CHAPTER 3.1.23.

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 exposure (*this is rarely seen in animals*) *and swelling of the regional lymph nodes. Oropharyngeal and pneumonic infections can be caused by ingestion of contaminated food and water or inhalation of aerosols, respectively. 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.*

The disease spreads through vectors such as mosquitoes, horseflies, deer flies, and ticks. Humans have a high risk of acquiring the disease through direct contact with sick animals, infected tissues, consumption of infected animals, drinking or direct contact with contaminated water, and inhalation of bacteria-loaded aerosols. Infection control precautions (*including personal protective equipment*) *based on an assessment of the risks should be in place when handling suspected or confirmed infective materials. All laboratory manipulations with live cultures or potentially infected or contaminated material must be performed at an appropriate biosafety and containment level as determined by biological risk analysis* (*see Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities).

Detection of the agent: *Polymerase chain reaction is a safe and convenient method of detection and identification of* F. tularensis *in clinical specimens. The bacterium can be demonstrated in impression smears or in fixed specimens of organs using a specific 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, e.g. isolation from tissues or carcasses, the use of selective medium containing antibiotics 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 for diagnosing human infection, but are of limited value in the more susceptible animal species that usually die before developing antibodies. Serological surveys can be conducted in relatively resistant species that survive the infection and develop antibodies, such as sheep, cattle, pigs, dogs, cats, wild ungulates, foxes and wild boars. Relatively resistant species of rodents and lagomorphs* (*e.g. European brown hare in Central Europe*) *can also be included in serological surveys.*

Requirements for vaccines: *The attenuated* F. tularensis *subsp.* holarctica *live vaccine strain* (*LVS, NCTC 10857*) *was 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. Novel vaccines against tularemia are under development 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, and also in beavers. In addition, a wide variety of other mammals have been reported to be infected, and the organism has been isolated from birds, fishes, amphibians, arthropods, and protozoa (Anda *et al.,* 2001*;* Gyuranecz, 2012; Morner & Addison, 2001; Yeni *et al.,* 2020). 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 two clinically most relevant 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 and is primarily transmitted by ticks or biting flies or by direct contact with infected animals. 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 has also been found in possums in Australia. It is primarily transmitted by direct contact or by arthropods (primarily ticks and mosquitoes) but may also 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 (Morner & Addison, 2001). The course of the disease lasts approximately 2–10 days, 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 pets, *F. tularensis* infection can result in clinical illness in cats but less commonly in dogs (Feldman, 2003). Both have been implicated in transmission of the disease to humans. Transmission from cats to humans occurs most commonly via bites or scratches and from dogs via close facial contact, ticks, and retrieved carcasses, as well as bites (Kwit *et al.,* 2019).

At necropsy, animals that have died from acute tularemia are usually in good body condition, but signs of septicaemia characterised by whitish foci of necrosis randomly distributed in the liver, bone marrow and spleen, are evident (Morner & Addison, 2001). In addition, the spleen is usually enlarged. Necrotic foci vary in size, with some barely visible to the naked eye. The lungs are usually congested and oedematous, and areas of consolidation and fibrinous pneumonia or pleuritis may be present. Fibrin may be present in the abdominal cavity. Foci of caseous necrosis are often present in one or more lymph node(s); those most often affected are lymph nodes 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. Species that are moderately susceptible to tularemia, and maintain the infection for a prolonged time, may serve as reservoirs of infection to others (Hestvik *et al.,* 2015). Infection can occur by simple contact with sick animals, infected tissues, consumption of infected animals, drinking or direct contact of contaminated water and via inhalation of infective aerosols. Hunters and forest rangers should take precautions before opening dead animals as they are at risk of infection. Suitable personal protective equipment (e.g. gloves, particulate-filtering masks or respirators, and eye-shields) must be worn during any manipulation of pathological specimens or cultures 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 as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). 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
Detection of the agent[a]						
Bacterial isolation	–	–	–	++	–	–
Antigen detection	–	–	–	+++	–	–
Conventional PCR[c]	+	–	–	+++	+	–
Real-time PCR[b]	+	–	–	+++	+++	–
Detection of immune response[d]						
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.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]Versage *et al.,* 2003; [c]Barns *et al.,* 2005.
[d]Serology is of limited value in susceptible animals, which usually die before development of specific antibodies.

1. Detection of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections using specific immunological or immunohistochemical methods of identification. If reagents are not readily available, fixed specimens can be analysed at laboratories equipped with proper reagents and methods. Bacterial isolation followed by identification via immunological or molecular methods is also used, however, *F. tularensis* may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. Selective culture media or animal inoculation can be used to enhance recovery of the organism. Polymerase chain reaction (PCR) is a safe and convenient way to detect and identify *F. tularensis* in clinical samples.

