CHAPTER 3.1.22.

TULAREMIA

SUMMARY

Description of the disease: *Tularemia is a zoonosis caused by* Francisella tularensis*. The causative bacterium is a Gram-negative coccoid rod, 0.2–0.5 µm × 0.7–1.0 µm, non-motile and non-spore-forming organism that is an obligate aerobe with optimal growth at 37°C. It is oxidase-negative, weakly catalase-positive, and cysteine is required for growth. Tularemia is primarily a disease of the orders Lagomorpha and Rodentia, but a wide range of other mammals and several species of birds have also been reported to be infected. Haematophagous arthropods have a substantial role both in the maintenance of* F. tularensis *in nature and in disease transmission.*

The disease is characterised by fever, depression and often septicaemia. In humans, there may be ulcers or abscesses at the site of inoculation (*this is rarely seen in animals*)*, and swelling of the regional lymph nodes. On post-mortem examination, lesions may include caseous necrosis of lymph nodes and multiple greyish-white foci of necrosis in the spleen, liver, lungs, pericardium, kidneys and other organs. The spleen is usually enlarged in septicaemic cases.*

It is important to understand that there is a high risk of direct infection of humans by direct contact with this organism. Special precautions, including the wearing of gloves, masks and eyeshields, are therefore recommended when handling infective materials. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (*see Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities)*.*

Identification of the agent: *Polymerase chain reaction is a safe and convenient way for the detection and identification of* F. tularensis *in clinical samples. The bacterium can be demonstrated in impression smears or in fixed specimens of organs by the fluorescent antibody test or immunohistochemistry. With Gram staining, the bacteria appear as very small punctiform Gram-negative rods, often difficult to distinguish as bacteria.*

The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and Chapin medium, or Modified Thayer-Martin agar. In certain cases selective medium or mouse inoculation is needed to aid successful isolation. The colonies are small, round and transparent, and do not appear before 48 hours incubation at 37°C. If transportation is necessary, samples should be inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or on dry ice if transit is likely to be prolonged.

Serological tests: *Serological tests are useful diagnostic aids in human infection, but are of limited value in the more susceptible animal species that usually die before developing antibodies. Epidemiological surveys can be conducted in domestic animals, in relatively resistant species that survive the infection, such as sheep, cattle, pigs, dogs, cats, wild ungulates, foxes and wild boars as these species develop antibodies. Relatively resistant species of rodents and lagomorphs* (*e.g. European brown hare in Central Europe*) *can also be used in epidemiological surveys.*

Requirements for vaccines: *The attenuated* F. holarctica *live vaccine strain* (*LVS, NCTC 10857*) *has been used for decades as a tularemia vaccine, especially in laboratory workers handling large volumes of* F. tularensis *cultures. This vaccine is no longer used because of its overall limited efficacy and concern about reversion to virulence, although a derivative of LVS is still used to immunise people in endemic regions of Russia. Novel vaccines against tularemia are under development but not yet licensed for human or animal use.*

A. INTRODUCTION

Tularemia is a zoonosis caused by *Francisella tularensis.* It occurs naturally in lagomorphs (rabbits and hares) and rodents, especially microtine rodents such as voles, vole rats and muskrats, as well as in beavers. In addition, a wide variety of other mammals, birds, amphibians and invertebrates have been reported to be infected (Gyuranecz, 2012; Morner & Addison, 2001). Tularemia occurs endemically in the northern hemisphere. The disease can occur as epizootic outbreaks in many countries in North America and Europe, while it occurs only as sporadic cases in some other countries in Europe and Asia. It is rarely reported from the tropics or the southern hemisphere.

The clinically most relevant two types of *F. tularensis* are recognised on the basis of culture characteristics, epidemiology, and virulence. *Francisella tularensis* subsp. *tularensis* (Type A) is mainly associated with lagomorphs in North America. It is primarily transmitted by ticks or biting flies, or by direct contact with infected lagomorphs. It is highly virulent for humans and domestic rabbits, and most isolates ferment glycerol. *Francisella tularensis* subsp. *holarctica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in North America, and in lagomorphs (hares) and rodents in Eurasia. It is primarily transmitted by direct contact or by arthropods (primarily ticks and mosquitoes), but may be transmitted through inhalation or through infected water or food. It is less virulent for humans and domestic rabbits, and does not ferment glycerol (Ellis *et al.*, 2002; Keim *et al.*, 2007; Morner & Addison, 2001).

