

TULAREMIA

SUMMARY

Tularemia is a zoonosis caused by *Francisella tularensis*. The causative bacterium is a Gram-negative coccoid rod, 0.2–0.5 μm \times 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. It occurs naturally in lagomorphs (rabbits and hares), and in rodents, especially microtine rodents (such as voles, vole rats and muskrats), and beavers. A wide range of other mammals and several species of birds have also been reported to be infected. Among domestic animals, the cat seems to be able to act as a carrier of the bacterium.

Two types of *F. tularensis* are recognised on the basis of cultural characteristics, epidemiology, and virulence in some hosts. *Tularemia* is largely confined to the Northern Hemisphere and is not normally found in the tropics or the Southern Hemisphere. *Francisella tularensis* subsp. *tularensis* (Type A) is associated with lagomorphs in North America. It is transmitted primarily by ticks and biting flies, is highly virulent for humans and domestic rabbits, and most of the isolates ferment glycerol. *Francisella tularensis* subsp. *palaeartica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) in northern North America, and in hares and small rodents in Eurasia. It may be water- or arthropod-borne, is less virulent to humans and rabbits, and does not ferment glycerol. In addition to vector transmission, tularemia may be spread contact with infected animals or environmental fomites by inhalation, or by ingestion of the poorly cooked flesh of infected animals or contaminated water.

The disease is characterised by fever, depression and 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, bone marrow and lungs. The spleen is usually enlarged.

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. The facility should meet the requirements for Containment Group 3 pathogens (see Chapter 1.1.2 Biosafety and Biosecurity in the veterinary microbiological laboratory and animal facilities).

Identification of the agent: The bacterium can be demonstrated in impression smears or in fixed specimens of organs, such as liver, spleen, bone marrow, kidney and lung, as well as in blood smears. Immunological methods, such as the fluorescent antibody test (FAT) are the most reliable way to identify the bacterium. With Gram staining, the bacteria appear as very small punctiform Gram-negative rods, often difficult to distinguish as bacteria. They can also be stained with May–Grunwald–Giemsa or phenol thionin.

The organism is highly fastidious. For growth it is necessary to use Francis medium, McCoy and Chapin medium, or Modified Thayer-Martin agar. The colonies are small, round and transparent, and do not appear before 48 hours incubation at 37°C. On Francis medium, the colonies may be confluent and have a milky appearance. If transportation is necessary, samples should be inoculated into sterile nutrient broth and stored at 4–10°C for a few hours or at –70°C if transit is likely to be prolonged.

In the past mice or guinea-pigs were experimentally inoculated with infected tissue material or with cultures to aid in the diagnosis of tularemia. Animal inoculation has been replaced by polymerase chain reaction (PCR) protocols for the identification of *F. tularensis*. The FAT demonstrates *F. tularensis* in pathological specimens.

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 and wild ungulates, as these species develop antibodies. Also relatively resistant species of rodents and lagomorphs can also be used in epidemiological surveys. Serological surveys can and are conducted in a number of wildlife species, such as moose, that are exposed to *F. tularensis*.

Requirements for vaccines and diagnostic biologicals: A live attenuated vaccine strain is available for vaccination of humans at high risk of exposure to virulent *F. tularensis*. However, the vaccine is only available for restricted use. The outcome of vaccination can be estimated by the demonstration of specific antibodies and the ability of lymphocytes to proliferate in the presence of *F. tularensis* antigen.

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 (19, 20). 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. Several epizootic outbreaks have been reported as a result of importation of subclinically infected lagomorphs.

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. *palaeartica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) and voles in northern North America, and in lagomorphs (hares) and rodents in Eurasia. It is primarily transmitted by direct contact or by 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 (1, 7, 14, 15, 17, 22).

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 (1, 17). Among domestic animals, the cat has been reported to be able to act as a carrier of the bacterium (6) and the disease is occasionally spread from cats to humans (8).

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 histological picture can resemble that of tuberculosis with chronic granulomas in liver, spleen, lungs and kidneys.

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. The facility should meet at least the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities. Countries lacking access to such 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

1. Identification of the agent

Francisella tularensis can be demonstrated in smear preparations or in histological sections. It can also be identified by culture or animal inoculation. However, *F. tularensis* may be difficult to isolate from dead animals and carcasses due to overgrowth of other bacteria. 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, such as the OIE Reference Laboratory (see Part 3 of this *Terrestrial Manual*).

a) 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 (13, 16, 18).

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.

b) Histological sections

Bacteria can be demonstrated in sections using immunohistochemical methods, such as the fluorescent antibody test (FAT) (16). The test is normally performed on specimens from liver, spleen or bone marrow, fixed in neutral buffered formalin and paraffin embedded. Slides are treated with rabbit anti-tularemia serum, washed and thereafter treated with sheep fluorescein-isothiocyanate-conjugated anti-rabbit serum. The samples are examined under a fluorescence microscope. Large numbers of bacteria can be seen in necrotic lesions and in the blood.

c) 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% CO₂. Heart blood, liver, spleen and bone marrow from moribund animals should be used for culture. It is necessary to use special culture media, such as:

- i) *Francis medium*: Peptone agar containing 0.1% cystine (or cysteine) and 1% glucose, to which is added, before solidification, 8–10% defibrinated 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 Iso VitaleX.

