# WHO GUIDELINES ON Tularaemia

World Health Organization

EPIDEMIC AND PANDEMIC ALERT AND RESPONSE

# WHO Guidelines on **Tularaemia**

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ALERT AND RESPONSE

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# Abbreviations

| | |
|---|---|
| BCYE | buffered charcoal yeast extract |
| BSA | bovine serum albumin |
| CA | enriched chocolate agar |
| CFU | colony-forming unit |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| FITC | fluorescein isothiocyanate |
| IHR | International Health Regulations |
| LD50 | The amount of a material, given all at once, which causes the death of 50% of a group of test animals |
| LPS | lipopolysaccharide |
| LVS | live vaccine strain |
| MIC | minimum inhibitory concentration |
| MBC | minimum bactericidal concentration |
| OD | optical density |
| OIE | World Organisation for Animal Health |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| rpm | revolutions per minute |
| RT | room temperature |
| SBA | sheep blood agar |
| TGBA | thioglycollate-glucose-blood agar |
| Type A | *F. tularensis* subspecies *tularensis* (Jellison type A) |
| Type B | *F. tularensis* subspecies *holarctica* (Jellison type B) |
| WHO | World Health Organization |

# 1. Introduction

Arne Tärnvik

Tularaemia is a bacterial zoonotic disease of the northern hemisphere. The bacterium (*Francisella tularensis*) is highly virulent for humans and a range of animals such as rodents, hares and rabbits. It may cause epidemics and epizootics. *F. tularensis* is transmitted to humans (i) by arthropod bites, (ii) by direct contact with infected animals, infectious animal tissues or fluids, (iii) by ingestion of contaminated water or food, or (iv) by inhalation of infective aerosols. There is no human-to-human transmission.

Tularaemia is reported from most countries in the northern hemisphere, although its occurrence varies widely from one region to another. In some countries, endemic regions with frequent outbreaks are close to regions that are completely free of tularaemia. There is also a wide variation with time. In an endemic area, tularaemia may occur annually within a 5-year period, but may also be absent for more than a decade. The reasons for this temporal variation in the occurrence of outbreaks are not well understood. When, after a long lapse, the first case of a new outbreak appears, the disease may be more or less forgotten and is therefore not easily diagnosed.

*F. tularensis* subspecies tularensis (type A) is one of the most infectious pathogens known in human medicine. The infective dose in humans is extremely low: 10 bacteria when injected subcutaneously and 25 when given as an aerosol (McCrumb, 1961; Saslaw et al., 1961a; Saslaw et al., 1961b). For example, on Martha's Vineyard in the United States of America, two adolescents contracted respiratory tularaemia after mowing a grassed area (Feldman et al., 2001). It is believed that an aerosol of *F. tularensis* subspecies *tularensis* was generated after the carcass of a rabbit which had died of tularaemia was accidentally shredded by the lawnmower.

The risk posed by tularaemia can be properly managed, provided the public health system is well prepared. In order to avoid laboratory-associated infection, safety measures are needed and consequently clinical laboratories do not generally accept specimens for culture. However, since clinical management of cases depends on early recognition, there is an urgent need for diagnostic services. In addition to its natural occurrence, *F. tularensis* causes great concern as a potential bioterrorism agent.

The present guidelines on tularaemia (i) provide background information on the disease, (ii) describe the current best practices for its diagnosis and treatment in humans, (iii) suggest measures to be taken in case of epidemics and (iv) provide guidance on how to handle *F. tularensis* in the laboratory. The target groups for these guidelines include clinicians, laboratory personnel, public health workers, veterinarians, and any other person with an interest in zoonoses.

The guidelines are the result of an international collaboration, initiated at a WHO meeting in Bath, United Kingdom of Great Britain and Northern Ireland in 2003, continued in Umeå, Sweden, in 2004 and finalized in Geneva, Switzerland, in 2005.

Each chapter of the guidelines was developed by a selected group of scientists with extensive developmental work and general experience relevant to the field covered. Chairs of each group met regularly throughout the process to ensure consistency and for critical review. The document was also subject to internal external peer reviews.

As with many other areas, understanding of the nature of tularaemia and its causative agent is evolving rapidly across all aspects discussed in the guidelines, including taxonomy, epidemiology, epizootiology, detection, diagnostics, therapy and prophylaxis. It is envisaged therefore that modifications to these guidelines will become necessary every three years.

# 2. The infectious agent

Tina Broman, Mats Forsman, Jeannine Petersen, Anders Sjöstedt

Historically, tularaemia was a public health problem in the former Soviet Union and in the USA. For example, in the USA some 1400 cases were reported from 1990 to 2000, as compared to more than 14 000 cases from 1920 to 1945 (Hayes et al., 2002). In the former Soviet Union, extensive outbreaks occurred during and after the Second World War. During the winter of 1941–42, 67 000 cases were reported from the region surrounding Rostov-on-Don (Jusatz, 1952). There are no data available that explain the decline in cases since the 1950s. However, it is assumed that this may be due to less-frequent exposure of humans to rodents, rabbits and hares which in turn may be related to a decrease in the number of hunters and a decrease in the percentage of the population living in rural settings. In the Russian Federation, vaccination programmes may have contributed to the decline. Experiences from the former Soviet Union during the Second World War and from Bosnia and Herzegovina, and United Nations Administered Province of Kosovo (Serbia) during the recent civil wars suggest that tularaemia can increase significantly during and after warlike conditions, or after natural disasters that disrupt the normal hygiene and sanitary conditions of a society (Jusatz, 1952; Reintjes et al., 2002). There are indications that the epidemiology of the disease may be changing. In Canada, for example, contact with rabbits was the most common source of infection before the 1950s, while contact with muskrats appears to be of greater importance today (Martin et al., 1982).

*Francisella tularensis*, the infectious agent of tularaemia, is a potential bioterrorist agent (Dennis et al., 2001). During the Cold War, the pathogen was possibly developed into biological warfare weapons in both the east and the west (references in World Health Organization, 2004a). The pathogen is attractive for these developments because of its very high infectivity and relative stability in aerosols which facilitates its dissemination.

On solid culture media, bacterial colonies become visible within 2–3 days of incubation at 37 °C and a preliminary confirmation can be easily and rapidly obtained by agglutination of the bacteria in immune serum. Due to scientific achievements of the past decade, molecular techniques are now available for rapid and final identification of *F. tularensis*, and for differentiation of subspecies.

## 2.1 Taxonomy

*F. tularensis* is not closely phylogenetically related to any pathogen or commensal of the human flora. It belongs to a group of intracellular bacteria, which includes mycobacteria *Listeria*, *Legionella*, *Brucella*, *Coxiella* and *Rickettsia*.

*Francisella tularensis* is one of two species in the genus *Francisella*, which is the only genus of the family Francisellaceae, a member of the gamma-subclass of Proteobacteria (Sjöstedt, 2005). The two species, *F. tularensis* and *F. philomiragia*, show a 16S rRNA sequence similarity of ≥98.3% (Forsman, Sandström & Sjöstedt, 1994). The family is distinguished by a unique set of phenotypic characteristics including a coccoidal morphology, Gram-negativity, a capability to degrade only a limited number of carbohydrates resulting in acid but no gas production, a growth requirement for cysteine, and a unique fatty acid composition (Sjöstedt, 2005).

Based on 16S rRNA gene sequence analysis, several tick endosymbionts are closely related to *F. tularensis* and have been preliminarily placed in the *Francisella* group (Sun et al., 2000; Scoles, 2004). *Caedibacter taeniospiralis*, a protozoan endosymbiont also shows 16S rRNA similarity to members of Francisellaceae (Beier et al., 2002). In addition, several potential new *Francisella* species, including several with high 16S rRNA similarity to *F. tularensis*, have been identified in soil samples in the Houston area of the USA (Barns et al., 2005).

## 2.2 Species and subspecies which differ in virulence and geographical range

With the recent reassignment of *Francisella novicida* as a subspecies of *F. tularensis*, there are currently four proposed subspecies, each of which displays several distinct biochemical, epidemiological, and virulence characteristics (Jellison, 1974; Sjöstedt, 2005). The 16S rRNA similarity is ≥99.8% among the subspecies (Forsman, Sandström & Sjöstedt, 1994).

The highly virulent *F. tularensis tularensis* (type A) is found in the USA, whereas the less virulent *F. tularensis holarctica* (type B) is found throughout the northern hemisphere. Subspecies are further discriminated into isolates and strains. An isolate is a population of bacterial cells in pure culture derived from a single colony on a primary isolation plate. In the case of *F. tularensis* and other fastidious organisms forming small-size colonies this definition is often violated. Several colonies are unavoidably sampled on a primary isolation plate. A strain is an isolate or group of isolates displaying specific genetic or phenotypic characteristics that set it apart from other isolates of the same species.

Strains of subspecies *mediasiatica* have been isolated only in Kazakhstan and Turkmenistan. Little work is known regarding these strains but experimental studies in rabbits have indicated a moderate virulence comparable to that of subspecies *holarctica*.

Subspecies *novicida* shows a low virulence in experimental models and causes disease in immunocompromised individuals (Hollis et al., 1989) but it has not been isolated from any animals. However, isolates of subspecies *novicida* have been linked to waterborne transmission in Australia, Spain and the USA (Hollis et al., 1989; Whipp et al., 2003). The isolate from Australia is the only *F. tularensis* strain originating from the southern hemisphere to date.

*F. philomiragia* is associated with salty water (Atlantic as well as Mediterranean) and is of low virulence, although it may cause disease in immunocompromised individuals. See section 4.1 for further information on the mode of transmission and clinical symptoms.

## 2.3 Seasonality, age and gender distribution

Although the age-related incidence rate of tularaemia is unknown, the disease is known to occur at all ages. Males have a higher incidence in all age categories. In Sweden, an overrepresentation among males has been attributed to their more frequent outdoor professional and leisure activities (Eliasson et al., 2002). In most countries where tularaemia is endemic, the disease is seasonal; its incidence seems to be highest during late spring, the summer months and early autumn (Olsufiev, 1977; Hayes et al., 2002; Tärnvik et al., 2004). Often, the number of cases shows wide variations from one year to another and this is probably related to climatic factors such as temperature and precipitation. However, there are virtually no data linking specific climatic conditions and outbreaks of tularaemia. This is an important area for future research that may yield important tools for predicting and possibly preventing outbreaks.

# 3. Epidemiology

Anders Sjöstedt

There is no evidence for human-to-human transmission of tularaemia. *F. tularensis* has been isolated from a variety of animals which may function as vectors for transmission to humans (Table 3.1). Transmission of *F. tularensis* subspecies *tularensis* is associated with rabbits, ticks and sheep, whereas *F. tularensis* subspecies *holarctica* is often isolated in association with streams, ponds, lakes and rivers. Beavers and muskrats in North America and lemmings and beavers in Scandinavia might play a role in maintaining the water association of the bacterium (Parker et al., 1951; Hopla, 1974; Hörnfeldt, Löfgren & Carlsson, 1986; Mörner et al., 1988a). There is also evidence that the bacterium can persist for months in watercourses, possibly in association with protozoa (Berdal et al., 1996). For further references see section 7.4.1. It should be noted, however, that the epizootology of tularaemia is not yet fully understood (Hopla, 1974). For instance, the respective importance of various susceptible animals as reservoirs for *F. tularensis* is still poorly known. Similarly, the role of various arthropods in the transmission of *F. tularensis* among animals and between animals and humans is not yet well understood.

## 3.1 Geographical distribution of tularaemia cases

Tularaemia has been reported in many countries of the northern hemisphere, but so far not from the southern hemisphere (Figure 3.1). An isolate of subspecies *novicida* has been found in Australia, but without the typical clinical presentation of tularaemia (Whipp et al., 2003). Endemic foci have existed for a long time in the Russian Federation, Kazakhstan and Turkmenistan (Olsufiev, 1977) as well as Finland and Sweden (Tärnvik, Priebe & Grunow, 2004). Annual cases are reported from most countries in eastern Europe whereas there are few reported cases in countries of continental western Europe. Outbreaks comprising hundreds of cases have occurred recently in Portugal, Spain, Sweden and UN Administered Province of Kosovo (Serbia) (de Cavalho et al., 2007; Pérez-Castrillòn et al., 2001; Eliasson et al., 2002; Reintjes et al., 2002). Cases are also reported in Japan and the northwestern and north-eastern regions of China. In the western hemisphere, cases regularly occur in the USA and Canada and a few cases have been reported from Mexico. In the USA, some 120 cases have been reported annually from 1990 to 2000 (Hayes et al., 2002). More than 50% of these cases occurred in Arkansas, Missouri, South Dakota and Oklahoma. Small outbreaks have been reported repeatedly from the island Martha's Vineyard in Massachusetts (Feldman et al., 2001; Feldman et al., 2003).

## 3.2 Vectors

### 3.2.1 Arthropods

A wide range of arthropods has been identified in the transmission of tularaemia between mammalian hosts. In the USA, ticks are considered as the most important vectors east of the Rocky Mountains, where *F. tularensis* has been detected in at least 13 species representing four genera: *Amblyomma*, *Dermacentor*, *Haemaphysalis*, and *Ixodes* (Hopla, 1974). Flies (*Chrysops* spp.) are the most common vectors in Utah, Nevada, and California. Several outbreaks of human tularaemia in Utah have been linked to transmission by *Chrysops discalis* (Jellison, 1950). In the former Soviet Union, the bacterium has been demonstrated to be transmitted by mosquitoes (*Aedes* spp., *Culex* spp., *Anopheles* spp.) and ticks (*Ixodes* spp.). However, in ticks

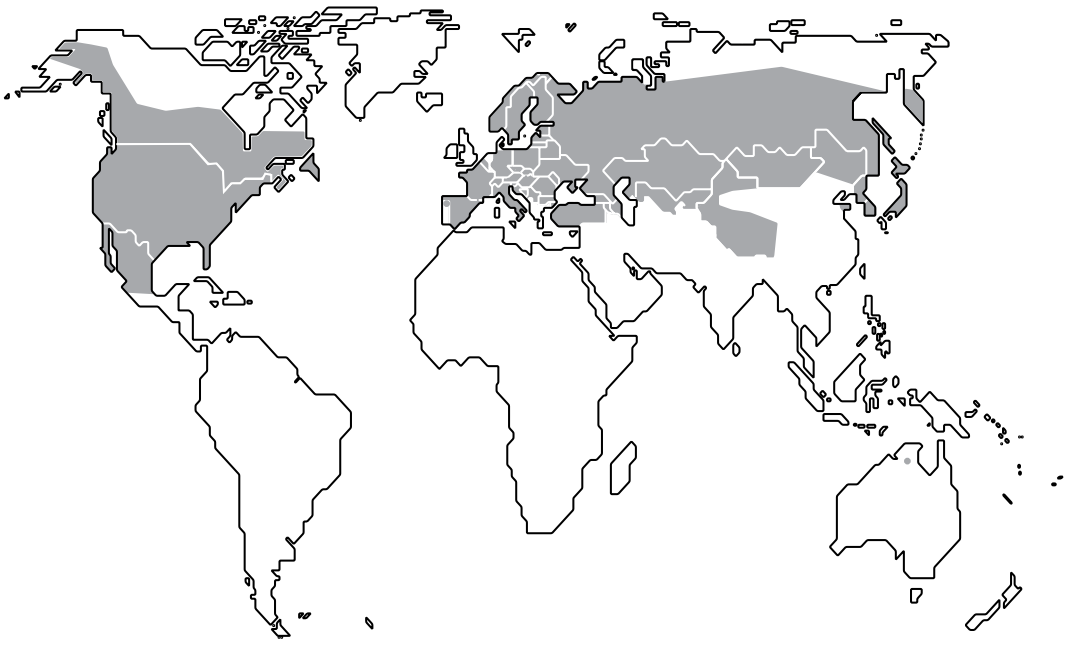


**Figure 3.1** The geographical distribution of tularaemia is indicated in grey. Locally, the disease often has an uneven distribution with recurrent epidemic outbreaks in geographically restricted areas. The data were compiled from publications in the medical literature between 1952–2006. Credit: Anders Johansson, Umeå University, Umeå, Sweden and from a recent publication (de Carvalho et al., 2007)

the prevalence appeared to be low; an investigation of 120 000 adult *Ixodes ricinus* in northwestern areas of the former Soviet Union between 1960 and 1964 resulted in identification of 0.01% positive ticks (Olsufiev & Dunayeva, 1970). In central Europe, the ticks *Dermacentor reticulatus* and *Ixodes ricinus* are important vectors. In areas of Austria and the Czech Republic, 2% of *D. reticulatus* ticks contained *F. tularensis* (Hubalek et al., 1997). In Finland and Sweden, clinical experience and epidemiological data support the role of mosquitoes as vectors for transmission of *F. tularensis* to humans (Christenson, 1984; Eliasson et al., 2002). In Sweden, mosquitoes of the species *A. cinereus* have been demonstrated to be infected (Olin,1942). Data reported both from the former Soviet Union and the USA show that flies of the family Tabanidae may serve as vectors for the disease (Krinsky, 1976). This family includes true horseflies (*Tabanus* spp. and *Chrysozona* spp.) and deer flies (*Chrysops* spp.). These flies are found in nearly all parts of the world and females of all species are blood-sucking. *F. tularensis* has been detected in at least 20 flea species of eight genera (*Amphipsylla*, *Cediopsylla*, *Ceratophyllus*, *Ctenophthalmus*, *Malaracus*, *Megabothris*, *Neopsylla*, and *Pulex*) but their role in the spread of *F. tularensis* is unclear (Olsufiev & Dunayeva, 1970).

Although the exact mechanism of transmission of tularaemia by flies and mosquitoes is still elusive, it should be noted that *F. tularensis* has never been demonstrated to reside in the salivary glands of any species. Maybe spread is simply mechanical, by contamination of mouthparts when a fly or a mosquito is biting an infected host. Alternatively, *F. tularensis* may be mechanically rubbed into the skin when the arthropod is swatted. Another source of infection of flies or mosquitoes has been suggested to be contaminated water (Pavlovsky, 1966).

### 3.2.2 Mammals

Tularaemia outbreaks have been described in hares (Mörner et al., 1988), prairie dogs (Petersen et al., 2004a), and mink (Henson, Gorham & Shen, 1978). It should be noted that although some studies suggest that mammals harbour and secrete the bacteria for longer periods of time (Bell, 1980), there is no evidence that they constitute a major natural reservoir for *F. tu-*

*larensis*. Nevertheless, *F. tularensis* is thought to be spread in the environment principally by various terrestrial and aquatic mammals such as ground squirrels, rabbits, hares, beavers, muskrats and, in particular, rodents such as meadow voles and water voles (Table 3.1). Systematic studies in the former Soviet Union revealed that among these animals, voles and mice were presumed to be the main source for the spread of *F. tularensis* to humans (Olsufiev, 1977). In particular, the most commonly-identified vectors were the water vole (*Arvicola terrestris*), the common vole (*Microtus arvalis*), the red-backed vole (*Clethrionomys* spp.) and the domestic mouse (*Mus musculus*). The role of non-rodent species such as rabbits and hares in the spread of tularaemia to other mammals and humans is not well known but it has been suggested that they may infect hunters in central Europe (Pfahler-Jung, 1989). In certain parts of the USA, transmission via ticks from the rabbit is an important cycle for the spread of *F. tularensis* subspecies *tularensis* to other mammals and humans (Taylor et al., 1991).

### 3.2.3 Rodent population size and transmission to humans

Outbreaks of the disease in humans often follow outbreaks of tularaemia in rodents. For example, outbreaks in humans in the Russian Federation were shown to be linked to epizootics of the disease in common voles (Mörner, 1992). In regions where tularaemia is endemic, antibodies to *F. tularensis* are frequently detected in the sera of trapped wild animals such as muskrats, beavers and common voles (Mörner, Sandström & Mattsson, 1988).