In sensitive animals, clinical signs of severe depression are followed by a fatal septicaemia. The course of the disease is approximately 2–10 days in susceptible species, and animals are usually dead when presented for diagnosis. Most domestic species do not usually manifest signs of tularemia infection, but they do develop specific antibodies to the organism following infection. Outbreaks with high mortality caused by the Type A organism have occurred in sheep (Morner & Addison, 2001). Among domestic animals, the cat has been reported to be able to act as a carrier of the bacterium and the disease is occasionally spread from cats to humans.

At necropsy, animals that have died from acute tularemia are usually in good body condition. There are signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen. In addition, the spleen is usually enlarged. Necrotic foci vary in size, and in some cases may be barely visible to the naked eye. The lungs are usually congested and oedematous, and there may be areas of consolidation and fibrinous pneumonia or pleuritis. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are often present in one or more lymph node(s). The lymph nodes that are most often affected are those in the abdominal and pleural cavities and lymph nodes draining the extremities. In less sensitive species, the macroscopic picture can resemble that of tuberculosis with subacute or chronic granulomas in the lungs, pericardium, kidneys, spleen and liver. Macrophages are the dominant constituent cell type in the granulomas, but other cells including lymphocytes, heterophil granulocytes, multinucleated giant cells and fibrocytes are also found occasionally. Focal or multifocal necrosis is often observed in the centre of these lesions (Gyuranecz, 2012; Gyuranecz *et al.*, 2010).

There is a high risk of human infection from *F. tularensis*, as the infective dose is extremely low and infected animals excrete bacteria in urine and faeces. Infection can occur by simple contact. Suitable precautions, such as the wearing of gloves, masks and eyeshields during any manipulation of pathological specimens or cultures, must be taken in order to avoid human infection. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Countries lacking access to a specialised national or regional laboratory should send specimens to the OIE Reference Laboratory. Experimentally inoculated animals and their excreta are especially hazardous to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of tularemia and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[1]						
Bacterial isolation	–	–	–	+++	–	–
Antigen detection	–	–	–	+++	–	–
Real-time PCR	+++	–	–	+++	+++	–
Detection of immune response						
SAT	+++	+++	+++	++	+++	_
TAT	++	+++	++	+++	+++	_
MAT	++	+++	++	+++	+++	_
ELISA	++	+++	++	++	+++	_

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; SAT = slide agglutination test; TAT = tube agglutination test;
MAT = microagglutination test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections. As the post-mortem picture is variable, diagnosis is sometimes difficult and immunological or immunohistochemical methods are preferable, although reagents may be difficult to obtain. It can sometimes be recommended, therefore, that fixed specimens be analysed at laboratories equipped with proper reagents or methods. It can also be identified by culture. However, *F. tularensis* may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. In these cases selective culture media or animal inoculation can be used. Polymerase chain reaction (PCR) is a safe and convenient way for the detection and identification of *F. tularensis* in clinical samples.

1.1. Smear preparations

Smear preparations are made on microscope slides as impression smears of organs such as the liver, spleen, bone marrow, kidney, lung or blood. The bacteria are abundant in such smears, but may be overlooked because of their very small size (0.2–0.7 µm). The bacteria can be demonstrated by direct or indirect fluorescent antibody staining. This is a safe, rapid and specific diagnostic tool (Karlsson *et al*., 1970; Morner, 1981).

Gram staining of smears reveals a scattering of small, punctiform Gram-negative bacteria near the limit of visibility. The use of oil microscopy increases the visibility of the bacteria. The bacteria may be difficult to distinguish from precipitates of stain.

1 A combination of agent identification methods applied on the same clinical sample is recommended.

1.2. Histological sections

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (Morner, 1981) or immunohistochemistry (Gyuranecz *et al.,* 2010). The tests are performed on organ samples fixed in neutral buffered formalin and paraffin embedded. Slides are treated with a primer rabbit or mouse anti-tularemia serum, washed and thereafter treated with a seconder fluorescein-isothiocyanate-conjugated or a horseradish peroxidase-labelled anti-rabbit or anti-mouse serum. The samples are examined under a fluorescence or light microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

1.3. Culture

Francisella tularensis will not grow on ordinary media, although an occasional strain can sometimes, on initial isolation, grow on blood agar. Incubation is at 37°C in ambient air or in 5% CO_2. Heart blood, liver, spleen, bone marrow or tularemic granulomas (from lungs, pericardium, kidney, liver, spleen, etc.) from moribund animals should be used for culture. It is necessary to use special culture media, such as the media given below.

1.3.1. Francis medium

Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defribrinated rabbit, horse or human blood.

1.3.2. McCoy and Chapin medium

This consists of 60 g egg yolk and 40 ml normal saline solution, carefully mixed and coagulated by heating to 75°C.