1.1. Smear preparations for antigen detection

Impression smears of organs, such as the liver, spleen, bone marrow, kidney, lung or blood, are made on microscope slides. 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.

1.2. Histological sections for antigen detection

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 first treated with rabbit or mouse anti-tularemia serum, washed and thereafter treated with a 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. Bacterial isolation

The bacteria can be isolated from heart blood, liver, spleen, bone marrow or tularemic granulomas (from lungs, pericardium, kidney, liver, spleen, etc.) from moribund animals, however it is highly fastidious; it will not grow on ordinary media, although an occasional strain can sometimes grow on blood agar on initial isolation. Culture preparations are incubated at 37°C, in ambient air or in 5% CO_2.

1.3.1. The culture media listed below are all appropriate for isolating *F. tularensis*

i) 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.

ii) 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.

iii) Modified Thayer–Martin agar

Glucose cysteine agar (GCA)-medium base supplemented with haemoglobin and a commercially available enrichment additive containing nicotinamide adenine dinucleotide with other factors.

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.

1.3.2. Selective media

Cystine heart agar broth with blood (CHAB) 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) is commonly used for complex clinical specimens. Growth in CHAB medium enables the presumptive identification of *F. tularensis* by characteristic growth at 24–48 hours of round and smooth green opalescent shiny colonies, 2–4 mm in diameter.

1.3.3. Identification of isolates

The bacteria are nonmotile, nonsporulating, bipolar staining, and of uniform appearance in 24-hour cultures, but pleomorphic in older cultures. Biochemical tests can provide a presumptive identification of isolates, but confirmation using immunological or molecular methods is typical. Type A subspecies may be biochemically distinguished from Type B subspecies by the fact that most Type A ferment glycerol.

Francisella tularensis species can be identified using matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) if corresponding reference spectra are available (Lopez-

Ramos *et al.*, 2020). The preparation is carried out using the ethanol/formic acid method. After 20 minutes in 70% ethanol, the bacteria are killed. The preparation is centrifuged and the supernatant removed. Equal volumes of formic acid and acetonitrile are then added and centrifuged again and the supernatant is dropped on the metal sample plates for spectrometry. Picking and direct application of the colonies without ethanol inactivation is not recommended.

1.4. 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 include conventional PCR (Barns *et al.*, 2005; Sjostedt *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'
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 and up to 40 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 detect specifically 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'
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.

Appropriate methods for differentiation of *F. tularensis* subspecies and genotypes include certain PCR assays (Birdsell *et al.*, 2014; Johansson *et al.*, 2000; Kugeler *et al.*, 2006; Tomaso *et al.*, 2007), canonical single nucleotide polymorphism analysis (canSNP; Vogler *et al.*, 2009a), typing of canonical insertions and deletions (canINDELs; Svensson *et al.*, 2009), DNA microarray analysis (Broekhuijsen *et al.,* 2003) and multi-locus variable-number tandem repeat analysis (MLVA; Johansson *et al.*, 2004; Vogler *et al.*, 2009b).

1.5. Animal inoculation

Animal inoculation is not recommended because of welfare and biosafety concerns. It should only be undertaken when culture enhancement in a laboratory animal is considered unavoidable, and where proper animal 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).

2. Serological tests

Serology is carried out for diagnosis of tularemia in humans but is of limited value in susceptible animal species, which usually die before specific antibodies can develop. Serology may be conducted 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. The most commonly used serological test for diagnosis of tularemia is the microagglutination test. Other tests (immunofluorescence and enzyme-linked immunosorbent assay [ELISA]) have comparable sensitivity and specificity, but may detect antibody earlier than agglutination tests (Maurin, 2020).

2.1. Agglutination tests

The antigen used in agglutination tests is typically 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 typically inactivated by suspension in saline with 0.5% formaldehyde. After centrifugation, the pellet is resuspended in an equal volume of saline containing 0.5% formaldehyde and 0.005% safranine (Sato *et al.,* 1990). 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* spp. have to be taken into consideration. Agglutination tests primarily detect IgM, although IgG contributes to the agglutination.

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 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 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 immunoglobulins IgA, IgM and IgG. Two weeks after the onset of tularemia, specific antibodies can be detected in the serum (Chaignat *et al*., 2014; Fulop *et al*., 1991). Because IgM is sustained for a long period, it cannot be used as an indicator of 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 the 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) was developed in the 1950s and used for decades to protect laboratory workers handling large volumes of *F. tularensis* cultures, however, it is no longer used because of its overall limited efficacy and concern about reversion to virulence. A number of novel vaccines against tularemia are under development using different approaches, but none have yet received regulatory approval for human or animal use (Carvalho *et al.,* 2014; Conlan, 2011).

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NB: At the time of publication (2022) there was no WOAH Reference Laboratory for tularemia (please consult the WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2022.