Widespread epizootics of tularaemia have also been associated with a preceding increase in the density of the rodent population size. The results from a recently published investigation showed that the number of human cases of tularaemia in the Novosibirsk region of the Russian Federation from 1956–2000 was correlated to the density of the water rat population (Efimov, Galaktionov & Galaktionova, 2003). In Sweden, a strong correlation between peaks in vole and hare populations and outbreaks of tularaemia in humans has been reported during the 1960s and 1970s (Hörnfeldt, Löfgren & Carlsson, 1986). However, in recent years, no correlation with the size of the rodent populations was found in Sweden (Tärnvik, Sandström & Sjöstedt, 1996).

Tularaemia transmission patterns may also change over time. In Canada, for example, contact with rabbits was the most common route of spread of infection before the 1950s while more recently, the water-living muskrat appears to be of greater importance (Martin et al., 1982).

### 3.2.4 Reservoirs in the environment

Faeces from the field vole (*Microtus agrestis*) were demonstrated to be a source of human infection in the 1960s in Sweden (Dahlstrand, Ringertz & Zetterberg, 1971). Hay stored in barns was contaminated with the faeces and humans contracted tularaemia in the barns via inhalation. On Martha's Vineyard in the USA, two fatal cases of tularaemia were associated with grass mowing and exposure by aerosol formation to the carcass of an infected rabbit (Feldman et al., 2001).

In a few instances, it has been reported that *F. tularensis* has been isolated from mud (Parker et al., 1951). However, there are no recent reports on the isolation of the bacterium from mud or soil. It is unclear whether this means that the bacterium rarely exists in mud or soil or whether it is difficult to cultivate *F. tularensis* from such sample types. Nevertheless, 16S rRNA sequences highly similar to those previously reported for *F. tularensis* were recently found in soil samples in Houston, Texas, USA (Barns et al., 2005). Although no bacteria were cultivated, the gene sequences indicated that they were derived from bacteria that belong to the family Francisellaceae but were distinct from known species and formed several new clades potentially representing new species or genera. These findings will allow comparative analyses of samples from other geographical locations.

## 3.3 Disease and pathology in animals

Signs and symptoms of tularaemia in wild animals are not well documented and are mostly based on postmortem examinations. The most common finding upon necropsy is an enlarged spleen and pinpoint white necrotic lesions in the spleen and liver (Bell, 1980; Hopla & Hopla, 1994). The manifestations are likely to be dependent upon the susceptibility of the particular animal species to tularaemia. The best-documented clinical cases are in domestic cats and dogs, captive monkeys, prairie dogs and laboratory animals.

### 3.3.1 Case descriptions in naturally-infected animals

Tularaemia has been diagnosed in two cats that were examined because of pyrexia and lethargy; both had a history of exposure to wild rabbits (Woods et al., 1998). One cat was vomiting and the other was anorexic. Physical examination revealed dehydration, lymphadenopathy and hepatomegaly. Acute *F. tularensis* infection in three domestic cats was associated with marked signs of depression, oral/lingual ulceration and regional or generalized lymphadenomegaly (Baldwin et al., 1991).

One case of tularaemia in a dog was characterized by acute onset of anorexia, pyrexia, lymphadenopathy and tonsillitis (Gustafson & DeBowes, 1996). The disease was self-limiting and clinical signs resolved within five days with only supportive therapy. A second case demonstrated acute onset of lethargy, anorexia and weakness (Meinkoth, Morton & Meinkoth, 2004). The dog had eaten an adult rabbit 36 h earlier.

In a Canadian zoological garden, three black and red tamarins (*Sanguinus nigricollis*) and one talopoin (*Cercopithecus talapoin*) died of tularaemia. They collapsed and died quickly after showing few symptoms. A second talapoin developed abscesses in the tongue and submandibular area and recovered after treatment (Nayer, Crawshaw & Neufeld, 1979). A golden-headed lion tamarin (*Leontopithecus chrysomelas*) demonstrated profuse salivation, ocular and nasal discharge and severe tongue ulcerations (Hoelzle et al., 2004).

Prairie dogs affected during an outbreak showed rapid deterioration of their status and within two days developed dehydration, ataxia, and severe diarrhoea (La Regina, Lonigro & Wallace, 1986). In an outbreak of tularaemia among captive prairie dogs, unusual lethargy, severe dehydration, and grossly enlarged cervical lymph nodes were characteristic clinical features (Petersen et al., 2004a).

### 3.3.2 Course of disease in laboratory animals

A number of laboratory animals, such as guinea-pigs, rabbits, rats, mice and monkeys, have been used in studies of the course of experimental tularaemia. Experimental work showed guinea-pigs to be highly susceptible to subcutaneous, intraperitoneal, and intranasal challenge with strain SCHU S4 of subspecies *tularensis* (Downs et al., 1947). Rats appear to be much more resistant to *F. tularensis* than other commonly used experimental animals. The subcutaneous and intradermal LD50 of strain SCHU S4 for rats was found to be at least 10 000-fold higher than for mice, rabbits or guinea-pigs (Downs et al., 1947). After an intraperitoneal challenge with SCHU S4, rats developed pyogranulomatous, necrotic hepatitis within 24 h (Moe et al., 1975). Lesions containing mostly macrophages developed within the red pulp in the spleen.

#### Rabbits

Extensive experimental work on tularaemia has been performed in rabbits. Aerogenic exposure resulted in necrosis of the bronchi by day 2 (Baskerville & Hambleton, 1976). Alveoli were distended by neutrophils and necrotic alveolar epithelial cells and the cervical and bronchial lymph nodes were enlarged. Necrotic foci were found in the liver and the white pulp of the spleen. By day 3, necrotic foci had increased considerably in size in lung, spleen and liver, and cervical and bronchial lymph nodes were necrotic.

### Mice

Mice have been widely utilized in the experimental model of tularaemia. Generally, few differences in susceptibility among various inbred strains to challenge with the live vaccine strain (LVS) or strain SCHU S4 have been detected (Anthony, Skamene & Kongshavn, 1988; Shen, Chen & Conlan, 2004). Studies before 1970 mostly used outbred mice whereas a number of studies employing inbred mice have been published after 2002. All available mouse strains appear to be highly susceptible to subcutaneous or aerosol infection with low doses of subspecies *tularensis* or *holarctica* and death occurs within one week at most (Shen, Chen & Conlan, 2004). Conlan and colleagues (2003) found that histopathological changes in the lung were inconspicuous and only small areas of necrosis were visible. Changes in the spleen were more marked and, by day 5, showed almost complete loss of follicle structures and abundant necrotic areas. Liver changes were observed within one day of infection and were characterized by inflammatory necrotic foci. These increased by day 4 with vast areas of necrosis but very little inflammatory response. Many of these changes were similar for subspecies *holarctica* and subspecies *tularensis* (Conlan et al., 2003). Overall, it appeared that the histological findings did not distinguish an infection caused by subspecies *holarctica* from that caused by subspecies *tularensis*.

### Monkeys

Historically, monkeys have been used to study both the pathogenesis of tularaemia and to evaluate vaccines. Grivet monkeys administered strain SCHU S4 intranasally became moribund from day 4 (Baskerville, Hambleton & Dowsett, 1978). Most of the pulmonary infection was confined to the upper respiratory tract as evidenced by copious mucopurulent oculonasal discharge. On histological examination, all monkeys developed extensive necrotizing rhinitis and pharyngitis. The lower airways only became infected on day 6 after administration. Concomitantly, regional lymph nodes, spleen and liver became heavily infected. In the liver, numerous macroscopic surface lesions were present by day 4. These consisted mostly of necrotic hepatocytes and Kupffer cells. By day 7, granulomas were beginning to develop at foci of liver infection. In the spleen extensive necrosis of the white and red pulp occurred.

Two studies examined tularaemia in Rhesus macaques challenged by an aerosol of a strain of subspecies *holarctica* (Schricker et al., 1972; Hall, Kovatch & Schricker, 1973). Mortality (3–18%) correlated to the number of bacteria inoculated ($10^3$–$10^6$ CFU). Infectious foci in the lungs showed mixed infiltrates of polymorphs, lymphocytes and histiocytes and progressed within 6 to 21 days to focal liquefactive necrosis followed by granuloma development. Hepatic changes consisted of circumscribed areas of mononuclear infiltrates, accumulation of mixed inflammatory cells in the sinusoids and enlargement of Kupffer cells. Splenomegaly was observed during the symptomatic period. Congestion of the red pulp and neutrophil accumulation in the sinuses were noted. Thus, tularaemia in monkeys has more similarities to tularaemia in humans than in rodents. See also section 8.2.

### 3.3.3 Serological evidence of tularaemia in wild animals

Seroconversion has been documented in free-living animals, primarily in carnivores such as kit foxes and coyotes (McCue & O'Farrell, 1988; Gese et al., 1997). Other animals which may survive infection and develop immunity also may demonstrate seroconversion (class II in Olsufiev & Dunayeva, 1970; Table 3.1). For example, 21% of the investigated sera from Scandinavian beaver showed increased antibody titres to *F. tularensis* (Mörner, Sandström & Mattsson, 1988). It is likely that seroconversion is indicative of infection with subspecies *holarctica* since subspecies *tularensis* in most animal species causes a uniformly fatal disease.

**Table 3.1**
Animals found to be infected with *F. tularensis*: their sensitivity to tularaemia and epizootiological significance (modified from Olsufiev and Dunayeva, 1970)

| Animal species | Geographical distribution | Potential[a] | References |
|---|---|---|---|
| RODENTS | | | |
| Muskrat (*Ondatra zibethica*) | North America, Europe and northern Asia | Class 1 | Dunaeva & Emel'Ianova, 1950; Parker et al., 1951; Young et al., 1969 |
| Common vole (*Microtus arvalis*) | Central Europe | Class 1 | Nekrasov et al., 1985 |
| Water vole (*Arvicola terrestris*) | Northern, central and eastern Europe | Class 1 | Tropin, 1969; Bell & Stewart, 1975; Nekrasov et al., 1985 |
| Red-backed vole (*Clethrionomys glareolus*) | Central Europe | | Gurycova et al., 2001 |
| Domestic mouse (*Mus musculus*) | Worldwide | | Nekrasov et al., 1985 |
| Mice (*Mus*; *Micromys*; some *Apodemus* spp.) | Worldwide | Class 1 | Gurycova, 2001 et al.; Nekrasov et al., 1985 |
| Lemmings (*Lemmus* spp.) | Alaska; northern Scandinavia | Class 1 | Algazin & Bogdanov, 1978; Berdal et al., 2000 |
| Rats (*Rattus* spp.) | Worldwide | Class 2 | Feldman et al., 2001; Nekrasov et al., 1985 |
| Dormouse (*Dryomys* nitedula) | Central, southern and eastern Europe | Class 2 | Friedl, Heinzer & Fankhauser, 2005 |
| True beaver (*Castor fiber*) | Europe; northern Asia | Class 3 | Mörner, Sandström & Mattsson, 1988 |
| American beaver (*Castor canadensis*) | North America | Class 2 | Bell et al., 1962; Parker et al., 1951 |
| Field mouse (*Apodemus agrarius*) | Eastern Europe and Asia | Class 2 | Gurycova et al., 2001; Nekrasov et al., 1985 |
| Hamsters (*Cricetulus*; *Cricetus*; *Mesocricetus*; *Phodopus* spp.) | Central Asia | Class 1 | MMWR, 2005; Baitanaev & Aikimbaev, 1977 |
| LAGOMORPHS | | | |
| Cottontail rabbits (*Sylvilagus* spp.) | North America | Class 1 | Woolf, Shoemaker & Cooper, 1993 |
| Hares (*Lepus* spp.) | Worldwide | Class 1 | Cerny, 2001 |
| Jack-rabbits, black-tailed hare, white-tailed hare | California, USA | Class 1 | Simons, Stevens & Reeves, 1953 |
| INSECTIVORES | | | |
| Red-toothed shrews (*Sorex* spp.) | North America; Europe and northern Asia. | Class 1 | Gurycova et al., 2001; Khlyap, 1989 |
| Hedgehog (*Erinaceus* spp.) | Europe; Asia | Class 2 | Farhang-Azad, Mescerjakova & Neronov, 1973 |
| Steppe hedgehogs (*Hemiechinus* spp.) | Mediterranean; Cyprus; Asia Minor – south-east Asia. | Class 2 | Difference vs Erinaceus |
| Water shrews (*Neomys* spp.) | Europe; Asia | Class 2 | Mikhailovskii, Litvinova & Sadovnikova, 1981 |
| Moles (*Talpa* spp.) | Europe; Japan | Class 1 | Fujita, Takada & Ohara, 1985 |

[a] Potential sensitivity of animal species to tularaemia classified as: class 1: acute disease after inoculation of 1–10 bacteria; rapid multiplication in blood and tissues; class 2: death after inoculation of $10^8$–$10^9$ bacteria; survival may occur at lower doses and then provide immunity; in the article by Olsufiev (Olsufiev and Dunayeva, 1970), it was suggested that a class 3 also exists, comprising genera resistant to *F. tularensis*; *Canis*, *Felis*, *Meles*, *Nyctereutes*, *Vulpes*, *Mustela*. Since there exists no experimental data on these genera, they have not been included in the Table.

# 4. Clinical expression in humans

Pedro Anda, Andrew Pearson, Arne Tärnvik

Tularaemia is transmitted to humans by arthropod bites (ticks, flies, mosquitoes), by direct contact with infected animals, by contact with tissues or fluids from infectious animals, by ingestion of contaminated water or food, or by inhalation of infective aerosols. There is no human-to-human transmission. Early diagnosis and treatment may be difficult in regions where tularaemia occurs rarely because the clinical expression of the disease is similar to a wide variety of acute infectious diseases. After acquisition, the bacteria replicate in lymphoid tissues of the host. In the acute phase of disease, the bacteria multiply rapidly. The severity of the disease depends on a capability of the patient to mobilize an immunological response. This response depends on cell-mediated immunity rather than on antibodies. Antibodies are good indicators of exposure to the bacterium but do not play a crucial role in protection. See also section 8.2. The mean incubation period of tularaemia is 3–5 days, but may range from 1–21 days. The onset of disease is abrupt, including rapid development of fever with chills, fatigue, general body aches and headache. This is the same for type A and type B tularaemia.

## 4.1 Source of infection and clinical outcome according to *F. tularensis* subspecies

### 4.1.1 Subspecies *tularensis* (type A)

*F. tularensis* subspecies *tularensis* occurs in the USA. It is one of the most infectious pathogens known in human medicine. The bacterium is transmitted among animals and from animals to humans by ticks and occasionally deerfly bites, or by aerosols. Human-to-human transmission by arthropods has never been described. Humans may also become infected by handling infectious animals.

Subspecies *tularensis* can cause serious clinical manifestations and significant mortality if untreated (Dienst, 1963). It may occasionally lead to rhabdomyolysis and septic shock (Kaiser et al., 1985; Provenza, Klotz & Penn, 1986; Klotz, Penn & Provenza, 1987). Before the advent of antibiotics, the fatality rate of type A tularaemia was 5–15% and in its most severe forms as high as 30–60%. Currently, it is less than 2% (Dennis et al., 2001). High fever is accompanied by progressive weakness, malaise, anorexia and loss of weight. Respiratory symptoms, including a dry cough, a sore throat and substernal pain may occur whether or not the disease is acquired by inhalation. Gastrointestinal symptoms such as nausea, vomiting and diarrhoea are more likely to occur in type A than in type B tularaemia.

### 4.1.2 Subspecies *holarctica* (type B)

Type B tularaemia occurs in Eurasia and in North America. In contrast to type A, type B is mainly associated with streams, ponds, lakes, rivers, and semi-aquatic animals such as muskrats and beavers. Therefore, some authors consider type B tularaemia to be a waterborne disease. In favour of this, subspecies *holarctica* has recently been shown to survive and replicate in protozoa (Abd et al., 2003). Nevertheless, subspecies *holarctica* has been found also in hares and other animals. The bacterium is transmitted to humans by direct contact with infectious animals, arthropod bites, aerosols, or ingestion of contaminated food or water.

Type B tularaemia is much less severe than type A tularaemia and fatal cases are rare. In type B tularaemia, fever predominates and is accompanied by focal symptoms and generally milder symptoms than those of type A tularaemia. However, it is frequently associated with suppurative complications (see section 4.2.9) and requires a considerable period of convalescence.

Worldwide, the prevalence of disease caused by type B is much higher than that caused by type A. An increase in prevalence of type B tularaemia has been observed during times of war. During the Second World War, at least 100 000 cases occurred each year. More recently, war-associated outbreaks occurred in UN Administered Province of Kosovo (Serbia) in 2000 and 2003, each comprising more than 300 cases (Reintjes et al., 2002). In contrast to the large Second World War outbreaks in the former Soviet Union, the UN Administered Province of Kosovo (Serbia) outbreaks occurred in areas that were not recognized as tularaemia-endemic. A possible intentional release was therefore suspected; however this was not indicated by a recently developed model to evaluate such suspicions based on scores for a variety of social, political, and clinical criteria (Grunow & Finke, 2002).

### 4.1.3 Subspecies *mediasiatica*

*F. tularensis* subspecies *mediasiatica* is only rarely reported as a cause of human disease. With regard to virulence, it relates more to subspecies *holarctica* than to subspecies *tularensis* (Olsufjev & Meshcheryakova, 1982).

### 4.1.4 Subspecies *novicida*

*F. tularensis* subspecies *novicida* is of low virulence. It occasionally causes disease in immunocompromised individuals. Subspecies *novicida* was first isolated in 1951 from water samples in Utah, USA, and has subsequently been reported as a cause of tularaemia in at least four patients, all from the USA (Hollis et al., 1989; Clarridge et al., 1996). Three patients all had underlying immunocompromising conditions. They presented with high fever, and blood cultures yielded growth of subspecies *novicida*. A fourth patient slowly developed an enlarged granulomatous neck gland, from which the bacterium was isolated. Subspecies *novicida* has also been isolated from patients with various clinical manifestations in Canada, Australia and Spain (Bernard et al., 1994; Escudero et al., 2003; Whipp et al., 2003) which indicates a broad range of clinical manifestations and a wide geographical distribution for this subspecies.

### 4.1.5 *Francisella philomiragia*

*F. philomiragia* is of low virulence and occasionally causes disease in immunocompromised individuals. In such individuals, *F. philomiragia* may cause pneumonia or fever with or without organ manifestations, such as meningitis or peritonitis. Epidemiological data suggest that the source of infection with *F. philomiragia* is associated with salt water. Five out of the 16 cases reported to date (Wenger et al., 1989; Sicherer et al., 1997; Friis-Møller et al., 2004) had a history of sea-water exposure in relation to near-drowning and were treated with high-dose corticosteroids. Seven patients had chronic granulomatous disease, a condition in which the phagocytic host defence is severely compromised.

## 4.2 Signs and symptoms

The clinical manifestations of tularaemia depend also on the route of acquisition (Table 4.1). When the bacteria are acquired through skin or oral mucous membranes, the result is usually a conspicuous enlarged tender node. When bacteria are inhaled, the infection will result in deep lymph-node enlargement.