1.3.3. Modified Thayer–Martin agar

Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and IsoVitaleX.

Media can be stored for up to 8–10 days at 4°C. Colonies that form on McCoy and Chapin medium are small, prominent, round and transparent. A more abundant growth is obtained on Francis medium and modified Thayer–Martin agar, with confluent colonies that have a milky appearance and a mucoid consistency. On either medium, colonies do not appear until after 48 hours' incubation at 37°C.

The following selective medium can be used in addition to the non-selective media: *Cystine heart agar* supplemented with 7.5 mg colistin, 2.5 mg amphotericin, 0.5 mg lincomycin, 4 mg trimethoprim and 10 mg ampicillin per litre (WHO, 2007).

Differential criteria for the identification of *F. tularensis* include absence of growth on ordinary media, distinctive cellular morphology, and specific fluorescent antibody and slide agglutination reactions. The bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures.

Francisella tularensis can be identified in stained smears, by agglutination with tularemia hyperimmune antiserum, or by animal inoculation. In areas of North America where both types of *F. tularensis* may occur, Type A may be distinguished from Type B by the fact that most Type A ferment glycerol.

The bacteria can also be identified by PCR.

1.4. Animal inoculation

Animal inoculation is not recommended because of welfare concerns. It should only be undertaken when isolation in a laboratory animal is considered unavoidable, and where proper biosafety facilities and cages are available (see chapter 1.1.4).

Tularemic granuloma or a piece of septicaemic organ (e.g. spleen, liver) is excised and about 1 g of tissue sample is homogenised and suspended in 2 ml of normal saline. A laboratory animal (preferably mouse) is injected subcutaneously with 0.5 ml of suspension. Diseased animals will die after 2–10 days of injection. Heart blood and bone-marrow samples are inoculated on culture media on the day of the laboratory animal's death (Gyuranecz *et al.*, 2009).

1.5. Molecular techniques

PCR-based assays are useful for the detection of *F. tularensis* DNA directly from human, animal and environment samples. They can also determine the *F. tularensis* subspecies or genotypes, either from isolated strains or directly from clinical samples.

Methods for detection of *F. tularensis* DNA that have been used include classical PCR (Barns *et al.*, 2005; Sjöstedt *et al.*, 1997) and real-time PCR systems (Versage *et al.*, 2003). It is to be noted that PCR testing of ticks must use specific gene targets or PCR fragment sequencing to differentiate *F. tularensis* from *Francisella*-like endosymbionts (Kreizinger *et al.*, 2013; Kugeler *et al.*, 2005; Michelet *et al.*, 2013).

A conventional PCR system targeting the 16S rRNA gene followed by PCR product sequencing was designed to detect *F. tularensis* and *F. philomiragia* as well as the *Francisella*-like tick endosymbionts by Barns *et al.* (2005) with the following primer pair: Fr153F0.1: 5'-GCC-CAT-TTG-AGG-GGG-ATA-CC-3' and Fr1281R0.1: 5'-GGA-CTA-AGA-GTA-CCT-TTT-TGA-GT-3'. Cycling conditions consist of initial denaturation for 10 minutes at 95°C followed by 30 amplification cycles of denaturation for 30 seconds at 94°C, primer annealing at 60°C for 1 minute and extension at 72°C for 1 minute.

A real-time PCR system targeting the *tul4* gene was designed by Versage *et al.* (2003) to specifically detect only *F. tularensis* with the following primers and probe: Tul4F: 5'-ATT-ACA-ATG-GCA-GGC-TCC-AGA-3', Tul4R: 5'-TGC-CCA-AGT-TTT-ATC-GTT-CTT-CT-3' and Tul4P: FAM-5'-TTC-TAA-GTG-CCA-TGA-TAC-AAG-CTT-CCC-AAT-TAC-TAA-G-3'-BHQ. The probe is synthesised with a 6-carboxy-fluorescein reporter molecule attached to the 5'end and a black hole quencher attached to the 3' end. The PCR consists of initial denaturation for 10 minutes at 95°C followed by 45 amplification cycles of denaturation for 15 seconds at 95°C, primer annealing at 60°C for 30 seconds.

Certain PCR systems (Birdshell *et al.*, 2014; Johansson *et al*., 2000; Kugeler *et al.*, 2006), canSNP (canonical single nucleotid polymorphism) (Vogler *et al.*, 2009a), canINDELs typing (canonical insertions and deletions) (Svensson *et al.*, 2009) and MLVA (multi-locus variable-number tandem repeat analysis) (Johansson *et al.*, 2004; Vogler *et al.*, 2009b), are appropriate methods for differentiation of *F. tularensis* subspecies and genotypes.

