* antigen as a biorecognition component was found useful for the serological diagnosis of tularaemia infection; however, the use of HRP labelled secondary antibodies was unavoidable in the assay format (Pohanka and Skladal, 2007b).

Some investigators consider microarray technologies as biosensors, hence these devices are included in this chapter. Microarray techniques are intriguing ones using a multichannel ordering. Gene microarrays and antibody microarray are very useful tools of modern research. An analysis based on antibody microarray is quite similar to classical immunological methods. On the other side, gene microarrays (some authors call them gene chips) are devices enabling to recognize specific sequences through collected nucleic acids spots immobilized on an optical slide. Antibody microarray based on lipopolysaccharides immobilized on the nitrocellulose-coated glass slides was presented for antibodies against *Escherichia coli*, *Salmonella typhimurium*, and *F. tularensis* with the limit of detection of 10 ng/ml (Thirumalapura et al., 2005).

Gene microarrays were proved to be a useful tool of discrimination of *F. tularensis* strains (Broekhuijsen et al., 2003) as well as a detection tool based on 16 S rRNA (Ramachandran et al., 2004).

7. Other instrumental techniques

Many other analytical methods could be designed for *F. tularensis* whole cell detection or serological diagnosis. Some innovative works have appeared. Nevertheless, many instrumental methods seem to be a challenge for *F. tularensis* assay.

Even chromatography techniques such as rapid immunochromatography were described (Berdal et al., 2000). Commercial BTA (BioThreat Alert) test strips produced by Alexeter Technologies (Chicago, Illinois, USA) are designed for multiple biological warfare agents including *F. tularensis* detection. The strips are based on the affine chromatography principle when colloidal gold-labelled antibodies adsorbed on the strip surface possess proper recognition capability. Screening of markers could be another effective way because lipids from the outer membrane are feasible for detection by gas chromatography (Abel et al., 1963) and complete analysis of fatty acids was made by the tandem of gas chromatography – mass spectrometry (Nichols et al., 1985). In another study, fast protein liquid chromatography was designed for the isolation of cell wall proteins (Belyi et al., 1995). Solid phase extraction (SPE) based on protein L and protein G affinity to serum antibodies in mice suffering from tularaemia was performed for the estimation of immunoglobulin levels (Pohanka, 2007). However, this model was not aimed at tularaemia specific antibodies.

8. Conclusions

The present work summarizes current strategies and methods suitable for the detection and/or identification of the pathogenic bacterium *F. tularensis* as well as proper serological diagnosis. Described methods are logically divided into several chapters according to important analytical features. The authors tried to introduce and order the approachable field of analytical methods from classical cultivation tests to highly sophisticated instrumental methods. Although analytical possibilities are extensive, the authors believe that main tendencies were mapped.

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