### 4.2.1 Ulceroglandular and glandular forms

In terms of numbers of epidemics, ulceroglandular and glandular tularaemia are by far the most frequent forms of the disease. In European countries, they may comprise more than 95% of the outbreaks. These forms are acquired by vector-borne transmission, direct contact with an infected animal, or indirect contact such as from tools used while dressing animals. Although acquisition by direct contact may occur in the absence of visible skin lesions, skin injuries will facilitate infection (Anda et al., 2001). Moreover, tularaemia associated with ani-

**Table 4.1**
Clinical forms of tularaemia

| Form | Route of acquisition |
|---|---|
| Ulceroglandular or glandular | Vector-borne and direct contact (touching infected animals or material contaminated with *F. tularensis*) |
| Oculoglandular | Touching the eye with contaminated fingers or possibly from infective dust |
| Oropharyngeal | Ingesting contaminated food or water |
| Respiratory | Inhaling contaminated dust or laboratory-acquired infection |
| Typhoidal | Unknown (probably oral or respiratory) |

mal bites is occasionally reported (Arav-Boger, 2000; Friedl, Heinzer & Fankhauser, 2005).

In ulceroglandular tularaemia, a primary ulcer develops at the site of bacterial exposure. The primary tularaemia ulcer is most often innocent (Figure 4.1) and may not even be noticed until the patient is medically examined. In subjects showing a number of mosquito bites, it may be difficult to decide which bite was infected. At about the time of onset of fever, a small papule appears and within a few days, it develops into a pustule surrounded by a zone of inflammation. Although the ulcer is usually solitary, several papules and pustules may occasionally be found. The ulcer soon heals, leaving a red thin area of 1 $cm^2$ eventually developing into a scar which resembles that of the bacilli Calmette-Guérin (BCG) vaccination. The term glandular tularaemia is used only to indicate that in some cases the primary ulcer is non-detectable.

Within a few days of onset of fever, the patient will perceive a regional lymph node enlargement. The lymph node soon becomes tender and palpable and often even visible. The overlying skin may be reddened and oedematous. Besides fever and unspecific symptoms, the lymph node enlargement is a major cause of concern and will often be the reason why the patient seeks medical attention (Figure 4.2 ). Provided appropriate therapy is started within a week of onset of disease (i.e. fever), the swelling of the lymph node resolves without further complications. However, when there is a treatment delay of more than 2 weeks, the risk of lymph node suppuration is as high as 30–40% (Kavanaugh, 1935; Stuart & Pullen, 1945). In an outbreak of tularaemia in Spain, where the ulceroglandular form was associated with a mean treatment delay of 43.4 ± 29.1 days, the presence of a constitutional syndrome without rigors was observed in most patients (Pérez-Castrillòn et al., 2001). In these cases, the lymph node remained enlarged and became soft upon palpation indicating an insidious melting process. Incision within the first weeks of infection should be avoided because of the risk of local spread of the infection (Kavanaugh, 1935).

### 4.2.2 Oculoglandular tularaemia

By touching the eye with a contaminated finger or possibly by exposure to *F. tularensis*-containing dust, the oculoglandular form of tularaemia may be acquired (Kavanaugh, 1935; Foshay, 1940; Guerrant et al., 1976; Steinemann et al., 1999). This form comprises less than 1% of all human cases of tularaemia, although higher figures (4.2%) have been described in more recent outbreaks (Pérez-Castrillòn et al., 2001). Together with fever and unspecific symptoms, the patients present with a unilateral conjunctivitis, usually expressed as an intense red conjunctiva with granulomatous lesions on the palpebral conjunctiva, swelling of the eyelids, excessive lacrimation, photophobia, and mucopurulent discharge (Chappell, Brainard & Shock, 1981). A large, tender preauricular lymph node will develop, sometimes changing the contour of the cheek.

### 4.2.3 Oropharyngeal tularaemia

Through ingestion of contaminated water or food, the oropharyngeal form may be contracted. A study in Finland between 1967 and 1983 included about 1100 cases of tularaemia of which 127 presented with disease localized in the head and neck region. Of these 127 cas-

es, 32 were compatible with the oropharyngeal form (Luotonen et al., 1986). In Turkey, the oropharyngeal form is common; in a literature survey of Turkish outbreaks between 1936 and 2004, 387 of 507 cases (77%) were referred to as the oropharyngeal form (Karadenizli et al., 2005). The patient presents with an ulcerative-exudative stomatitis and pharyngitis, with or without tonsillar involvement (Foshay, 1940; Hughes & Etteldorf, 1957; Luotonen et al., 1986). Physical examination shows redness and pustular changes in the mouth and pharyngeal mucous membranes. The disease is associated with an excessive regional neck lymphadenitis, although remarkably the lymph node enlargement is most often unilateral. These signs are easily misinterpreted as being caused by streptococci. In that case, patients will receive penicillin which does not affect *F. tularensis*. For instance, in a Turkish outbreak of oropharyngeal tularaemia, diagnosis was delayed and suppurating lymph nodes developed in 40% of the cases (Helvaci et al., 2000).

Before a case of tularaemia can be diagnosed as oropharyngeal tularaemia, ulceroglandular tularaemia needs to be excluded. This is not always easy, because patients with tularaemia acquired by mosquito or tick bites in the head and neck region may show neck lymph node enlargement in the absence of a primary skin ulcer. An epidemiological investigation of the cases' food and water sources may allude to oropharyngeal tularaemia.

### 4.2.4 Respiratory tularaemia

The respiratory form of tularaemia is contracted by inhalation of aerosolized *F. tularensis*. Most frequently, this occurs during farming activities. Outbreaks occur less frequently than those of ulceroglandular tularaemia, but may involve a high number of cases. The source of bacterial aerosols is believed to be carcasses of rodents or lagomorphs remaining in the fields after death from tularaemia. Respiratory tularaemia may present with symptoms of pneumonia, including cough, chest pain, and an increased respiratory rate. It may present with high fever and unspecific symptoms such as nausea and vomiting. Respiratory symptoms may be absent. Pneumonia may occur as a primary manifestation of the respiratory form but may also appear as a complication in any form of tularaemia, due to bacteraemic spread. In respiratory tularaemia, the course of disease differs markedly between type A and type B (Tärnvik & Berglund, 2003).

In type A disease, pneumonia is a fulminant condition which, prior to the advent of antibiotic therapy, was associated with a fatality rate of 30–60%. The onset is sudden with chill, high fever, dyspnoea, dry or productive cough, pharyngitis, chest pain, headache, profuse sweating, drowsiness and general weakness. The condition is extremely severe and may simulate symptoms and signs of typhoid fever, including mental deterioration and a pulse-temperature dissociation (Stuart & Pullen, 1945; Dienst, 1963; Avery & Barnett, 1967; Evans et al., 1985; Gill & Cunha, 1997).

Volunteers exposed to aerosols of virulent *F. tularensis* type A developed acute illness 3–5 days later, including systemic symptoms. Less than half of them had radiological evidence of pneumonia (McCrumb, Snyder & Woodward, 1957; Saslaw et al., 1961b). Obviously, in these studies, the development of pneumonic changes may have been suppressed by an early application of antibiotic treatment. In a majority of patients contracting natural respiratory type A tularaemia, radiographic changes are demonstrable. The changes are highly variable. In the early phase, discrete peribronchial infiltrates may be discerned, followed by bronchopneumonial infiltration in one or more lobes. The X-ray appearance may simulate a wide variety of conditions, including pneumococcal pneumonia, tuberculosis, lymphoma, or carcinoma of the lungs. An intentional aerosol release of *F. tularensis* type A would be expected to result in clinical manifestations similar to those recognized in natural respiratory tularaemia.

Respiratory type B tularaemia has been described in large outbreaks in Sweden and Finland: during the outbreak in Sweden caused by contaminated hay, only 7% of the 140 patients had symptoms of pneumonia (Dahlstrand, Ringertz & Zetterberg, 1971). In contrast, in an outbreak among farmers in Finland, 80% of the 53 cases showed radiological pulmonary

changes (Syrjälä et al., 1985a). Hilar adenopathy was the most common finding and occurred in 13 of 38 cases, followed by pneumonic infiltration (5 of 38 cases) and pleural effusion (1 of 38 cases). See Figure 4.3 for respiratory type B tularaemia with hilar lymphadenopathy and Figure 4.4 for respiratory type B tularaemia with extended pulmonary consolidation.

### 4.2.5 Typhoidal tularaemia

Historically, the typhoidal form was defined as tularaemia devoid of skin or mucous membrane lesion and/or a remarkable lymph node enlargement (Francis & Callender, 1927). This definition dates back to the time when modes of transmission of *F. tularensis* were barely known. The classification of clinical symptoms and signs is now based on a combination of clinical expression and route of acquisition of the infection. A continued use of the term "typhoidal" seems unjustified and confusing (Avery & Barnett, 1967; Syrjälä et al., 1985a). Only when no route of infection can be established may the term still be acceptable.

### 4.2.6 Tularaemia in childhood

Although conforming to general features of the disease, there are some differences in clinical symptoms and signs between children and adults. In Arkansas, USA, where type A tularaemia is prevalent, lymphadenopathy has been reported to occur more frequently in children than in adults, with a more frequent cervical localization and a tendency for delayed suppuration (Jacobs & Narain, 1983; Jacobs, Condrey & Yamauchi, 1985). Also pulmonary manifestations have been observed more frequently (Jacobs, Condrey & Yamauchi, 1985). A relative increase in degree of severity, reported in children in Scandinavia (Uhari, Syrjälä & Salminen, 1990), probably relates to an increased delay of diagnosis of tularaemia in childhood.

### 4.2.7 Tularaemia in immunosuppressed patients

Tularaemia occurs mainly among previously healthy subjects. The experience of tularaemia in immunosuppressed patients is limited. Since cell-mediated immunity is mandatory to host resistance against tularaemia, a fulminant course of disease may be expected in patients with T-cell deficiency, such as lymphoma, HIV disease and in those undergoing corticosteroid or cytostatic treatment.

### 4.2.8 Extraordinary clinical expression of tularaemia

*F. tularensis* has been isolated occasionally from patients hospitalized because of febrile disease of unknown origin. A majority of these patients were compromised by neutrophil deficiency (Maranan et al., 1997; Sarria et al., 2003), transplantation-associated immunosuppressive therapy (Naughton et al., 1999; Khory et al., 2005), stomach cancer (Han, Ho & Safdar, 2004), HIV disease (Gries & Fairchok, 1996) or the presence of a prosthetic medical device (Pittman, Williams & Friedman, 1996; Cooper et al., 1999). Although these subjects lived in areas where *F. tularensis* is endemic, illness was not preceded by obvious exposure events and the route of transmission of *F. tularensis* remained unknown. Thus, *F. tularensis* has to be kept in mind when fastidious Gram-negative coccobacilli are isolated from blood or other deep sites.

### 4.2.9 Complications

Tularaemia is associated with a variety of complications. In ulceroglandular type A tularaemia, pneumonia may present with parenchymal infiltrates and often also with pleural effusion. In an overview from the USA, ulceroglandular type A tularaemia was associated with pneumonia in 30% of the cases (Evans et al., 1985). Only occasionally, hilar lymphadenitis was found. In North America, where type A tularaemia is prevalent, fulminant manifestations are reported, including severe septicaemia (Provenza, Klotz & Penn, 1986), meningitis (Rodgers et al., 1998), endocarditis (Tancik & Dillaha, 2000), hepatic failure (Gourdeau et al., 1983; Evans et al., 1985; Ortego et al., 1986) and renal failure (Tilley, Garman & Stone, 1983; Penn

**Figure 4.1** Primary ulcer in a human case of tularaemia (reproduced with the courtesy of Department of of Infectious Diseases, Umeå University, Sweden).

**Figure 4.2** Lymph node enlargement in a human case of glandular tularaemia (reproduced with the courtesy of Department of of Infectious Diseases, Umeå University, Sweden).

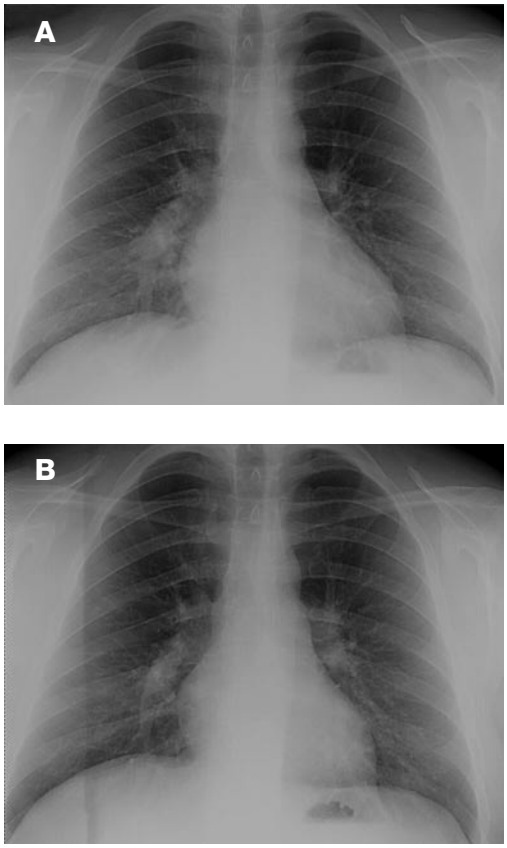



**Figure 4.3** Hilar enlargement in a 24-year-old farmer with fever but with no lower respiratory tract symptoms. Tularaemia was serologically verified and radiography was performed (A) 13 days, and (B) 10 weeks after onset of disease and successful treatment with oral doxycycline (Tärnvik & Berglund, 2003, reproduced with permission from the *European Respiratory Journal*).

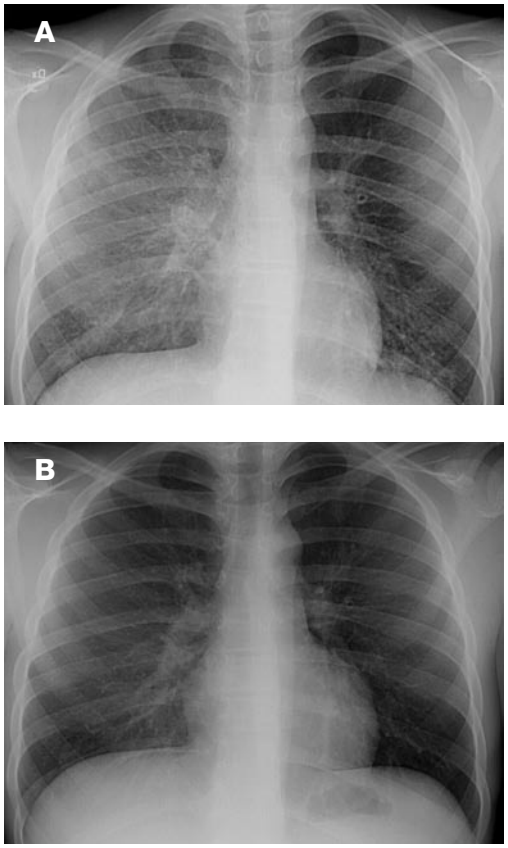



**Figure 4.4** Extended right-sided pulmonary consolidation in a 16-year-old male with fever and a productive cough. Tularaemia was serologically confirmed and radiography was performed (A) 11 days, and (B) 2 months after the onset of disease and successful treatment with oral ciprofloxacin. (Tärnvik & Berglund, 2003, reproduced with permission from the *European Respiratory Journal*).

& Kinasewitz, 1987). Septicaemia associated with type A tularaemia is usually extremely severe and potentially fatal. High fever, abdominal pain and diarrhoea may occur early in the course of disease and may be followed by changes in mental state deteriorating from confusion to coma. A disseminated intravascular coagulation syndrome or an acute respiratory disease syndrome may occur and the patient may succumb to septic shock.

In type B disease, meningitis (Hill et al., 1990) and septicaemia (Tärnvik et al., 1989; Hoel et al., 1991) are occasionally reported. All patients included in these reports recovered. As a complication of type B tularaemia, pneumonia seems to occur only rarely. In a Swedish outbreak in 1981, mainly of ulceroglandular disease, pneumonia was diagnosed in 1 of 400 cases (Christenson, 1984). However, a delay in the diagnosis can increase the incidence of such complications. In a Spanish outbreak, a considerable delay in diagnosis occurred (mean 47.5 pm 31 days) and 5 of the 142 patients developed pneumonia (Pérez-Castrillòn et al., 2001).

In both type A and type B tularaemia, immune-mediated skin reactions occur frequently, including erythema nodosum and erythema multiforme (Perman & Maclachlan, 1931; Syrjälä, Karvonen & Salminen, 1984; Evans et al., 1985; Greco et al., 1987, Akdis et al., 1993).

### 4.2.10 Blood chemistry

Routine blood chemical analyses show sparse abnormalities. In type A tularaemia, the white blood cell count may be normal or raised up to 20 x $10^6$/$mm^3$ and the differential count typically shows a relative increase of lymphocytes. Liver enzymes may show slightly increased values (Evans et al., 1985).

A study of type B tularaemia in Finland comprised laboratory data on 52 cases with pulmonary changes and 42 cases with the ulceroglandular and glandular forms (Syrjälä, 1986). The mean leukocyte count was 8.3 x $10^6$/$mm^3$ and the differential count unremarkable. The mean C-reactive protein value peaked at 53 mg/l, which is remarkably low for an invasive disease. The sedimentation rate, however, remained increased at 30–50 mm/h for the first month after onset of disease.

## 4.3 Differential diagnoses

Tularaemia may be mistaken for a range of other diseases (Table 4.2). Among these are a wide variety of conditions presenting with fever and lymph node enlargement. When the epidemiology is suggestive, tularaemia should be considered in any case of fever of unknown origin.

**Table 4.2**
Differential diagnosis by route of acquisition

| Disease | Route Ulc[1] | Res[2] | Difference from tularaemia |
|---|---|---|---|
| Staphylococcal | x | | Staphylococcal disease such as furunculosis is less often associated with general symptoms |
| Streptococcal | x | | Streptococcal disease is usually associated with intense local inflammation, including erysipelas or more skin lesions or less extensive impetiginous lesions, whereas the tularaemia skin lesion is remarkably mild and limited in size. |
| Anthrax | x | x | Cutaneous anthrax is associated with a skin blister which undergoes necrosis. Although similar to the tularaemia ulcer in that the anthrax lesion is typically painless, it is assocated with extensive tissue damage. Respiratory anthrax develops more rapidly than tularaemia into a toxic fatal state, and this occurs irrespective of antibiotic therapy. In anthrax the radiological examination shows a mediastinal widening due to oedema and bleeding which may not be easily distinguished from mediastinal lymphadenitis such as present in tularaemia. |
| HIV | x | | Highly variable clinical expression, including fever and lymph node enlargement; antibody and/or antigen assays are confirmative. |
| CMV disease | x | | Febrile disease with lymph node enlargement; antibody and/or antigen assays are confirmative. |
| EBV disease | x | | Febrile disease with lymph node enlargement; antibody assays are confirmative. |
| Pasteurellosis | x | | Pasteurellosis usually results from cat and dog bites or scratches and is associated with intense local inflammation, including erythema and oedema. The local inflammation is more pronounced and the lymph node enlargement less prominent than in tularaemia. *Pasteurella multocida* is easily isolated from wound specimens. |
| Mycobacteriosis | x | | Mycobacterial disease may present with the same kind of local lymphadenopathy as tularaemia, although at a much slower rate of development. Moreover a cytological or histological specimen may show indistinguishable patterns in mycobacterial disease and tularaemia, including lymphocytes, epitheloidal cells and multinuclear giant cells. Mycobacterial disease is confirmed by demonstration of acid-fast bacilli; culture of the bacteria and PCR. |
| Rickettsiosis | x | | The spotted fever group of rickettsiosis, including Rocky Mountain fever in North America and fièvre boutonneuse in the Mediterranean region and Africa, present with fever and skin manifestations and are spread by ticks. Rocky Mountain typically involves an exanthema and the fièvre boutonneuse the development of eschar. Rickettsiosis is usually confirmed by demonstration of serum antibodies. |
| Toxoplasmosis | x | | Toxoplasmosis is a febrile zoonosis which may show lymph node enlargement and fever and which is usually confirmed by demonstration of serum antibodies. |
| Cat-scratch disease | x | | Cat-scratch disease is caused by *Bartonela henselae* and acquired by contact with cats; most often involving scratches. The primary skin lesion and lymph node enlargement are similar to tularaemia, but fever and other systemic symptoms are less pronounced. Laboratory confirmation is not easy although culture from blood may be successful. |
| Plague | x | x | Bubonic plague develops within 2–6 days of exposure such as by a flea bite. Buboes, which are enlarged lymph nodes, become extremely swollen and tender. The course of disease is usually more rapid and general symptoms more fulminant than those of ulceroglandular tularaemia. Septic shock may ensue, resembling shock associated with Gram-negative septicaemia. Plague pneumonia is rapidly fulminant, producing copious watery or purulent sputum production, haemoptysis, respiratory insufficiency and shock. It is almost invariably fatal. Plague is suspected by epidemiological circumstances and demonstrated by isolation of the causative agent. |

***Table 4.2*** *(contd)*

| Disease | Route Ulc[1] | Res[2] | Difference from tularaemia |
|---|---|---|---|
| Lymphoma | x | | Lymphoma is an obvious differential diagnosis of concern both in glandular and respiratory tularaemia. Hilar lymphadenopathy in tularaemia may be radiologically indistinguishable from lymphoma (see Figure 4.2). |
| Brucellosis | x | | Brucellosis is a febrile zoonosis which may show few specific signs and symptoms. It may be confirmed by blood culture and also by serology. |
| Hantavirus disease | x | | Hantavirus pulmonary syndrome is a severe febrile noncardiogenic pulmonary oedema which is spread by aerosols from rodents and occurs in the Americas. Hantavirus disease is confirmed by serology. |
| Leptospirosis | x | | Leptospirosis is a febrile zoonosis which is predominantly spread by rodents. It is usually confirmed by serology. |
| Influenza | x | | Influenza is rapidly diagnosed by direct immunofluorescent assay of respiratory secretions. PCR is a more sensitive method performed to an increasing extent in microbiological laboratories. |
| Atypical pneumonia | x | | Pneumonia caused by *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* or *Legionella pneumophila* may resemble respiratory tularaemia. Epidemiological circumstances including spread among humans (*Mycoplasma*, *Chlamydia*) and association with irrigation and air-conditioning systems and showers (*Legionella*) may be suggestive. Usually serology will be confirmative. |

[1] Ulceroglandular; [2] Respiratory.