2. Serological tests

Serology is carried out for diagnosis of tularemia in humans, but is of limited value in sensitive animal species, which usually die before specific antibodies can develop. Serology may be employed, either on sera or on lung extracts (Morner *et al*., 1988), in epidemiological surveys of animals that are resistant or relatively resistant to infection, such as sheep, cattle, pigs, moose, dogs, foxes, wild boars, birds or the European brown hare in Central Europe (Gyuranecz *et al.*, 2011; Morner *et al*., 1988; Otto *et al.*, 2014). As there is no antigenic difference between Type A and Type B strains, the less virulent *F. tularensis* ssp. *holarctica* and its attenuated live vaccine strain (LVS, NCTC 10857) could be used as antigen in all serological tests.

2.1. Agglutination tests

The most commonly used serological tests are the agglutination tests. The antigen is a culture of *F. tularensis* on Francis medium. The culture is harvested after 5–6 days. Younger cultures yield a poorer antigen. The colonies are suspended in 96% alcohol, giving a thick suspension that can be stored for 1–7 days at room temperature. The sediment is washed with normal saline and resuspended in an equal volume of normal saline. Crystal violet powder is added to a final concentration of 0.25%. The bacteria are stained by adding crystal violet and incubating at 37°C for at least 24 hours and at most 7 days.

After the supernatant fluid has been discarded, the deposit is suspended in normal saline with or without thimerosal (merthiolate) at a final concentration of 1/10,000, or formaldehyde at a final concentration of 0.5%. The suspension is calibrated with positive and negative sera, and adjusted by adding normal saline to provide an antigen that when tested on a slide gives readily visible stained agglutination reactions against a clear fluid background.

Possible cross-reactions with S-type *Brucella* species and *Legionella* sp. have to be taken into consideration.

2.1.1. Slide agglutination

Slide agglutination is a useful field method (Gyuranecz *et al.*, 2011). In the slide agglutination test 1 drop of whole blood (approx. 0.04 ml) is mixed with 1 drop of antigen and the reaction is considered positive if flakes appear within 1–3 minutes at 20–25°C.

2.1.2. Tube agglutination

The test is performed in tubes containing a fixed amount of antigen (0.9 ml) and different dilutions of serum commencing with 1/10, 1/20, 1/40, etc. The results are read after 20 minutes of shaking, or after 1 hour in a water bath at 37°C followed by overnight storage at room temperature. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive tubes are those that have a clear supernatant fluid.

2.1.3. Microagglutination

The test is performed in microtitre-plates. Serial two-fold dilutions of sera (25 µl) are mixed with an equal volume of formalin-inactivated whole cell suspension (Chaignat *et al.*, 2014). The plates are read out after incubation at 37°C for 18 hours. The agglutinated sediment is visible to the naked eye or, preferably, by using a hand lens. The positive wells are those that have a clear supernatant fluid.

2.2. Enzyme-linked immunosorbent assay

Another serological test, the enzyme-linked immunosorbent assay (ELISA), also allows an early diagnosis of tularemia (Carlsson *et al*., 1979 Chaignat *et al.*, 2014). Different antigens, whole bacteria as well as subcellular components (e.g. purified lipopolysaccharide), have been used as recall antigens against immunoglobulines IgA, IgM and IgG; 2 weeks after the onset of tularemia, specific antibodies can be detected in the serum (Chaignat *et al.*, 2014; Fulop *et al*., 1991). IgM is sustained for a long period and cannot be used as an indication of a recent infection (Bevanger *et al.,* 1994). For routine diagnosis, whole heat-killed (65°C for 30 minutes) bacteria can be used as antigen. Bacteria can be coated to plastic plates, using the usual procedures (Carlsson *et al*., 1979) followed by serial dilutions of serum to be tested. Positive reactions can be visualised by anti-antibodies labelled with enzyme. The test should also be read in a photometer with positive and negative sera as controls.

C. REQUIREMENTS FOR VACCINES

The attenuated *F. tularensis* subsp. *holarctica* live vaccine strain (LVS, NCTC 10857) has been used for decades as a tularemia vaccine, especially in laboratory workers handling large volumes of *F. tularensis* cultures. This vaccine is no longer used because of its overall limited efficacy and concern about reversion to virulence, although a derivative of LVS is still used to immunise people in endemic regions of Russia. Novel vaccines against tularemia are under development but not yet licensed for human or animal use (Conlan, 2011).

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NB: At the time of publication (2016) there were no OIE Reference Laboratories for tularemia (please consult the OIE Web site: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2016.