Media can be stored for up to 8–10 days at 4°C. Colonies that form on McCoy 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.

- iv) *GCA agar with thiamine (BBL)*: When used with added blood, the medium is commonly referred to as GBCA and can be substituted for the original, noncommercial medium described by Down *et al.* (5). Suspend 58 g of the dry material in 1 litre of distilled or demineralised water, and mix thoroughly. Heat with frequent agitation and boil for 1 minute. Dispense into tubes and sterilise by autoclaving at 118–121°C for 15 minutes.

For larger volumes (up to several litres) of culture medium, autoclave at the same temperatures for 30 minutes. Cool to 45–48°C. Aseptically add 25 ml of packed human blood cells or 50 ml of defibrinated rabbit or sheep blood. Mix thoroughly and pour into plates. Incubate at 37°C for 24 hours before use to decrease surface moisture and to test for sterility (5).

The following selective medium can be used in addition to the non-selective media: *Cystine heart agar* (DIFCO) with 5% rabbit blood, and penicillin (100,000 units), Polymyxin B sulphate (100, 000 units), and cycloheximide (0.1 ml of a 1% stock solution) per litre.

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 hybridisation with probes specific to the 16S rRNA of *F. tularensis*, *F. tularensis* Type A, and *F. tularensis* Type B (10), or by polymerase chain reaction (PCR) with primers targeting specific regions of the 16 rDNA molecules. The PCR will allow identification at the genus, species and subspecies level (11).

d) Capillary tube precipitation test on pathological samples

Tissues, such as spleen, liver or bone marrow, are ground with sterile sand in three-to-five times their volume of normal saline. The suspension is transferred to a tube and two volumes of ethyl ether are added. After shaking, the mixture is allowed to stand for 4–5 hours at room temperature. It is again shaken and then allowed to stand overnight.

The aqueous phase is drawn off and centrifuged at 2000 **g** for 30 minutes. The supernatant fluid, containing the antigen, is drawn off and distributed into capillary tubes to which tularemia antiserum is added.

The tubes are incubated at 37°C for 3 hours, then kept at 4°C overnight. A positive result is the formation of a ring of precipitate.

e) Animal inoculation

Animal inoculation is extremely hazardous and is only recommended for agent identification in cases when culture is negative and agent identification is needed for epidemiological reasons. It should only be undertaken where proper biosafety facilities and cages are available (see Chapter 1.1.2). PCR techniques should be used for identification of *F. tularensis*.

Laboratory animals (preferably mice) are inoculated with culture material to confirm the nature of an isolate. Pathological specimens may be inoculated for the direct detection of *F. tularensis*. Inoculated mice usually die before lesions can form.

Intraperitoneal injection is sufficient for passage of pure cultures. All routes of administration in mice, such as subcutaneous, percutaneous, or intravenous, will lead to an infection that is invariably fatal within 2–10 days.

f) Molecular techniques

The PCR has recently been developed for the diagnosis of *F. tularensis* and is an excellent method for identifying the agent in wound specimens from humans (12).

A real-time PCR has proven to be a highly sensitive and specific assay and is a major diagnostic improvement, as all other methods for the specific identification of *F. tularensis* subsp. *tularensis* are very time-consuming (23).

A DNA microarray has also been developed that is capable of distinguishing the highly virulent subspecies *F. tularensis* subsp. *tularensis* and the moderately virulent subspecies *F. tularensis* subsp. *holarctica* (3).

2. Serological tests

Serology is currently 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 (18), in epidemiological surveys of animals that are resistant to infection, such as sheep, cattle, pigs, moose, dogs or birds (18, 20). As there is no antigenic difference between Type A and Type B, the less virulent *F. tularensis palaeartica* could be used as antigen in all serological tests.

a) Tube agglutination

The most commonly used serological test is the tube agglutination test. 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.

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. Possible cross-reactions with *Brucella abortus*, *B. melitensis* and *Legionella* sp. have to be taken into consideration.

b) Enzyme-linked immunosorbent assay

Another serological test, the enzyme-linked immunosorbent assay (ELISA), also allows an early diagnosis of tularemia (4). This method is now widely used for clinical purposes. Different antigens, whole bacteria as well as subcellular components (9), 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. IgM is sustained for a long period and cannot be used as an indication of a recent infection (2). 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 (4) 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 AND DIAGNOSTIC BIOLOGICALS

Vaccines have been produced with the aim of protecting humans, but as all vaccine development involves testing in animals, it is clear that some of the vaccines could be used to protect animals. However, in most countries, there is no vaccine licensed for use in animals.

Before 1940, attempts to vaccinate against tularemia were performed by use of whole killed bacteria or bacterial extracts. None of these vaccines induced protection against highly virulent strains of *F. tularensis*. Instead, viable attenuated vaccines were developed. Attenuation was performed by repeated culture of bacteria on various media with or without antiserum. Such live attenuated vaccines have been used in mass vaccinations of people in the former Soviet Union since 1946, either as monocultures or as a mixture of strains.

An attenuated live vaccine strain of *F. tularensis* biovar palaeartica is available and can be used for restricted vaccination of individuals at high risk (21).

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NB: There is an OIE Reference Laboratory for Tularemia (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).