# 5. Treatment

Pedro Anda, Andrew Pearson, Arne Tärnvik

Despite improvements in therapy over the past decades, type A tularaemia is still associated with fatal outcome. Type B tularaemia, although much less virulent than type A, is frequently associated with suppurative complications and a considerable period of convalescence. In patients with prolonged fever of unknown origin, an empiric trial of medication with beta-lactam antibiotics is often initiated. However, these will not affect *F. tularensis* and will thus have no effect on the course of disease. Currently, no vaccine against tularaemia is available (see section 8.2). Early recognition and appropriate antibiotic treatment of the disease is essential. The following recommendations are derived from Dennis et al., 2001.

## 5.1 Recommendations for treatment and prophylaxis

### 5.1.1 Adults

In severe tularaemia which requires hospitalization, parenteral administration of an aminoglycoside is the first choice for treatment. Gentamicin is preferred at 5 mg/kg daily, divided into two doses and monitored by assay of serum concentrations of the drug. If streptomycin is available, it is an alternative given by intramuscular injection 2 g daily, divided in two doses, for 10 days. In severe cases the treatment period will depend on clinical response, and may comprise more than 10 days. In less severe cases or in a mass casualty setting, oral ciprofloxacin or doxycycline is preferred. Ciprofloxacin 800–1000 mg daily, divided into two doses, may be given intravenously or by oral administration. The treatment period should be 10–14 days. An alternative is doxycycline, 200 mg daily, divided in two oral doses and, due to the bacteriostatic nature of the drug, given for at least 15 days.

### 5.1.2 Children

In cases which require hospitalization, parenteral administration of an aminoglycoside is preferred. The drug of choice is gentamicin, 5–6 mg/kg divided into two or three doses and monitored by assay of serum concentrations of the drug. If available, streptomycin 15 mg/kg twice daily (up to 2 g daily) is an alternative. In milder cases, particularly in areas endemic for the less virulent type B tularaemia, ciprofloxacin 15 mg/kg twice daily (up to 1 g daily), is a feasible alternative. Irrespective of the antibiotic chosen, treatment should be continued for at least 10 days.

### 5.1.3 Pregnant women

This is a situation where potential side-effects have to be weighed against the benefits of treatment of a severe infection. Even though gentamicin and ciprofloxacin are not approved for administration in pregnancy by the Food and Drug Administration of the USA, their use in pregnancy in tularaemia has been recommended by a working group on civilian biodefence (Dennis et al., 2001). Ciprofloxacin is an option and a brief course of gentamicin treatment is an alternative. Doses are the same as for non-pregnant subjects and the treatment period should be individualized.

### 5.1.4 Immunocompromised patients

In severe bacterial diseases, bactericidal agents are usually preferable for treatment in immunodeficiency. In tularaemia, however, there is little experience in this category of patients. An aminoglycoside should be the first-line drug, and ciprofloxacin an alternative.

Doses are the same as for non-compromised subjects and a treatment period of 14 days may be required. In case of the need for prolonged treatment, an initial aminoglycoside administration may be followed by a period of oral or parenteral ciprofloxacin treatment.

### 5.1.5 Post-exposure prophylaxis

Three situations are to be considered:

1. Accidental exposure of laboratory personnel: antibiotic treatment should be initiated within 24 h and a treatment period of 14 days is recommended with either ciprofloxacin 1000 mg daily divided in two doses, or oral doxycycline 200 mg daily, divided in two doses.
2. Exposure most likely did not occur (in the laboratory): an increased vigilance may be sufficient, including daily measurement of body temperature for 14 days and a readiness to treat if symptoms appear.
3. Incidental spread of *F. tularensis* by aerosol: potentially exposed persons should be instructed to be alert to the development of fever within 14 days of exposure, and treatment initiated if necessary according to schedule above.

## 5.2 Antibiotic agents

Table 5.1 gives the minimal inhibitory concentrations (MICs) for various antibiotic agents against *F. tularensis*.

**Table 5.1**
Minimum inhibitory concentrations (MIC) (in mg/l) of various antimicrobial agents for *F. tularensis* in three studies

| Agent | Baker[a] | Scheel[b] | Johansson[c] | |
|---|---|---|---|---|
| | | | Type A | Type B |
| Streptomycin | ≤0.5–4 | 2–16 | 0.064–2 | 0.032–1 |
| Gentamicin | 0.25–2 | 2–4 | 0.032–0.25 | 0.016–0.125 |
| Tetracycline | ≤0.25–2 | – | – | – |
| Doxycycline | – | <0.25–2 | 0.125–2 | 0.125–2 |
| Chloramphenicol | ≤0.25–4 | 1–8 | 0.5–1 | 0.25–1 |
| Rifampin | ≤0.03–1 | – | 0.25–2 | 0.125–1 |
| Ciprofloxacin | – | 0.031–0.125 | 0.016–0.064 | 0.016–0.064 |

[a] Including 13 type A and 2 Type B isolates; the reference is: Baker, Hollis & Thornsberry, 1985.
[b] Including 20 type B isolates; the references is: Scheel et al., 1993.
[c] Including 8 type A and 16 Type B isolates; the reference is Johansson et al., 2002.

### 5.2.1 Aminoglycosides

Streptomycin became established early as the drug of choice for treatment of tularaemia (Foshay, 1946). However, it is no longer widely used in human medicine because of its potential to cause vestibular toxicity and a frequent appearance of hypersensitivity reactions among the personnel involved in its administration. Therefore, streptomycin is no longer easily available and has largely been replaced by other aminoglycosides. In tularaemia, gentamicin is currently the preferred alternative. Nevertheless, streptomycin is still the drug of choice for tularaemia meningitis.

#### Streptomycin

Streptomycin is bactericidal in vitro and highly efficacious against *F. tularensis*. Out of 224 streptomycin-treated cases documented in the literature, 217 were cured (Enderlin et al., 1994). No relapses occurred and failures were restricted to severe cases, most frequently com-

plicated by renal dysfunction. In a study cited by Enderlin et al. (1994), of 141 patients treated with streptomycin, a mortality of 3% was reported, compared to 33% before the advent of antibiotic treatment.

#### Gentamicin

Although in a murine model, streptomycin was found to be more effective than gentamicin (Mason et al., 1980), most experience in human tularaemia suggests that gentamicin is a useful alternative. Gentamicin is the preferred aminoglycoside for parenteral treatment of severe cases which require hospitalization.

A concern raised with gentamicin is the relatively poor penetration of the drug into cells under in vitro conditions. Nonetheless, gentamicin is internalized by pinocytosis although slowly, and susceptibility assays by in vitro cell systems show that gentamicin is capable of killing intracellular *F. tularensis* (Maurin, Mersali & Raoult, 2000).

In a review of the literature, among 36 patients treated with gentamicin, 31 were cured (Enderlin et al., 1994). Of two relapses, one patient had been treated for only 6 days; the other was subjected to treatment after a delay of 43 days. This patient responded to a 9-day course, but developed tularaemia meningitis after withdrawal of treatment.

### 5.2.2 Chloramphenicol

Chloramphenicol is bacteriostatic. It is now seldom used because it is associated with relapse and with rare but severe side-effects. The only advantage of chloramphenicol is a relatively high penetration into the cerebrospinal fluid which may be of value in treatment of tularaemia meningitis.

### 5.2.3 Tetracyclines

Tetracyclines were widely used to treat tularaemia. Their disadvantage is their bacteriostatic nature and thus the risk of relapses (Sawyer et al., 1966; Enderlin et al., 1994), but tetracyclines are still valuable alternatives for oral treatment of tularaemia. For pharmacokinetic reasons tetracycline, which was used in the 1960s, has now been replaced by doxycycline.

#### Tetracycline

In tularaemia, cell-mediated immunity, and CD4 and CD8 T cells in particular, are mandatory to contain the infection. The peripheral blood T-cell response to *F. tularensis* usually becomes demonstrable in vitro 12–14 days after the onset of disease. Before that time, tetracycline can only be expected to inhibit the bacteria. Consequently, the bacteria will remain alive until bactericidal host mechanisms develop and become able to cope with the infection (Syrjälä et al., 1984; Tärnvik, 1979). To minimize the risk of relapse in case of treatment with bacteriostatic agents such as tetracycline, the treatment period needs to be long enough to allow the cell-mediated immune response to develop.

A relationship between relapse and bacteriostatic action has been confirmed experimentally. When soldiers in the USA were voluntarily infected by an aerosol containing 25 000 SCHU-S4 (type A) organisms (Sawyer et al., 1966), they developed tularaemia within 2–7 days of exposure. Treatment with streptomycin, 2 g daily, divided into two doses, was started on the day of onset of fever and given for 6 days. Rapid cure occurred. When tetracycline was given, 0.5 g four times daily for 6 days, defervescence occurred as rapidly as with streptomycin but was followed by relapse within a week of withdrawal of treatment. In treatment trials, a daily dose of 2 g of tetracycline for 10 days or 1 g for 15 days was insufficient to prevent relapses. Only by increasing the dose to 2 g daily for 15 days, was the disease suppressed completely. These experimental data have been corroborated by experience from treatment of natural tularaemia. Among 50 cases reviewed, tetracycline treatment resulted in relapse in six cases (Enderlin et al., 1994). In at least three of these cases, the treatment period was less than 7 days.

Tetracycline has been tested as a prophylactic agent against tularaemia in volunteers. When initiated 24 h after exposure, oral tetracycline at a daily dose of 2 g for 14 days or 1 g for 28 days was sufficient to prevent disease, whereas 1 g daily for 14 days was not (Sawyer et al., 1966).

#### Doxycycline

A daily dose of 200 mg doxycycline is believed to correspond to 2 g of tetracycline. Based on experimental and clinical data on older formulations of tetracycline and by taking data on the immune response into consideration, 200 mg of doxycycline daily, divided into two oral doses, for at least 15 days is recommended in adults. On such a regimen, a mean serum concentration of 4 mg/l will be reached (Welling et al., 1977). This dose is expected to result in optimal antibacterial activity, since an MIC of 0.25–2 mg/l is reported by various laboratories (Table 5.1). A dose of 100 mg daily for 3 weeks might be an alternative, but is less convincingly supported by data from the literature. The side-effects of doxycycline are mild, mostly limited to gastrointestinal effects and mitigated by taking the drug with food. Unfortunately, doxycycline and other tetracyclines are not recommended for use in children under the age of 8 years, due to possible adverse effects on developing teeth.

### 5.2.4 Quinolones

More recently, quinolones have been introduced as new options for oral treatment of tularaemia. Most data are so far restricted to ciprofloxacin and to clinical use in type B tularaemia. Agar dilution assay and E-tests have shown MIC values for quinolones in *F. tularensis* in the range of 0.02–0.1 mg/l (Table 5.1) and broth dilution tests a MIC value of 0.25 mg/l and a minimum bactericidal concentration (MBC) also of 0.25 mg/l (Maurin, Mersali & Raoult, 2000), verifying the bactericidal activity of the agent. In Scandinavia, a first clinical report included four patients treated with oral ciprofloxacin 750 mg twice daily and one patient treated with norfloxacin 400 mg twice daily; all recovered within a few days without relapse (Syrjälä, Schildt & Raisainen, 1991). In further studies, 12 children were successfully treated (Johansson et al., 2000a) and 41 of 43 adults (24 with ulceroglandular, 3 with pulmonary, and 14 with typhoidal tularaemia) showed excellent responses to oral ciprofloxacin given for 10 days (Johansson et al., 2001a). In a tularaemia epidemic in north-western Spain, comprising 142 patients, the efficacy of ciprofloxacin was reported to be higher than that of streptomycin or doxycycline and moreover, ciprofloxacin treatment was associated with fewer side effects (Pérez-Castrillòn et al., 2001). In another report from Spain, relapse was recorded in seven of 14 patients treated with ciprofloxacin (Chocarro, Gonzalez & Garcia, 2000), although in that group of patients, treatment failure may have been due to a considerable delay from onset of disease to start of treatment.

Apart from ciprofloxacin, only sporadic cases have been described using quinolones for tularaemia treatment. Two subjects were reported to be successfully treated with levofloxacin; both were acutely ill patients and neither relapsed within 12 months of follow-up (Limaye & Hooper, 1999).

Altogether, quinolones offer new options for treatment of tularaemia. As far as type B tularaemia is concerned, they seem to be useful for children. Although in early studies, quinolones were shown to cause arthropathy in immature animals, the risk is currently considered to be low in humans and the drug to be safe for children. Obviously, the lack of oral alternatives has hampered a rational handling of tularaemia in childhood. A review of 67 children suffering from tularaemia showed a mean duration of symptoms as long as 26 days (range, 8–92 days), probably related at least in part to the fact that 20 patients received drugs known to be ineffective against *F. tularensis* (Uhari, Syrjälä & Salminen, 1990).

The therapeutic use of quinolones has so far been largely restricted to type B tularaemia. In vitro, type A (*F. tularensis* subspecies *tularensis*) also seems to be susceptible to quinolones. Isolates from various areas in the USA were found to have MIC values of ciprofloxacin as low as 0.016–0.064 mg/l (Johansson et al., 2002) (Figure 5.1). Moreover, these type A isolates and

24 type B isolates all showed MIC values < 0.125 mg/l for six different quinolones. It needs to be emphasized, however, that the efficacy of ciprofloxacin for treatment of type A tularaemia has not been proven. On the contrary, there is experience of treatment failure in children in the USA (Jacobs RF, personal communication). It should be recalled also that ciprofloxacin and other quinolones are not approved for use in tularaemia by the Food and Drug Administration of the USA.

### 5.2.5 Other antibiotics

Beta-lactams such as penicillin are ineffective against *F. tularensis*. Although ceftriaxone has been found to be active in vitro, several cases of therapeutic failure following the use of this drug have been experienced (Cross & Jacobs, 1993; Enderlin et al., 1994). Neither is erythromycin a reliable drug for tularaemia, despite a susceptibility of type A organisms. Rifampicin is active in vitro but is not recommended for clinical use, due to a potential for induction of resistance (Bhatnagar et al., 1994; Johansson et al., 2000a). Co-trimoxazole and clindamycin are ineffective.

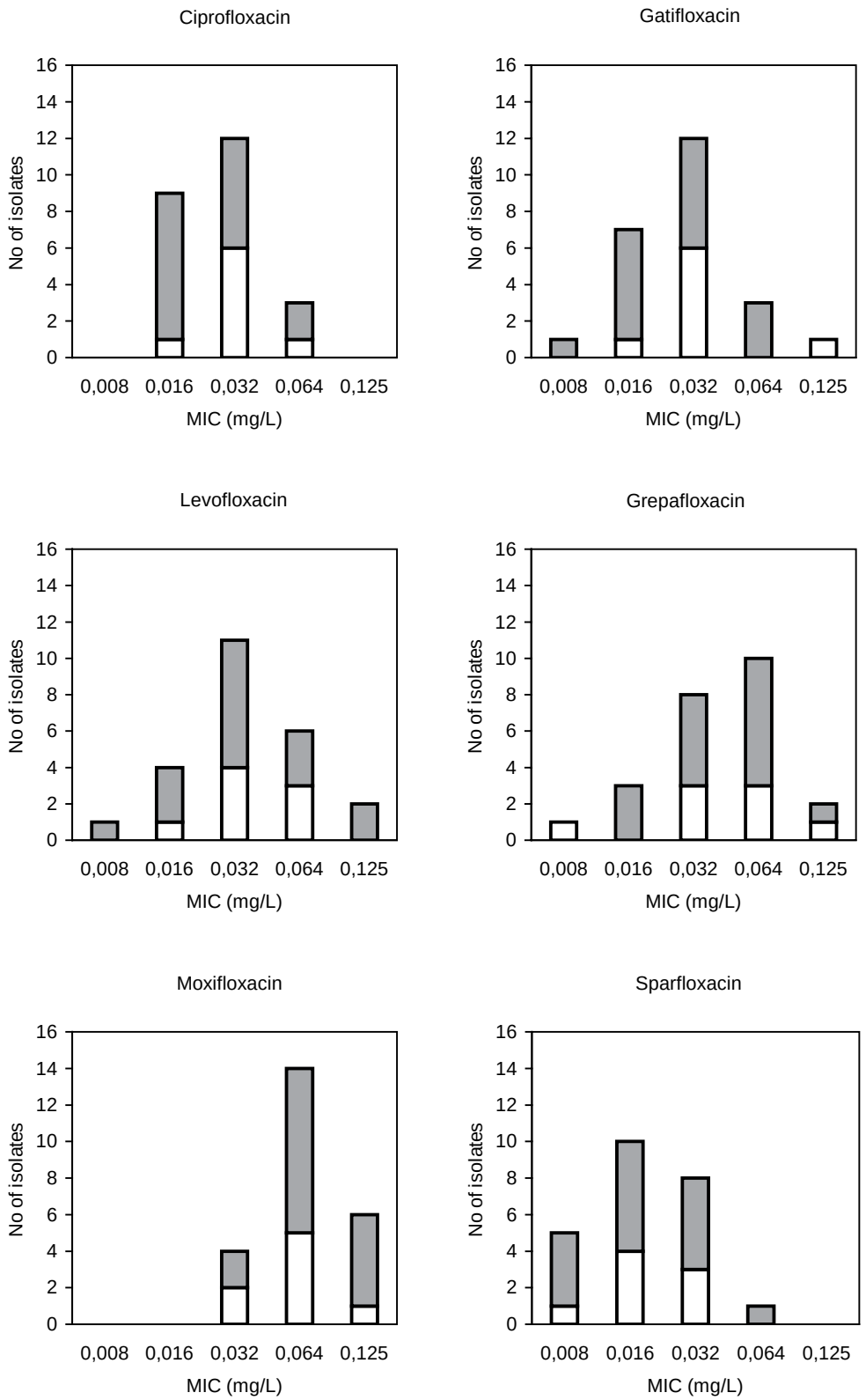
## 5.3 Antibiotic resistance of *F. tularensis*

No natural resistance in *F. tularensis* to antibiotics used for clinical therapy has been demonstrated. This is true for aminoglycosides, tetracyclines, chloramphenicol, and quinolones. Erythromycin resistance, however, is prevalent in Europe but not in North America. Although erythromycin is not included among agents used for treatment of tularaemia, erythromycin resistance may be used as an epidemiological marker.

For experimental purposes, streptomycin- and tetracycline-resistant strains of *F. tularensis* have been developed and most probably, quinolone resistance is easily introduced as well. Yet, the risk for development of antibiotic resistance of importance in clinical practice is low. The reason is that tularaemia in humans is the end-stage of the infection. Most evidence indicates that *F. tularensis* is not transferred from one family member to another or from patients to hospital staff. Neither are there any data to suggest that tularaemia might be spread among humans by mosquitoes.

Due to the potential use of *F. tularensis* for bioterrorism, antibiotic resistance remains of concern. Consequently, methods for rapid determination of the susceptibility of *F. tularensis* to various antibiotics, including aminoglycosides, tetracyclines, chloramphenicol, quinolones, and rifampicin are needed.

**Figure 5.1** Distribution of MIC values for six quinolones. Open bars represent isolates of *F. tularensis* subspecies *tularensis*, and grey bars represent *F. tularensis* subspecies *holarctica*. (Johansson et al., 2002, reproduced with permission from the *Scandinavian Journal of Infectious Diseases*).



# 6. Laboratory diagnostics and discrimination of subspecies and strains

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## 6.1 Case definitions

The case definitions that describe the criteria for diagnosis of tularaemia are: suspect, presumptive and confirmed. The criteria for each are:

*Suspect.* An exposure history consistent with risks known to be associated with tularaemia together with clinical symptoms compatible with tularaemia.

*Presumptive.* Suggestive clinical symptoms and a clinical sample that tests positive for tularaemia by antigen or DNA detection. A single positive serum is also considered presumptive.

*Confirmed.* Recovery of an isolate and identification of the culture as *F. tularensis* by antigen or DNA detection. Alternatively, paired serum specimens with a fourfold difference in titre (tube or microagglutination assay) or significantly (ELISA), with at least one serum positive, are also considered confirmatory.

## 6.2 Diagnostic specimens

### 6.2.1 Human

In principle, specimens for recovery of live bacteria should be collected before antibiotics are administered. In cases with preceding or ongoing therapy, culture may still be useful, particularly when beta-lactam antibiotics or other agents inactive against *F. tularensis* were used. The choice of specimen for diagnostic testing is dependent on the form of clinical illness; ulceroglandular, glandular, oculoglandular, oropharyngeal, respiratory, or typhoidal. The following specimens are acceptable for the various forms of illness as specified:

*Blood.* Whole blood (for all clinical forms of illness).

*Serum.* Serum is preferred; plasma and whole blood dired on paper filter may be an acceptable alternative. A first specimen should be collected as early in the course of infection as possible, followed by a second specimen taken in the convalescent period (at least 14 days later and preferably 3–4 weeks after onset of symptoms). Serum is acceptable for all clinical forms of illness.

*Respiratory secretions.* Pharyngeal swabs, bronchial/tracheal washes or aspirates, sputum, transthoracic lung aspirates, or pleural fluid collection (for respiratory, typhoidal, oropharyngeal forms of illness).

*Swabs.* Swabs of visible lesions or affected areas should be collected (for ulceroglandular and oculoglandular forms of illness).

*Aspirates.* Aspirates from lymph nodes or lesions (for ulceroglandular, glandular, and oropharyngeal forms of illness).

*Tissue biopsies.* Tissue samples from lymph nodes (for ulceroglandular, glandular, and oropharyngeal forms of illness). Invasive sampling, such as incision of an affected lymph node, should be avoided during the acute stage of disease. Experience indicates that such intervention may further the spread of the infection. There is little experience on the value of biopsies for diagnostic purposes.

*Autopsy materials.* Samples from visible abscesses and from lymph node, lung, liver, spleen, cerebrospinal fluid, and bone marrow.

### 6.2.2 Mammals

*Serum.* Serum is the preferred specimen but plasma and whole blood dried on filter paper may be acceptable alternatives. A second specimen should be taken at least 14 days, and preferably 3–4 weeks after the onset of symptoms (serum is acceptable for all clinical forms of illness).

*Aspirate.* Aspirates from lymph nodes or lesions (for ulceroglandular, glandular, and oropharyngeal forms of illness).

*Autopsy materials.* Samples from visible abscesses and from lymph node, lung, liver, spleen, cerebrospinal fluid, and bone marrow are acceptable specimens. See section 7.4.4 for recommendations on the collection of samples from dead animals.

### 6.2.3 Arthropods

Ticks, deer flies and mosquitoes have all been implicated in the transmission of tularaemia. For recovery of live organisms, vectors should be transported live or frozen at -80 °C. Mouse inoculation can be used to isolate pathogenic *F. tularensis* subspecies from infected ticks and deerflies (Klock, Olsen & Fukushima, 1973; Hubalek et al., 1996). Molecular assays have also been described for testing ticks (Goethert, Shani & Telford, 2004; Kugeler et al., 2005). Multiple targets should be assayed and amplification products sequenced. *Dermacentor* spp. have been shown to harbour *Francisella*-like endosymbionts that cross-react with molecular targets used for the detection of *F. tularensis* (Scoles, 2004; Kugeler et al., 2005).

### 6.2.4 Environmental specimens

Despite the fact that *F. tularensis* is often referred to as living for months in mud or contaminated soils, environmental samples such as water, soil and rodent faeces should only be collected in the context of an epidemiological investigation. Optimal methods for recovery and identification of *F. tularensis* from environmental samples do not yet exist. Prior to application of any methodology for the detection of *F. tularensis* in environmental samples, the sensitivity and specificity of the test should be determined on spiked samples. If samples are collected in the context of an epidemiological investigation, samples should be split into two fractions. One set of samples should be spiked with a known number of *F. tularensis* in order to test the purification protocol prior to diagnostic testing of unspiked samples. In addition, molecular assays must also be evaluated for cross-reactivity with *Francisella*-like organisms present in soil and water (Barns et al., 2005).

### 6.2.5 Collection and transport of human specimens

Every effort should be made to collect and preserve specimens so that viable bacteria can be recovered. Decontaminate the surface area prior to specimen collection since contamination of the sample with normal flora could interfere with interpretation of culture results. Ensure that adequate volumes (depending on type of sample) are collected to avoid false negatives as a result of insufficient sample volume. Specimens should be labelled clearly with the patient's name, identification number, source, specific site, date, time of collection, and the initials of the collector. To minimize loss in viability, specimens should be delivered to the laboratory within 24 h, preferably within 2 h. In case of prolonged transport (i.e. > 24 h), survival of *F. tularensis* is uncertain. Provided the appropriate transport medium is utilized (contact with laboratory recommended), molecular diagnostic techniques can still be applied.

*Blood.* Two or more venous blood samples should be obtained, preferably from separate sites and comprising in total 30–40 ml, into bottles of a conventional blood culture system, such as the BACTEC system. Transport directly to the laboratory at room temperature. Hold at room temperature until placed onto the blood culture instrument or incubator. Do not refrigerate, but keep at ambient temperature (10–37 °C). If whole blood is to be tested by PCR, it is recommended that heparin is not used as an anticoagulant as it may inhibit the PCR reaction.

*Serum.* Serum is obtained by drawing the blood into a serum separator tube containing no additives or anticoagulants, allowing it to clot, and centrifuging to separate the serum. If serum is required for testing, separation from blood should take place as soon as possible after collection, preferably within 24 h at ambient temperature. Centrifuge for 10 min at 3000 revolutions per min and transfer the serum into a plastic transport tube. Sera may be stored at 2–8 °C for up to 10 days. If testing is delayed for a long period, serum samples may be frozen. If separation on site is not possible, or is inadvisable for safety reasons, the blood sample should be stored at 2–8 °C. Do not freeze.

*Biopsy.* Obtain a tissue specimen. If the lesion is large or there are multiple lesions, collect multiple specimens from representative sites. Submit tissue or scraping in a sterile container. For small tissue samples, add several drops of sterile normal saline to keep the tissue moist. Transport at room temperature for immediate processing. If processing of specimen is delayed, keep specimen chilled (2–8 °C). Invasive sampling, such as incision of an ascending lymph node, should be avoided during the acute stage of disease. Experience indicates that such intervention may further the spread of the infection. There is little experience on the value of biopsies for diagnosis.

*Swabs.* Take samples of fluid with a sterile swab. Try to get as much fluid as possible onto the swab. If using a swab transport carrier, the swab should be reinserted into the transport package and the swab fabric moistened with the transport medium inside the packet. For transport of specimens for PCR analysis, a buffer solution containing a nuclease inhibitor should be used (Johansson et al., 2000b). For culture, a rayon-tipped plastic applicator and a tube containing Amies agar with charcoal (Copan Italia, Brescia, Italy) showed good preservation (Johansson et al., 2000b). Transport at 2–8 °C; room temperature is acceptable. If processing of the specimen is delayed, store it at 2–8 °C.

*Respiratory or sputum specimens.* Collect specimens in screw-capped containers and transport them to the laboratory as quickly as possible to reduce overgrowth by commensal oral flora. For transit periods up to 24 h, transport bacterial specimens at ambient temperature. If processing of the specimen is delayed, keep it chilled (2–8 °C).

*Aspirate.* Aspirate the fluid from the abscess with a sterile needle and syringe. Transfer the aspirate aseptically into a sterile tube. Transport the sample at room temperature for immediate processing. Keep the specimen chilled (2–8 °C) if processing is delayed.

*Autopsy specimens.* Obtain a tissue specimen. If the lesion is large or there are multiple lesions, collect specimens from representative sites. Submit tissue or scraping in a sterile container. For small tissue samples, add several drops of sterile normal saline to keep the tissue moist. Transport at room temperature for immediate processing. If processing of specimen is delayed, keep the specimen chilled (2–8 °C). Formalin-fixed specimens can also be prepared and must be packaged separately from unpreserved autopsy specimens for bacterial isolation.

### Regulations for transport of diagnostic and infectious specimens

Specimens to be shipped to diagnostic laboratories require special attention to safe packing of the material. See Annex E for transport guidelines.

## 6.3 Bacteriology

*F. tularensis* is a tiny, faintly-staining, Gram-negative, non-motile, non-spore-forming bacterium (Chu & Weyant, 2003; Sjöstedt, 2005). Cells are pleomorphic, typically appearing as short rods or coccoid forms. A double-layered cell wall surrounded by an outer layer and electron-transparent capsule encases the bacterium. Large amounts of lipids are contained in both the cell wall and capsule. The fatty acid composition is unique, being high in saturated

even-chain acids ($C_{10:0}$, $C_{14:0}$, $C_{16:0}$) and two long-chain hydroxyl acids (3-OH $C_{16:0}$, 3-OH $C_{18:0}$) (Jantzen, Berdal & Omland, 1979). The lipid A portion of the lipopolysaccharide (LPS) is modified by the addition of a phosphate-linked galactosamine in at least one strain of subspecies *holarctica* (Phillips et al., 2004). Electron micrographs have shown the presence of pili on the surface of *F. tularensis* (Gil, Benach & Thanassi, 2004). *F. tularensis* is aerobic and considered a fastidious organism due to its requirement of cysteine for enhanced growth (Chu & Weyant, 2003; Sjöstedt, 2005). The organism is relatively inert biochemically, with only a few sugars (glucose, maltose, sucrose and glycerol) utilized. *F. tularensis* does not reduce nitrate, is oxidase and urease negative and weakly catalase positive. Personnel handling diagnostic cultures of *F. tularensis* are at considerable risk of infection and need to practise appropriate precautionary measures (see sections 6.6 and 8.1).

## 6.4 Diagnostic tests

Diagnosis of tularaemia is based on recovery of an isolate, antigen or molecular detection, and serology. Protocols for these methods can be found in Annex A. Accurate identification of *F. tularensis* in diagnostic laboratories relies heavily on the use of specialized reagents or media. Commercial biochemical identification systems available in clinical diagnostic laboratories cannot be relied upon to accurately identify *F. tularensis*. If a diagnosis of tularaemia is suspected, the physician should notify the laboratory in order to increase the likelihood of detection.

### 6.4.1 Serology

Serology is commonly used for confirmation of tularaemia. Antibody responses against *F. tularensis* are generally detectable in patients 10–20 days post-infection (Koskela & Salminen, 1985). Agglutination, either microagglutination or tube agglutination, is the standard serological test used for determining the presence of antibody to *F. tularensis* (Brown et al., 1980). The protocol for microagglutination is given in section A.5.1. Antigens used to measure immune response include FopA, LPS, and an outer membrane carbohydrate-protein fraction (OMP) and whole killed cells. ELISA tests have also been utilized, with the caveat that IgM responses may be sustained for long periods and are thus not indicative of recent infection (Viljanen, Nurmi & Salminen, 1983; Bevanger, Maeland & Kvan, 1994). More recently an ELISA (directed against LPS) combined with Western blot (against antigen extracted from whole killed cells) showed very good sensitivity and specificity for diagnosis of infection (Schmitt et al., 2005). The ELISA protocol is in section A.5.2.

### 6.4.2 Culture

Culture provides a conclusive diagnosis of infection and an invaluable resource for molecular epidemiology, subtyping and discovery of novel species and subspecies. Whenever possible, culture should be attempted. *F. tularensis* grows well on several types of cysteine/cystine-supplemented agar including enriched chocolate agar (CA), cystine heart agar with 9% chocolatized blood (CHAB), buffered charcoal yeast extract (BCYE), thioglycollate-glucoseblood agar (TGBA), and GC Agar II with 1% haemoglobin and 1% IsoVitaleX. For media formulations see section C.1. *F. tularensis* can be isolated from nutrient enriched specimens (tissues) on sheep blood agar (SBA), but cysteine-enriched media are strongly recommended for subculture as the organism will usually fail to thrive with continued passage on SBA. Additionally, growth on CHAB provides presumptive identification of *F. tularensis* as the organism shows characteristic growth on this medium (green, opalescent, raised, shiny colonies at 24–48 h). For growth characteristics by subspecies see secion 6.5.2. Mouse inoculation can also be used for recovery of an isolate. Mice are very sensitive to infection by *F. tularensis* and typically become ill within 3–4 days of inoculation. Tissue samples (spleen and liver) taken from moribund mice can be cultured on agar media to recover live organisms (see protocol A.2.1).

Samples from ulcers frequently yield pure cultures of *F. tularensis* while other specimens may contain contaminant bacteria. CHAB medium supplemented with antibiotics (CHAB-A) significantly improves recovery rates from tissue sources contaminated or overgrown by other flora (Petersen et al., 2004b). In specimens where the presence of mixed flora is likely, the use of CHAB-A should be considered. For blood culture, the BACTEC system or an equivalent system is recommended.

### 6.4.3 Antigen detection

Antigen detection can be useful for either direct identification of *F. tularensis* in clinical specimens or for confirmatory identification of isolates recovered in culture. Direct fluorescent antibody staining, using a FITC-labelled rabbit antibody directed against whole killed *F. tularensis* cells, is a rapid assay for identification of *F. tularensis* in primary specimens or for confirmation of recovered isolates (Petersen et al., 2004a, see protocol A.3.1). Slide agglutination can be used for the rapid confirmation of recovered isolates (see protocol A.3.2). Immunohistochemical staining, using a monoclonal antibody directed against the LPS, can be used to visualize *F. tularensis* in formalin-fixed tissues (Zeidner et al., 2004). In addition, antigen detection can be performed by antigen capture ELISA (Grunow et al., 2000, see section A.3.3).

### 6.4.4 Molecular detection

A variety of PCR methods have been described for the molecular detection of *F. tularensis*. PCR can be a valuable diagnostic tool when organisms are non-cultivable or when culture is not recommended due to biosafety concerns. The majority of PCR tests for *F. tularensis* have been conventional PCR assays targeted at the genes *fopA* or *tul4* encoding the outer membrane proteins (Fulop, Leslie & Titball, 1996; Sjöstedt et al., 1997; Higgins et al., 2000; Johansson et al., 2000b). The *tul4* PCR assay has been validated with specimens from wounds from patients with ulceroglandular tularaemia (Johansson et al., 2000b; see protocol A.4.1). A multitarget real-time TaqMan PCR assay for *F. tularensis* has also been developed (Versage et al., 2003). This assay has increased specificity and rapidity over conventional PCR assays and can provide added sensitivity when testing specimens in which the number of organisms is expected to be quite low. The assay targets include the *ISFtu2* element, *23kDa* and *tul4* genes. This assay has been evaluated with animal tissues, and a limited number of human specimens, but further evaluation with human specimens is needed (Versage et al., 2003; Kugeler et al., 2006).

In cases where diagnostic efforts are not directed specifically towards *F. tularensis*, 16S rDNA sequence identification can be particularly useful (Clarridge, 2004). Universal 16S rDNA primers can be used to accurately identify isolates recovered in culture. *Francisella*-specific 16S rDNA primers should be used for contaminated samples (Forsman, Sandström & Sjöstedt, 1994; see protocol A.4.1).

## 6.5 Supplemental characterization

Once an isolate has been confirmed as *F. tularensis*, a variety of supplemental tests can be used for additional characterization. Protocols for these methods can be found in Annex B. These tests can be extremely useful for determining the source of infection, understanding transmission cycles or identifying species or subspecies.

### 6.5.1 Antimicrobial susceptibility

Antimicrobial susceptibility testing is not routinely performed since naturally occurring resistance to the antibiotics used for clinical treatment of tularaemia has not been reported. Broth microdilution using cation-adjusted Mueller-Hinton broth (CAMHB) with 2% defined growth supplement can be used for susceptibility testing (Clinical and Laboratory Standards

Institute, 2005). The E-test, a quantitative gradient diffusion test, has also been utilized to determine MICs of various antibiotics for *F. tularensis* (Ikäheimo et al., 2000; see protocol B.1.1).

### 6.5.2 Differentiation of species and subspecies

Methods to discriminate subspecies of *F. tularensis* allow the distinction of isolates of subspecies *tularensis* from the other subspecies. This is crucial in the USA where both subspecies *tularensis* and subspecies *holarctica* occur concurrently.

### 6.5.3 Growth characteristics

*F. tularensis* subspecies *tularensis*, *holarctica*, and *mediasiatica* are slow-growing, fastidious organisms generally requiring sulfhydryl compounds for good growth. These three subspecies may be isolated from tissue on SBA. For subculture, cysteine/cystine-supplemented media are generally required as the organism usually fails to thrive with continued passage on SBA. *F. tularensis* subspecies *novicida* and *F. philomiragia* are both non-fastidious organisms and can be isolated on general microbiological solid media, including SBA. The presence of cysteine/cystine in the media can enhance the growth. Most isolates of *F. philomiragia* and *F. tularensis* subspecies *novicida*, but not other subspecies, can grow in nutrient broth containing 6% NaCl.

### 6.5.4 Biochemical biotyping

Differences in biochemical reactivity exist between the species and subspecies (Table 6.1) (Chu & Weyant, 2003; Sjöstedt, 2005). The oxidase test can be used to differentiate between *F. tularensis* and *F. philomiragia*. *F. tularensis* subspecies *tularensis* and *holarctica* differ in citrulline ureidase activity and glycerol fermentation and conventional biochemical assays can be utilized for biochemical differentiation based on glycerol fermentation or citrulline ureidase activity (Sandström et al., 1992). Alternatively, the automated system, BIOLOG, may also be used to detect glycerol fermentation (Petersen et al., 2004a).

### 6.5.5 Molecular biotyping

Molecular differences have been exploited to develop PCR subtyping assays. These PCR assays can provide *F. tularensis* subspecies identification based on size differences in amplified DNA products. Molecular subtyping assays are directed against an upstream region of an RNA helicase (the Ft-M19 assay), the presence or absence of the *ISFtu2* insertion sequence element (ISFtu2 assay), the *F. tularensis* region of difference 1 (RD1 assay) and the *pdpD* gene (pdpD-2 assay) (Johansson et al., 2000c; Broekhuijsen et al., 2003; Johansson et al., 2004; Nano et al., 2004; Petersen et al., 2004a; Byström et al., 2005). Primer sequences and sizes of amplified gene products for the subspecies are given in Table 6.2 and protocol B.2. As many of these assays have only been validated with limited numbers of strains, it is recommended to verify the subspecies by an independent method. Real-time PCR assays have also been developed for classification of *F. tularensis* subspecies *tularensis* and *F. tularensis* subspecies *holarctica* (see protocol B.2.3).

### 6.5.6 Discrimination of strains

Methods to discriminate strains of *F. tularensis* (i) provide the ability to trace the origin of isolates or the source of an outbreak and (ii) enable progress in epidemiological research. A multi-locus variable number tandem repeat assay (MLVA) for *F. tularensis*, based on 25 different repeats in the genome, shows excellent discrimination of individual isolates and provides information regarding population structures and phylogenetic relationships within *F. tularensis* (Johansson et al., 2004). The method also allows accurate subspecies classification. At present, MLVA is the best method for obtaining high-resolution characterization of *F.*

**Table 6.1**
Discriminating characteristics of *Francisella* species and subspecies

| Characteristic | *F. tularensis* subspecies | | | | *F. philomiragia* |
|---|---|---|---|---|---|
| | *tularensis* | *holarctica* | *mediasiatica* | *novicida* | |
| Cysteine/cystine requirement | + | + | + | – | – |
| Carbohydrate fermentation: | | | | | |
| Maltose | + | + | – | weak | + |
| Sucrose | – | – | – | + | + |
| D-glucose | + | + | – | + | weak |
| Glycerol | + | – | + | weak | – |
| Citrulline ureidase production | + | – | + | + | nt |
| Oxidase production[a] | – | – | – | – | + |
| H2S production on TSI | + | | | | |
| Cell size (µm) | 0.2–0.7x0.2 | 0.2–0.7x0.2 | 0.2–0.7x0.2 | 0.7x1.7 | 0.7x1.7 |

[a] Tested with Kovacs reagent; +, positive; –, negative; nt, not tested; TSI, triple sugar iron agar.

**Table 6.2**
Polymerase chain reaction (PCR) methods to discriminate *F. tularensis* subspecies

| Assay primer sequence (5' to 3') | | Product size (base pairs) | | | | |
|---|---|---|---|---|---|---|
| | | *F. tularensis* subspecies | | | | *F. philomiragia* |
| | | *tul*[a] | *hol*[b] | *med*[c] | *nov*[d] | |
| Ft-M19 | F:aggcggagatctaggaaccttt<br>R:agcccaagctgactaaaatcttt | 250 | 220 | 250 | ±250 | na |
| ISFtu2 | Tuf1705:gatagatacacgccttgctcaa;<br>Tu B R431:acccagccaatgcctaaata | 390 | 1248 | nt | 390 | na |
| pdpD-2 | F:tgggttattcaatggctcag<br>R:tcttgcacagctccaagagt | 280 | na | nt | 136[e] | nt |
| RD1 | F:tttatataggtaaatgttttacctgtacca<br>R:gccgagtttgatgctgaaaa | 1522 | 924 | 1453 | 3322[e] | nt |

[a] *tularensis*.
[b] *holarctica*.
[c] *mediasiatica*.
[d] *novicida*.
[e] Only one strain of *F. tularensis* subspecies *novicida* was tested.
F, forward; R, reverse; nt, not tested; na, no amplification.

*tularensis*. Analysis of 192 *F. tularensis* strains indicates that *F. tularensis* subspecies *tularensis* shows more genetic diversity than *F. tularensis* subspecies *holarctica*, suggesting the former subspecies to be older in evolutionary terms. Furthermore, two distinct clades, designated A.I and A.II, within subspecies *tularensis* have been identified (Johannson et al., 2004). Pulsed-field gel electrophoresis (PFGE) has also been used to define two subpopulations of type A (type A-east and type A-west) in the USA. A comparative PFGE and epidemiological analysis of human tularaemia in the USA has provided data suggesting that type A-west infections are clinically less severe than both type B and type A-east infections (Staples et al., 2006).

## 6.6 Biosafety considerations

Human error, poor laboratory techniques, and misuse of equipment cause the majority of laboratory-acquired infections. Safety measures in the laboratory are discussed in section 8.1. A compendium of technical methods to avoid or minimize such problems can be found in Part IV of the WHO Laboratory Biosafety Manual (World Health Organization, 2004b).

### 6.6.1 Diagnostic laboratory-acquired infections

Personnel handling diagnostic cultures of *F. tularensis* are at considerable risk of infection and need to take precautionary measures. Due to the extremely low infectious dose, tularaemia has been one of the most commonly reported laboratory-associated bacterial infections (Pike, 1976; Centers for Disease Control and Prevention, 2000; Shapiro & Schwartz, 2002). Since in most routine laboratories, cultures from clinical samples are handled at the bench, there is an obvious risk of exposure. It is therefore essential that laboratory personnel are informed of the possibility of tularaemia as a differential diagnosis when samples are submitted for diagnostic tests, to ensure that the appropriate biosafety guidelines are followed. *F. tularensis* may be present in lesion exudates, respiratory secretions, blood, tissues from infected animals, fluids from infected animals, and fluids from infected arthropods. Recognized laboratory hazards include contact of skin or mucous membranes with infectious materials, accidental inoculation, ingestion, and exposure to aerosols and infectious droplets.

### 6.6.2 Diagnostic laboratory requirements (biosafety level)

The WHO Laboratory Biosafety Manual provides practical guidance on biosafety techniques for use in laboratories at all levels. For diagnostic laboratories, biosafety levels are also described in this Manual (World Health Organization, 2004b). For diagnostic laboratories biosafety level 2 practices, containment equipment, and facilities are recommended for activities involving clinical materials of human or animal origin suspected or known to contain *F. tularensis*. Biosafety level 3 and animal biosafety level 3 practices, containment equipment, and facilities are recommended, respectively, for all manipulations of suspect cultures, animal necropsies and for experimental animal studies. Preparatory work on cultures or contaminated materials for automated identification systems should be performed at biosafety level 3.

### 6.6.3 Decontamination and sterilization

Information on decontamination and sterilization is available in chapter 8.

# 7. Surveillance and outbreak management

Mats Forsman, Roland Grunow, Michael Kosoy, Gérard Krause

Tularaemia has been reported from most countries of the northern hemisphere. The epizootology of tularaemia is highly complex (Hopla, 1974). For instance, the distribution of cases in humans is patchy in the sense that, within a country or region, cases frequently occur at one location but are absent from surrounding areas. The reason for this distribution pattern is not yet fully understood. The differences in habitat and reservoir between subspecies *tularensis* and subspecies *holarctica* need to be considered in surveillance programmes and during outbreak investigations. Type A tularaemia (subspecies *tularensis*) occurs in the USA in relatively dry environments. It is highly virulent in humans but also in rabbits, sheep and other animals. Type B tularaemia (subspecies *holarctica*) occurs in Eurasia and in the USA. It is less virulent in humans and animals, although in humans it is often associated with severe complications. *F. tularensis* subspecies *holarctica* is mainly associated with streams, ponds, lakes, rivers, and semi-aquatic animals such as muskrats. *F. tularensis* is transmitted to humans by direct contact with infectious animals, arthropod bites, aerosols, or intake of contaminated food or water. Human-to-human transmission by aerosols or via arthropods has never been described. More information on the various vectors for transmission can be found in chapter 3.

## 7.1 Surveillance

### 7.1.1 Concept of surveillance

Surveillance of disease is the continuing scrutiny of all aspects of occurrence and spread of a disease that are pertinent to effective control. Its main purpose is to detect changes in trend or distribution of diseases in order to initiate investigations or control measures. Surveillance generally uses methods distinguished by their practicability, uniformity, and frequently their rapidity, rather than by their complete accuracy. This includes the systematic collection and evaluation of morbidity and mortality reports, special reports of field investigations of epidemics and individual cases, isolation and identification of the infectious agent by laboratories, and information regarding immunity levels (including serological prevalence) in segments of the population. A report summarizing these data should be prepared and distributed to all persons cooperating in the surveillance and others with a need to know the results of the surveillance activities (Last, 1988).

Surveillance needs to be based on clear case definitions (see section 6.1). As shown in analysing an outbreak of tularaemia in UN Administered Province of Kosovo (Serbia) in 1999–2000 (Reintjes et al., 2002), the interpretation of case counts may be complicated by unknown and/or rapidly changing population sizes. Case numbers need to be related to the population at risk of contracting the disease in order to compare disease occurrence over time or across geographical areas. Therefore, considerable effort needs to be made not only to collect reliable and complete data on cases but also to have recent population figures to hand. In outbreak situations, age-group and sex-specific incidence rates may provide hints to the source of infection.

### 7.1.2 Reasons for disease surveillance

A disease surveillance system, when adequately designed, allows not only the early detection of outbreaks and the identification of risk areas, but also assessment of the impact of actions targeted at reducing the size of the outbreak and at preventing further spread of the disease.

Such documentation may do much to mitigate panic and to re-establish normality in a population affected by an outbreak (Dennis et al., 2001; World Health Organization, 2004a). In any disease surveillance system, awareness of the respective disease is crucial. This is more difficult for rare or sporadically-occurring diseases such as tularaemia (Dembek et al., 2003). A surveillance system may also provide scientific insight into the epidemiology of a disease, such as the distribution patterns of *F. tularensis* (Gurycova et al., 2001).

## 7.2 Surveillance in humans

### 7.2.1 Routine surveillance based on notifiable disease reports

Mostly, public health surveillance is based on statutory reporting of notifiable diseases by physicians and/or laboratories to public health departments. This system allows the monitoring of large geographical areas at low cost and with relatively good geographical and socioeconomic representativeness. Reporting compliance among physicians and laboratories needs to be stimulated by regular information on the disease and easy reporting procedures. Reporting forms should be short, simple and unambiguous. A well-established notifiable disease reporting system may facilitate in-depth follow-up. More detailed and/or more reliable data may be collected by specially trained personnel. Such data may also include additional laboratory diagnoses. Tularaemia is a notifiable disease in many countries.

### 7.2.2 Sentinel surveillance

Sentinel surveillance relies on a set of reporting sources that agree to report all cases of one or more specific conditions. This is useful for diseases that are not notifiable and thus for which no routine surveillance system is in place. Sentinel surveillance may also be carried out in addition to routine surveillance in order to collect supplementary data. Due to a higher motivation and/or expertise of the reporting sources, sentinel surveillance is expected to provide qualitatively better data than routine surveillance based on notifiable-disease reports. However, due to a comparatively low number of reporting sources, the general population may not be well represented by the data collected.

### 7.2.3 International surveillance networks

WHO has established an operational function (Global Outbreak Alert and Response Operations) to support the implementation of the international aspects of the International Health Regulations (IHR 2005; World Health Organization, 2006). This function relies on a systematic follow-up of information on suspected outbreaks and provision of support to outbreak response activities. This function includes: "epidemic disease intelligence", which is the pro-active collection of unverified information on possible outbreaks from all available sources (e.g. news wires, web sites, etc); "outbreak verification", which implies verifying the existence of an outbreak and is generally done through official counterparts in ministries of health or UN agencies; and "outbreak response", which implies providing technical assistance to contain the national and international public health consequences of outbreaks and is offered immediately by WHO, but conducted only upon request or acceptance of Member States. From 1 January 2001 to 15 November 2005, WHO monitored four events concerned with tularaemia. Two of these events involved infected pet animals and their shipment to other countries. The other two events involved higher than expected numbers of infections among humans in areas where tularaemia is known to occur naturally. For one of these events, WHO assisted in obtaining the needed diagnostic laboratory materials.

## 7.3 Surveillance in animals

One of the main missions of the World Organisation for Animal Health (OIE), Paris, France is to report on the world animal health situation in all transparency. In order to fulfil its mandate in this respect, OIE manages the World Animal Health Information System, based on the

commitment of Member States to notify to OIE the main animal diseases, including zoonoses such as tularaemia (World Organisation for Animal Health, 2005a). Member States are committed to provide information (immediate notification and follow-up reports), six-monthly reports and annual information, as laid down in chapter 1.1.2 of the Terrestrial Animal Health Code entitled "Notification and epidemiological information" (World Organisation for Animal Health, 2005b) which describes conditions and reasons for notification.

The World Animal Health Information System has two components: first, the OIE Early Warning System which includes an alert procedure to warn the international community of exceptional epidemiological events in Member States. This alert system is aimed at the veterinary services of Member States and other stakeholders, enabling them to take any necessary protective measures as quickly as possible to prevent the introduction of pathogens originating from infected countries. Second, the OIE Monitoring System disseminates monthly and annual information provided by Member States on animal diseases and zoonoses (World Organisation for Animal Health, 2005a). In addition, the OIE publications *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* include standards for laboratory diagnostic tests for tularaemia and the production and control of biological products (principally vaccines) for veterinary use and contribute to the international harmonization of methods for the surveillance and control of this disease (World Organisation for Animal Health, 2005b).

For zoonotic diseases such as tularaemia, surveillance in animals may prevent or minimize outbreaks in humans. Evidence in support of a tularaemia epizootic is a number of carcasses of water rats, mice, muskrats, hares, or rabbits, more than "usual" (Mörner et al., 1988). Systematic surveys of natural foci of tularaemia would allow the early detection of an epizootic, but are highly demanding of labour and resources. Thus experience to date in efficacy of such approaches for tularaemia is very limited. Surveys in animals could be proposed in order to monitor changes in population structure and density of tularaemia susceptible lagomorphs and rodents. These surveys could be achieved by (i) systematic and directed investigation of susceptible mammals and arthropods in a region of interest; (ii) searching and testing carcasses and desiccated remnants (skin, bones) of dead animals; and (iii) examining water and mud samples collected close to places with dead animals or evident rodent activity. Contacting local mammalogists or parasitologists (academic, government) who have been engaged in research in the area may provide information about population trends or even archived blood or tissue samples for baseline determinations. In addition, these experts may provide invaluable logistical support and more "hands". It could be of interest to investigate antibody prevalence in sera of carnivores if available, e.g. within the frame of other prevalence studies. Carnivores can also be indicators of *F. tularensi*s prevalence as they are exposed by consuming infected moribund or dead animals. However, the utility of this approach needs to be investigated.

## 7.4 Outbreak management (humans)

The main tasks of an outbreak investigation are to discover or confirm the causative agent of the outbreak and to limit the risk of exposure. In most cases, tularaemia outbreaks are self-limiting, but large outbreaks may occur under poor hygienic conditions. For instance, this was the case after the war in UN Administered Province of Kosovo (Serbia) in 1999. The situation was worsened by financial, social, and administrative constraints which made it difficult to prevent further spread of tularaemia (Reintjes et al., 2002). In this section, the requirements for an investigation of a tularaemia outbreak will be described.

### 7.4.1 Natural outbreaks

Epidemics in humans may occur after direct contact with infected animals (Greco & Ninu, 1985; Andres-Puertas et al., 1999), vector transmission (Christenson, 1984; Markowitz et al., 1985; Eliasson et al., 2002), oral intake of infected water (Greco et al., 1987; Rogutskii et al., 1997; Anda et al., 2001; Pazdiora et al., 2002; Gurcan et al., 2004), or inhalation of bacteria-containing aerosols or dust (Dahlstrand, Ringertz & Zetterberg, 1971; Syrjälä et al., 1985a;

Feldman et al., 2001). In some outbreaks, multiple modes of acquisition may be involved concomitantly (Lopez et al., 1982; Tikhenko et al., 2001).

### 7.4.2 Outbreaks after deliberate release of *Francisella tularensis*

*Francisella tularensis* has been considered as a possible biological warfare agent and has been stockpiled or otherwise weaponized by States forces (references in World Health Organization, 2004a). Since tularaemia occurs naturally, in most cases a deliberate attack would be rather difficult to differentiate from the natural occurrence of the disease (Grunow & Finke, 2002). However, various "non-conclusive" criteria such as intelligence information may indicate indirectly the possible use of a biological warfare agent. The existence of a threat in terms of a political or terrorist environment or armed conflict, in connection with suspected or proven access to a biological agent and capabilities to deploy it as a weapon, could provide reasons to suspect an intentional release of *Francisella*. Further reasons to suspect a deliberate use of *Francisella tularensis* include: (i) the isolation and identification of genetically-manipulated *Francisella* or of genetic or biological variants uncommon in a given region (e.g. subspecies *tularensis* or *mediasiatica* as a causative agent in an outbreak in Europe would not be expected as a natural occurrence); (ii) an unusual antibiotic resistance in the pathogen; (iii) an unusual incidence of pneumonic or oropharyngeal manifestations which may be associated with a deliberate release of *Francisella* in aerosols or by contamination of food and drinking-water. The recent improvements in genetic identification of *Francisella* genomes and molecular typing may be helpful to differentiate between a deliberate release and natural occurrence of *F. tularensis*. See section 6.5.

### 7.4.3 Outbreak investigation

#### Situations in which an outbreak investigation may be helpful

The possibilities to recognize and investigate a tularaemia outbreak depend on various factors such as the political and socioeconomic situation in the affected area and the availability of laboratory resources and specialists. In general, field investigations may be helpful in the following situations:

- when the source of infection is unclear;
- when the outbreak occurs as an emerging disease in non-endemic areas;
- during the outbreak, when the effectiveness of measures targeted at the elimination of further sources of infection need to be verified or documented;
- when there is a concern of a deliberate release of the pathogen;
- when there are intentions and resources to collect more information about the epidemiology of tularaemia in the context of scientific research programmes.

A checklist of practical advice on how to carry out an outbreak investigation can be found in Annex F. It should be emphasized that the best results and most benefit may be achieved when the investigation is initiated at the earliest possible stage of the outbreak. The most common approach is an epidemiological investigation, e.g. a case-control study, to identify risk factors. This was done during the outbreak in UN Administered Province of Kosovo (Serbia) in 1999 (Reintjes et al., 2002). The study design and the questionnaire used in UN Administered Province of Kosovo (Serbia) are included in Annex F.

#### Confirmation of the outbreak by verification of cases

The confirmation of tularaemia cases should be based on the case definitions given in section 6.1. Any epidemiological investigations should be based on these case definitions. Depending on the situation, it may be necessary to send samples for confirmation to a specialized laboratory in a different country. Specimens to be shipped to diagnostic laboratories require special attention to the safe packing and transportation of the material (see Annex E).

### 7.4.4 Sample collection

In outbreak situations, an extended sample collection should be realized beyond the material that is required for direct diagnostic purposes. The aim is to detect the source(s) of infection, to determine the transmission route(s), and to perform the molecular characterization of the pathogen. Therefore, human diagnostic specimens as well as animal and environmental samples are helpful (see section 6.2).

#### Dead animals

When an outbreak is suspected or when an epizootic is recognized, collection of dead animals after die-offs becomes an important measure. The public should be encouraged by local authorities to report sick and dead animals observed. However, before examining dead animals for tularaemia, deliberate poisoning by humans must be ruled out as the cause of the die-off. When collecting dead animals, the following steps are recommended:

1. Handle animals wearing thick rubber gloves.
2. Place the carcass in double plastic bag.
3. Label the bag containing the carcass.
4. Transport the bag containing the carcass to the field station or to a laboratory authorized to accept specimens for tularaemia analyses. If a fresh carcass cannot be investigated within 24 h, it should be frozen, thereby possibly enabling the isolation of *F. tularensis* later.

#### Necropsy specimens from animals

1. Tissues (fresh frozen, unpreserved) from lung, liver, spleen, lymph nodes, skin lesions and/or kidney: suitable for culture in order to recover live organisms and for identifying DNA by PCR. When animals have died from tularaemia, the pathogen can be isolated most often from liver or spleen; tissues which are easy to obtain.
2. Formalin-fixed tissue (not to be frozen) is suitable for histopathology, immunohistochemistry and PCR. Formalin-fixed specimens must be packaged separately from unpreserved autopsy specimens for bacterial isolation.

#### Other samples

Several other animal or environmental specimens have been subjected to attempts to identify the source of infection with tularaemia. However, systematic studies of the best methods of specimen collection and of the performance of the diagnostic methods applied to such specimens have not yet been done. Observations at the location where dead animals are found are often as important as the carcass itself to determine the source of tularaemia. Observations at the site of collection of a sample should be recorded with reference to collected samples, such as filters, surface swabs, dry materials, etc. In addition, the following may be helpful:

1. Capture of animals/trapping of animals – this can be helpful in outbreak investigations to identify sources of infection and to type the pathogen when dead animals are not found.
2. Animal excrement: extensive die-offs of tularaemia-susceptible animals during epizootics may render the capture of living animals and collection of sufficient samples more difficult. In this situation, collection and examination of animal excrement for detection of *F. tularensis*-specific antigen may be performed.
3. Ticks from captured animals – for detection of *F. tularensis*, ticks may be collected from host mammals or with a drag. Removing ticks from captured animals is relatively simple and can be carried out simultaneously when collecting fleas and other ectoparasites. Whenever possible, ticks should be transported alive in moisturized tubes in an insulated box cooled with ice or cooling packs. If live transportation is not possible, transport ticks on dry ice. This may facilitate culture of *F. tularensis*.

4. Water samples from streams and wells in the affected area – when carcasses of infected mammals that have died of tularaemia remain in streams or wells, the water may become contaminated with *F. tularensis* and may remain contaminated for more than a month. Mammals drinking the water may become infected and local epizootics may occur. Contaminated water may also be highly infectious for humans and domestic animals.

### 7.4.5 Laboratory methods suitable under field conditions

Hand-held assays for detection and identification of *F. tularensis* are currently being developed (Grunow et al., 2000; Emanuel et al., 2003).

Depending on the location, size and duration of the outbreak, it can be helpful to establish a field laboratory. This may be a mobile laboratory which is brought in or a local laboratory which is additionally equipped for diagnosing tularaemia. A field laboratory for tularaemia should be able to perform microagglutination and/or ELISA for antigen and antibody detection (Syrjälä et al., 1986; Sjöstedt et al., 1997; Grunow et al., 2000; Porsch-Ozcurumez et al., 2004). In addition, simple instruments for genetic detection of pathogens under field conditions and biosensors are currently under development. The laboratory may also isolate *F. tularensis*, provided appropriate safety measures are in place (see section 8.1). In addition, the field laboratory will be the focal point for the collection and registration of the samples. It may also prepare samples for shipment to specialized laboratories for confirmation or further research such as molecular characterization (see section 6.4.4).

### 7.4.6 Measures for preventing the spread of tularaemia

During a tularaemia outbreak, the following precautions may be taken:

#### Oropharyngeal tularaemia

Waterborne infection:

— avoid drinking of unboiled water
— disinfect (chlorinate) water which is used for washing or for brushing teeth
— protect water sources from contact with animals such as rats, mice,: etc.

Foodborne infection:

— protect food stores from contact with animals (e.g. trap mice, rats or other rodents)
— avoid eating food which may be contaminated with animal faeces
— wash food with care as the aerosols and dust created can be infectious.

#### Respiratory or ulceroglandular tularaemia

Infectious aerosols, direct contact with infectious animals, and arthropod bites:

— avoid the hunting of hares and rabbits and consumption of hare and rabbit meat;
— wash hands after contact with wild and domestic animals;
— regularly inspect domestic animals for signs of disease; in outbreak situations, avoid close contact with domestic animals such as dogs and cats;
— avoid dust and aerosols (especially relevant for farmers and landscapers) by closing doors of tractors and avoid rooms where aerosols or dust are generated; if such exposure cannot be avoided, wear respiratory masks (protection class FFP3);
— avoid exposure to blood-sucking arthropods by wearing long-sleeved clothing, and using repellents or mosquito nets.

Although currently not available, vaccination of the human population against tularaemia in endemic regions would be most effective.

# 8. Considerations for handling *F. tularensis*

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## 8.1 Safety measures in the laboratory

### Laboratory-acquired infections

Although most naturally-occurring cases of tularaemia are the consequence of vector-borne transmission or from contact with infected animal tissues, the form of the disease which is of by far the greatest concern in the laboratory is respiratory tularaemia. This pathogen is so infectious by the airborne route that it has been stated that "The hazard of infection with *Francisella tularensis* is well recognized; few persons escape illness if they continue to work with the organism" (Overholt et al., 1961). Studies in the USA with human volunteers during the 1950s and 1960s showed that the infectious dose of a strain of *F. tularensis* subspecies *tularensis* was between 10 and 50 CFU (McCrumb, 1961), making this bacterium one of the most infectious pathogens by the airborne route (Feldman et al., 2001). Prior to 1970, the incidence of laboratory-acquired tularaemia was high. For instance, in one laboratory working with *F. tularensis* subspecies *tularensis* the incidence of typhoidal tularaemia was reported to be 5.7 cases per 1000 employees at risk (Burke, 1977). In a detailed analysis of 34 cases of laboratory-acquired infection it was reported that 20 showed pulmonary involvement (Overholt et al., 1961). For the vast majority of these cases, there was no obvious previous exposure of the individual to the pathogen, but the high incidence of pulmonary involvement clearly suggest that most of these individuals contracted the disease after exposure to airborne bacteria (Overholt et al., 1961). The reductions in the incidence of laboratory-acquired tularaemia since the 1970s appear to be due partly to the use of more specialized laboratories with appropriate microbiological containment cabinets, partly to the increased awareness of the risk of infection, and partly to the use of the LVS vaccine (Rusnak et al., 2004).

### 8.1.1 Laboratory biosafety level

There is no global agreement on the assignment of organisms to laboratory biosafety levels. WHO recommends that countries (and regions) should draw up a national (and regional) classification of microorganisms taking into account: (i) the pathogenicity of the organism; (ii) the mode of transmission and host range of the organism; (iii) the local availability of effective preventive measures; and (iv) the local availability of effective treatment. In most countries, the highly virulent subspecies *tularensis* will be classified as risk group 3 (high risk for the laboratory worker, but low community risk) while the other subspecies will be classified as risk group 2 (moderate risk for the laboratory worker, low community risk (World Health Organization, 2004b).

The degree of risk varies not only with the virulence of the organism but also with the material being handled. We recommend that all strains of *F. tularensis* subspecies *tularensis* should be handled at biosafety level 3 while strains of subspecies *novicida* and the LVS strain of *F. tularensis* can generally be handled at biosafety level 2. It is recommended that all handling of clinical samples and cultures suspected to be *F. tularensis* should be performed under biosafety level 3 conditions. In addition, any work which involves the culture of human-virulent strains of *F. tularensis* subspecies *holarctica* or subspecies *mediasiatica* where aerosols of the bacteria may be generated should be carried out at a minimum of biosafety level 3.

A key consideration when handling *F. tularensis* is not only the physical construction of the laboratory, but also the experience of the individuals who will be handling the bacterium. Because of the high infectivity of *F. tularensis* by the airborne route, we recommend that any

individuals who plan to work with *F. tularensis* receive formal training in the methodologies and procedures required for the safe handling of this pathogen. In particular, individuals should be made aware of the possible sources of aerosols and the procedures which minimize the generation of aerosols. In addition, we recommend that new workers are supervised by an experienced individual during their learning phase.

Since *F. tularensis* is one of the most infectious pathogens known, the importance of using appropriate biosafety practices and facilities cannot be overemphasized. Each laboratory should have defined procedures addressing the use of equipment (especially equipment that may generate aerosols); disinfection of equipment and contaminated materials; handling and processing samples; spill containment and clean-up; and waste handling. These procedures should be clearly and concisely written, easily accessible and rigorously followed. Guidance on biosafety level 3 containment and practices and disinfection is provided in the WHO Laboratory Biosafety Manual (World Health Organization, 2004b).

### 8.1.2 Physical requirements for a laboratory handling pathogenic *F. tularensis*

When handling cultures of any pathogenic *F. tularensis* subspecies, biosafety level 3 is prudent. A separate room is required with only one entrance; a biohazard notice prohibiting the entry of unauthorized persons should be prominently displayed at the entrance. Ideally, the room should have a double-door entrance designed to provide an airlock. The ventilation should be arranged to maintain the air pressure within the room at a slightly lower level than its surroundings. Air from the room should be discharged to the exterior, well away from air intakes and opening windows, otherwise it must be sterilized by filtration or heat treatment. The walls should be impermeable and all windows sealed to allow disinfestation and fumigation; it should be safeguarded against infestation with rodents or insects. The room must have a properly installed and tested Class II or III biological safety cabinet. The air exhaust from the cabinet should be so arranged as to avoid interference with the air balance in the room or within the cabinet when it is switched on. The room should have a sink, an autoclave and enough incubator space for all culture requirements. Hand-washing facilities must be provided near the exit.

Biosafety cabinets should be used for all procedures involving human pathogenic strains (including *F. tularensis* strain LVS) and especially when aerosols of bacteria might be generated. These procedures include the growth of bacteria in liquid culture, the growth of large numbers of bacteria (e.g. for research purposes), procedures which couple significant mechanical energy into cells (for example sonication and centrifugation of cultures), or animal experimental work. See the WHO Laboratory Biosafety Manual (World Health Organization, 2004b) for appropriate and useful information on selection and use of biological safety cabinets. Ideally, items of equipment which might generate aerosols should be contained within a purpose-built cabinet.

Centrifuges may cause dangerous aerosols, especially when tubes containing virulent bacteria break. Glass tubes should not be used for virulent materials, instead polycarbonate tubes with tightly-fitting screw-capped lids and rubber O-rings are recommended. When centrifuges are located outside a biosafety cabinet, the centrifuge rotors should incorporate some form of seal that allows the rotor to be removed and opened only within a safety cabinet. Additionally, during centrifugation procedures access to the laboratory should be limited only to individuals wearing some form of respiratory protection such as a respirator. Respiratory protection should only be removed when the rotor has been opened, and the integrity of the centrifuge tubes has been established.

### 8.1.3 Decontamination and sterilization

Materials which are potentially contaminated with *F. tularensis* should be sterilized before their disposal. The bacterium does not form resistant structures and is relatively sensitive to all standard inactivation procedures. Therefore the destruct cycle of inactivation used in auto-

claves is suitable for the inactivation of *F. tularensis*. The bacterium is sensitive to hypochlorite and other commonly-used chemical decontaminants. The thermal inactivation of the bacteria at temperatures below those experienced in an autoclave is possible, for example for the generation of killed cells for immunization, ELISA plate coating or the isolation of DNA or polysaccharides. Typically, heating to 60 °C for 1 h will inactivate bacteria in suspension, but the precise conditions for complete killing need to be ascertained in the laboratory. The temperature and time required for bacterial killing will depend not only on the strain being used but also on the density of the cell suspension and the suspending medium. Care must be taken with this type of low-temperature inactivation to ensure the statistically-significant possibility of killing all bacteria. Therefore it is necessary to first construct a killing curve which will inform the researcher of the conditions required for bacterial killing. Whatever method is used to inactivate *F. tularensis*, it is essential to ensure that samples leaving a biosafety level 3 laboratory, for handling at a lower level of containment, are free of viable *F. tularensis*.

*F. tularensis* is readily inactivated on exposure to UV irradiation. However, the sterilization of safety cabinets by exposure to an internal UV light source is not recommended as the primary method of bacterial killing. If there is a massive spill, safety cabinets used for the handling of *F. tularensis* should be sterilized using formaldehyde vapour, in accordance with the manufacturer's instructions. Formaldehyde decontamination is not necessary for routine work.

### 8.1.4 Health monitoring of laboratory workers

Some form of health monitoring is essential for staff working with *F. tularensis*. Baseline blood samples should be taken before any work commences and staff should carry an "at risk" card indicating that in the event of a febrile illness, accidental exposure to *F. tularensis* should be considered by the attending clinician. In the event of possible exposure to *F. tularensis*, the subsequent development of a fever is likely to provide one of the earliest indications of infection. For more details on the symptoms of tularaemia see chapter 4.

### 8.1.5 Treatment following a suspected exposure

Following likely exposure of an individual to *F. tularensis*, appropriate chemotherapy should commence immediately; at least 14 days of treatment is required. However, the isolation of the individual is not necessary since human-to-human transmission of the disease has not been reported. For details of chemotherapeutic regimes for the treatment of tularaemia see chapter 5.

## 8.2 Vaccines and vaccination

One focus of current research work in the USA and in Europe is to devise a vaccine which could be used to protect individuals from disease caused by the deliberate release of *F. tularensis*. This vaccine would also have a utility for the protection of researchers who are working with highly-virulent strains of *F. tularensis*. Vaccine development was initiated in the 1930s in the former Soviet Union. The most widely studied and used vaccine strain in recent times has been the attenuated Live Vaccine Strain (LVS), which was derived from a virulent strain of *F. tularensis* subspecies *holarctica*. The precise history of the LVS strain is uncertain but it is known to have been derived from strain 15, which was originally generated at the Gamaleya Institute in the former Soviet Union. A mixed ampoule of vaccine strains, probably including strain 15, was transferred to the USA in 1956, and the LVS strain was probably derived from this strain in the USA (Eigelsbach & Downs, 1961; Sandström, 1994). In the past, *F. tularensis* LVS has been studied as an investigational vaccine by the United States Department of Defence (Waag et al., 1992; Waag et al., 1995; Waag et al., 1996) but is not licensed for general use in the USA (Dennis et al., 2001). It is also unavailable in Europe and most other parts of the world, but a live attenuated vaccine is still in use some parts of the former Soviet Union

where it has been used to immunize millions of people against tularaemia (Sjöstedt, Tärnvik & Sandström, 1996). It is possible that future studies will allow either the LVS vaccine or alternative attenuated strains to be licensed. Therefore it is appropriate to provide a summary of the properties of the LVS vaccine.

Of the animal species which have been evaluated as models of disease, the non-human primates and rabbits appear to mimic human disease most accurately. In contrast, mice appear to be uniformly susceptible to disease caused by strains belonging to all subspecies, including *F. tularensis* subspecies *novicida*. Much of the research on mechanisms of virulence and protection which has been carried out in recent years has used the LVS strain of *F. tularensis* in mice. This model of disease is valuable because protective immune responses can be induced by the delivery of this strain intradermally or by scarification while mechanisms of virulence can be investigated when the strain is given by the intraperitoneal or intravenous routes. Many workers have combined these different routes to investigate the protective responses induced by LVS against a subsequent intraperitoneal or intravenous challenge with LVS. In addition, the risk of serious laboratory-acquired infection is significantly reduced when the LVS strain is used rather than a highly-virulent strain.

However, it is also important to consider the limitations of this disease model. There is good evidence that protection against an intraperitoneal or intravenous challenge with LVS can be mediated solely by antibody directed against LPS, whereas protection against highly-virulent strains of *F. tularensis* in mice requires CD4+ and or CD8+ T-cell responses. There is also good reason to believe that these findings reflect the nature of the protective responses in humans; previous studies have shown that the passive transfer of immune serum provides only very limited protection against tularaemia.

The nature of the attenuating lesion(s) in the LVS strain is not known. When delivered intradermally into the mouse, LVS is relatively avirulent and by the subcutaneous route the median lethal dose is approximately $10^7$ CFU (compared with < 10 CFU reported for virulent *F. tularensis* subspecies *tularensis* and subspecies *holarctica* strains). However, by the intraperitoneal route, LVS has a median lethal dose of < 10 CFU (Fortier et al., 1991). The reasons for this route-specific attenuation in the mouse are not known. In addition, different batches of the vaccine show different immunogenicity, a property ascribed to the different proportions of so-called blue and grey colony types (Eigelsbach & Downs, 1961).

Clinical trials with the LVS vaccine have not been carried out, but there are some data from studies with small numbers of individuals which suggest that vaccination by the intradermal route is able to either provide protection against a subsequent challenge with fully virulent *F. tularensis* or to reduce the severity of the disease (Saslaw et al., 1961a; Saslaw et al., 1961b). There is some evidence that the vaccine is effective when given by the airborne and oral routes of delivery (Saslaw et al., 1961a; Saslaw et al., 1961b; Hornick & Eigelsbach, 1966; Hornick et al., 1966). Trials in the USA during the 1950s and 1960s involved volunteers who were immunized with LVS and approximately one year later challenged by the airborne route with 200–20 000 CFU of *F. tularensis* strain SCHU S4. All of the control subjects developed tularaemia and at the earliest indication of disease were treated with streptomycin or tetracycline. All made a complete recovery from the disease. The majority of vaccinees challenged with up to 2000 organisms escaped major clinical illness. Immunized volunteers challenged with 20 000 organisms showed modified disease symptoms compared to non-immunized volunteers infected with a similar dose of SCHU S4 (Eigelsbach, Hornik & Tulis, 1967). In addition to these studies there is evidence that the LVS vaccine has had an impact on the incidence of laboratory-acquired tularaemia. A study published by Burke in 1977 reported the incidence of laboratory-acquired tularaemia before (1950–1959) and after (1960–1969) routine immunization of workers. The incidence of typhoidal tularaemia fell from 5.7 to 0.27 cases per 1000 employees at risk. The incidence of ulceroglandular tularaemia was unchanged but the clinical signs and symptoms of this form of the disease were moderated in vaccinated individuals. Vaccination of laboratory workers may reduce the risk of laboratory-acquired infections (Rusnak et al., 2004).

At this time it is not clear whether or when the LVS vaccine will be licensed. However, the data presented above indicate the value of this vaccine for the prevention of laboratory-acquired tularaemia. Should the LVS vaccine not be licensed, then development of an alternative licensable vaccine may require considerable time. The protective components of the LVS vaccine have not yet been identified, and very little is known about mechanisms of virulence. A rationally attenuated mutant to replace the LVS vaccine seems to be the most likely solution and some of the pathways that might be interrupted to provide attenuation have already been identified (Karlsson et al., 2000). However, techniques for the construction of defined mutants of *F. tularensis* are at an early stage and will need to be further developed before a rationally attenuated mutant can be devised and tested.

### 8.2.1 Genetic manipulation of *F. tularensis*

Although techniques for the genetic manipulation of *F. tularensis* are in their relative infancy, it is important to consider which replicons and antibiotic markers are acceptable for use. Genes encoding resistance to antibiotics that might be used prophylactically or therapeutically should be used with caution, and a full assessment of the risk associated with such manipulations carried out with special reference to the availability of alternative antibiotics for the treatment of disease. *F. tularensis* is naturally resistant to many beta-lactam antibiotics and therefore ampicillin resistance is not a useful marker for genetic manipulation of strains. Similarly, many strains are resistant to erythromycin, although this marker may be useful in erythromycin-susceptible strains. The markers used most frequently in *F. tularensis* include chloramphenicol resistance, tetracycline resistance and kanamycin resistance. Of these, tetracycline and kanamycin resistance markers offer the best selection. Because the tetracyclines are often used as drugs of first choice, the use of tetracycline resistance as a marker may not be acceptable in all countries. Additionally, care should be taken over the selection of kanamycin resistance markers. It is important to use a gene that encodes kanamycin-specific resistance rather than one which provides resistance to a range of aminoglycosides since this might provide resistance to streptomycin and gentamicin. The kanamycin resistance gene found in transposon Tn5 is kanamycin-specific and can be used in *F. tularensis*.

### 8.2.2 Sources of strains for research

Although strains of *F. tularensis* are in principle available from a number of national culture collections, the release of strains from these sources is now carefully controlled. In some countries additional legislation operates to regulate the acquisition of strains of *F. tularensis* from any source. For example, in the Russian Federation the export of killed or live bacterial cells or even of DNA to any other country is prohibited. The stringent controls on access are currently the subject of considerable discussion. For example, in the USA the American Type Culture Collection (ATCC) is currently reviewing the procedures for the release of *F. tularensis* to researchers. It is recommended that researchers should attempt to obtain strains from culture collections or other researchers working in their own country in the first instance.

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# Annexes

# A. Diagnostic protocols

The following protocols have been provided by the University of Umeå, Umeå, Sweden, Centers for Disease Control and Prevention, Fort Collins, CO, USA, the Bundeswehr Institute of Microbiology, Munich, Germany, and the Robert Koch Institute, Berlin, Germany.

*Biosafety.* Personnel handling diagnostic cultures of *F. tularensis* are at considerable risk of infection and need to take precautionary measures. Biosafety Level 3 and Animal Biosafety Level 3 practices, containment equipment, and facilities are recommended, respectively, for all manipulations of suspect cultures, animal necropsies and animal studies.

*Collection and transport of samples.* Every effort should be made to collect and preserve specimens so that viable bacteria can be recovered. Decontaminate the surface area prior to specimen collection. Ensure that adequate volumes are collected to avoid false negatives as a result of insufficient sample volume. To minimize loss in viability, specimens should be transported to the laboratory within 24 h. For collection and transportation procedures for specific specimen types, see section 6.2.5.

*Note.* Some of the protocols described below have been developed for use with the reagents listed. Reagents other than those listed in the protocol should be properly validated prior to use.

## A.1 Gram stain

*Perspective.* The Gram stain can be used for suspect cases of *Francisella tularensis*. Poorly-staining, Gram-negative (pink) short rods or coccoid forms from a patient with exposure and clinical symptoms compatible with tularaemia are considered suspect for *F. tularensis*.

*Application.* Smears may be prepared from fresh, unpreserved specimens including cultures, tissues, and primary specimens in which the number of organisms is expected to be high.

*Reagents.* Gram stain kit.

*Equipment/supplies*

1. Microscope slides.
2. Gas or alcohol burner.
3. Staining rack for slides.
4. Microscope with high power and oil immersion objectives.

*Procedure*

1. Smear a thin layer of the specimen onto the slide. Smears must be made with a light concentration of cells in order to visualize individual cells clearly. Thick smears containing too many cells may give an indistinct reading.
2. Let the smear air dry, then heat-fix the smear by passing the slide through a flame, then let the slide cool.
3. Cover the smear with crystal violet for 1 min, followed by iodine for 2 min; rinse with tap water.
4. Decolorize the smear with ethanol for 10 s; rinse with tap water.
5. Counterstain the smear with safranin for 1 min; rinse with tap water.
6. Allow the slide to air dry then examine using the oil immersion objective on the microscope.

*Interpretation.* In Gram-stained smears, *F. tularensis* appears as a very tiny (0.2–0.7 µm x < 1.0 µm), pleomorphic, poorly-staining Gram-negative (pink) short rods or coccoid forms usually seen as single cells.

*Quality control.* Staining of test specimens should be carried out in parallel with known Gram-positive and Gram-negative organisms to ensure proper staining results. Reagents should be fresh and the crystal violet stain kept free of contamination. Microscope lenses and objectives should be kept dust and oil-free.

## A.2 Culture on agar

*Perspective.* Culture is considered the "gold standard" for *F. tularensis*. Cystine heart agar enriched with chocolatized red blood cells (CHAB) is a practical medium for culture of *F. tularensis* as the organism displays characteristic morphology (green, opalescent, raised, shiny colonies). Other enriched media such as GCII agar base with 1% haemoglobin and 1% IsoVitaleX™, Mueller-Hinton with 1% IsoVitaleX™, buffered charcoal yeast extract (BCYE), or thiogylcollate-glucose blood agar (TGBA) can also be used for the cultivation of *F. tularensis*, however, the colony morphology is not distinctive on these media. For contaminated specimens, antibiotic-supplemented media are recommended.

*Application.* All clinical specimens.

*Media*

1. Cystine heart agar base with 9% chocolatized sheep blood (see section C.1).
2. Mueller-Hinton agar with 1% IsoVitaleX™.
3. Thioglycollate-glucose blood agar (TGBA).
4. GCII agar base with 1% haemoglobin and 1% IsoVitaleX™ (see section C.2).
5. Chocolate agar.
6. Buffered charcoal yeast extract (BCYE).
7. CHAB-A: CHAB supplemented with colistin 7.5 mg, amphotericin 2.5 mg, lincomycin 0.5 mg, trimethoprim 4 mg and ampicillin 10 mg per l.

*Equipment/supplies*

1. Sterile bacteriologic loops.
2. Incubator: 37 °C.

*Procedure*

1. Use established inoculation and plating procedures for various clinical specimens.
2. Incubate cultures at 35–37 °C; ambient atmosphere or 5% $CO_2$ is acceptable.
3. Incubate the primary plates for 7–10 days.

*Interpretation.* On general media such as chocolate agar, or BCYE, *F. tularensis* is a greywhite, opaque colony, usually too small to be seen at 24 h on primary isolation from clinical specimens. After incubation for 48 h or more, colonies are about 1–2 mm in diameter, white to grey to bluish-grey, opaque, flat, with an entire edge, smooth, and have a shiny surface. On CHAB, after 48 h the colonies are 2–4 mm in size, greenish-white, dense with a butyrous consistency. A characteristic opalescent sheen is evident on the surface of the colonies, especially if the plate has been incubated for 48–72 h. *F. tularensis* will not grow on MacConkey agar.

**Figure A.1** *F. tularensis* on CHAB agar after 48 h incubation. Image provided by the Centers for Disease Control and Prevention, Fort Collins, CO, USA

*Quality control.* Each batch of plates must be checked for sterility by incubating plates at 37 °C for at least 24 h. Each batch of plates must be checked for the ability to support growth of *F. tularensis*.

### A.2.1 Mouse inoculation

*Perspective.* Inoculation of laboratory mice is a useful method for recovery of *F. tularensis* from contaminated specimens. Prior to any work with animals in the laboratory, protocols must be reviewed and approved by the appropriate animal use committee.

*Application.* Specimens or cultures contaminated with other organisms.

*Materials*

1. Specimen suspected to contain *F. tularensis*.
2. Pathogen-free mice (Swiss-Webster outbred mice, 6–9 weeks old).
3. Saline (0.85% NaCl).
4. Sterile sand or latex particles.

*Facilities/supplies/equipment*

1. Animal-care facilities (negative-pressure room).
2. Sterile mortar and pestle.
3. Syringes (1 ml) with 25 x 1/2″ needles.
4. Sterile gloves.
5. Discard container ('Sharps bin') for needles.
6. Sterile scissors, forceps.
7. Alcohol, 70%.
8. Sterile Petri dish or shallow container.

*Procedure*

1. Sample preparation: tissue specimens are prepared by excising small pieces into a mortar with a few drops of saline. A small amount of sterile abrasive material (sand or latex) is added to the mixture in the mortar before grinding with the pestle. Cultures are resuspended in saline and mixed prior to inoculation. Aspirates and respiratory specimens should be diluted in saline only if too viscous to inoculate.
2. Inoculation of the animal: inject 0.1–0.5 ml of the prepared samples subcutaneously in the lower abdomen. Typically, two mice are each injected with 0.1 ml of prepared inoculum from culture or other purified material, whereas for tissue specimens, up to 0.5 ml of suspension should be injected.
3. Observation: mice are checked twice daily for signs of illness. Surviving mice are kept for 3 weeks (21 days) before reporting as negative.
4. Processing mouse tissue: all moribund mice are sacrificed. Animals are necropsied and spleen and liver tissues are removed for culture on agar plates.
5. Interpretation: *F. tularensis* is easily isolated when passaged through susceptible, laboratory-raised, specific-pathogen free mice. Inoculated mice typically become ill within the first 3–4 days post-inoculation. Tissue samples (liver, spleen) taken from the mice are good sources of nearly pure cultures of *F. tularensis* as mice will selectively amplify pathogenic strains of *F. tularensis*. Recovered liver and spleen are enlarged and haemolytic and sometimes contain multiple focal abscesses.

*Quality control.* Since the mouse inoculation test is dependent on visible morbidity, mouse colonies should be inoculated with a known virulent *F. tularensis* isolate to test their sensitivity to infection prior to use. Sacrificed mice must be processed as quickly as possible or stored in the freezer at -20 °C to prevent deterioration of the material. When performing necropsy of the mice, take care not to cause unwanted enteric contamination by touching the lower abdominal surfaces. Use a freshly cut tissue for culture.

## A.3 Antigen detection

### A.3.1 Direct fluorescence assay (DFA)

*Perspective.* The direct fluorescent antibody (DFA) assay is based on the interaction between fluorescently (FITC)-labelled rabbit polyclonal anti-*F. tularensis* antibody and *F. tularensis*-specific antigens. This antibody is generated against whole killed *F. tularensis* cells. DFA is a rapid assay that can be used for both presumptive and confirmatory detection of *F. tularensis*. The DFA test is considered presumptive when a primary specimen is DFA-positive and confirmatory when a recovered isolate is DFA-positive.

*Application.* Slides may be prepared from cultures, ulcer swabs, tissues, blood and sputum specimens.

*Reagents*

1. FITC-labelled rabbit anti-*F. tularensis* conjugate (CDC, Fort Collins, CO; section D, Table D.1).
2. Phosphate-buffered saline (PBS).

*Equipment/supplies*

1. Microscope slides, two-etched ring.
2. Gas or alcohol burner.
3. Coplin jars or other jars capable of holding slide/s.
4. Fluorescence microscope, with FITC filter.

*Procedure*

1. Primary specimens: smear a thin layer of the specimen onto the slide. Smears must be made with a light concentration of cells in order to visualize individual cells clearly. Thick smears containing too many cells may give an indistinct reading.
2. Cultures: from a 24 h culture, use a 1 µl inoculating loop to pick up cells in the 3rd quadrant of growth. Prepare a suspension equivalent to 0.5 McFarland standard in saline. Mix well. Use a sterile swab to smear onto the slide.
3. Let the smear air dry.
4. Heat-fix the smear by passing the slide through a flame and then allowing it to cool.
5. Add 20 µl of conjugate (enough to cover the smear area) and incubate for 30 min at ambient temperature. Use a humidified chamber for incubation to prevent evaporation.
6. Fill a Coplin jar with PBS and soak the slide for 10 min. Discard the PBS from the container and refill with fresh PBS. Soak the slide for a further 10 min.
7. Remove the slide from PBS and rinse in distilled H2O to remove remaining salt.
8. Allow the slide to air-dry.
9. View the slide using a fluorescence microscope and FITC filter; slides can be analysed using 40X, 63X or 100X objectives.

*Interpretation.* A positive DFA result is seen as bright, intense green staining over the entire surface of the bacterial cell. *F. tularensis* cells are tiny, (0.2–0.7 x ≤ 1.0 µm) pleomorphic cells and generally appear as single cells in short rod or coccoid form.

*Quality control.* Each DFA test should be performed in parallel with a positive and negative control to ensure proper interpretation. Positive and negative controls can be prepared in advance and stored at -20 °C for future use. Process controls and test samples together. To maintain integrity of the conjugate, avoid freeze-thawing FITC-labelled antibodies. Upon receiving reagents, small aliquots should be made and the stock stored (in the dark) at -20 °C. The reagent should be checked annually to ensure reactivity.

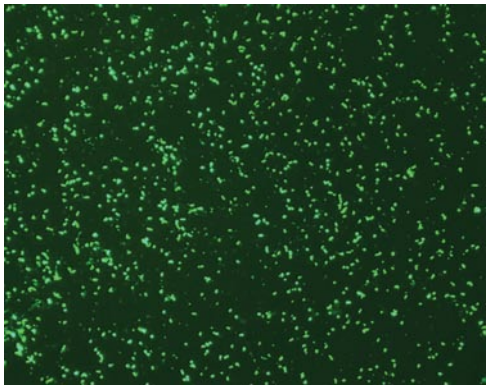

**Figure A.2** Isolate of *F. tularensis* stained by the direct fluorescence method (magnification 630X). Image provided by Centers for Disease Control and Prevention, Fort Collins, CO, USA.

### A.3.2 Slide agglutination

*Perspective.* The slide agglutination assay is based on the interaction between high-titre anti-*F. tularensis* antibody and *F. tularensis*-specific antigens. This interaction produces immediate clumping of the cells, visible to the naked eye. A positive slide agglutination test on an isolate, with characteristic *F. tularensis* growth on agar, is confirmatory for *F. tularensis*.

*Application.* Suspect cultures of *F. tularensis*.

*Reagents*

1. Hyperimmune rabbit-anti *F. tularensis* antibody (Difco, Becton Dickinson and Company; Annex D).
2. Normal rabbit serum.
3. Suspect culture.

*Equipment/supplies*

1. Bacteriological loops, 1 µl size.
2. Glass slides, two-etched ring.
3. Pipettor, 10–200 µl.

*Procedure*

1. Mark and section slide with wax pencil into two compartments.
2. Place one drop of positive serum in one compartment and one drop of negative serum in the next compartment.
3. Add equal amounts (i.e. one small loopful) of isolate to the positive and negative control serum; make sure cells are resuspended evenly.
4. Rock slide back and forth for 1 min, or according to manufacturer's recommendations.
5. Examine spots for visible clumping.
6. Discard the used slide in the proper disposal container and decontaminate by autoclaving.

*Interpretation.* A positive reaction (clumping of cells) is visible if the suspect culture is *F. tularensis*. A negative result is visualized as an absence of clumping. Each test should be performed in parallel with a positive and negative control to ensure proper interpretation. Process controls and test samples together.

*Quality control.* To ensure specificity, positive and negative reagents may be tested against other killed bacterial cell preparations such as *Brucella* spp., *Yersinia* spp., *Bacillus* spp., and *Escherichia coli*.

### A.3.3 Capture enzyme-linked immunosorbent assay (cELISA)

*Perspective.* The capture enzyme-linked immunosorbent assay (cELISA) is based on antigen binding to a monoclonal antibody (mAb) coated onto a solid phase (microtitre plate) and detection of the bound antigen using the same mAb (peroxidase labelled) (Grunow et al., 2000). The mAb is specific for the LPS of *F. tularensis*, including subspecies *tularensis*, *holarctica*, and *mediasiatica*. The cELISA is considered confirmatory when a recovered isolate, with characteristic growth on agar, is positive. Either whole cells or LPS extracted cells (final dilution of at least 1:4) can be used in the assay. The sensitivity of the assay is about $10^4$–$10^3$ CFU/ml.

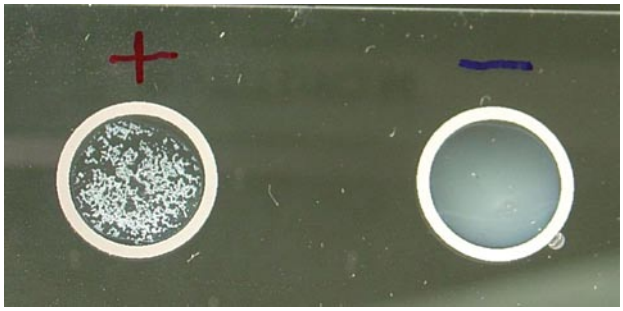


**Figure A.3** Slide agglutination for *F. tularensis* +, positive serum; –, negative serum. Image provided by Centers for Disease Control and Prevention, Fort Collins, CO, USA.

*Application.* This protocol has been developed primarily for confirmation of suspect cultures and presumptive testing of animal tissues.

*Reagents (sufficient for one microtitre plate, i.e. 40 unknown samples)*

1. Monoclonal antibody anti-*F. tularensis* (e.g. Senova GmbH, Germany, Annex D).
2. Monoclonal antibody anti-*F. tularensis*-HRP conjugate (e.g. Senova GmbH, Germany, Annex D).
3. Positive control (e.g. *F. tularensis* LVS $OD_{560nm}$= c. $10^9$ cells/ml), 0.01% thiomersal added for long-term storage, optional: solubilization of LPS using LPS extraction buffer.
4. Tetramethyl benzidine (TMB) ready to use (12 ml).
5. Phosphate buffered saline (PBS), isoton, pH 7.2, 1l.
6. Dry skimmed milk.
7. Tween-20.
8. Coating buffer: sodium carbonate pH 8.5–9, 0.025 M.
9. 0.25 M sulphuric acid.
10. Optional: thiomersal 1%.
11. Optional: LPS extraction buffer 10 ml (Chlamydia Assay, Abbott, Solna, Sweden, or prepared to the following formulation: for 100 ml, chenodeoxycholic acid 0.099 g EDTA trisodium salt 0.354 g, sodium azide 0.100 g, sodium chloride 0.231 g, disodium hydrogen phosphate-7-hydrate 1.764 g, sodium hydrogen phosphate 0.454 g, add to 100 ml distilled water).

*Equipment/supplies*

1. ELISA-reader.
2. 8 or 12 multi-channel pipette.
3. Reservoirs to handle fluids for multi-channel pipette.
4. 1 channel pipette 10–100 µl.
6. Microcentrifuge tubes 1.5 ml.
7. 1l glassware, 1 l and 10 ml beakers or tubes.
8. 10 ml pipettes and appropriate pipettor.
9. ELISA-Washer (optional).

10. Incubator at 37 °C.
11. Water-bath at 8 °C.
12. Water-bath at 60 °C.
13. pH meter.

*Preparation of solutions (to process one plate)*

1. The wash buffer is prepared by mixing PBS powder in 1 l distilled water (0.15 M) and 0.5 ml Tween-20, and adjusting the pH to 7.4 (this buffer can be stored at room temperature for 3 days).
2. The blocking buffer is prepared by adding 1.0 g of dry skimmed milk (4%) to 25 ml wash buffer (prepared fresh before use).
3. The dilution buffer is prepared by mixing 10 ml of blocking buffer with 30 ml of wash buffer (prepared fresh before use).
4. The coating buffer is prepared by adding 0.8 g sodium carbonate and 1.46 g sodium hydrogen carbonate to 250 ml distilled water, 0.025 M. The pH should be adjusted to pH 8.5–9.0.

*Procedure (for one plate, up to 40 unknown samples)*

1. Dilute stock solution of mAb to a final concentration of 5µg/ml in 10.5 ml coating buffer.
2. Add 100 µl of diluted mAb per well and cover with cover tape or plate seal.
3. Incubate for 1 h at 37 °C or overnight at 4 °C.
4. Preparation of unknown samples: Liquid samples should be diluted 1:2 with dilution buffer. Solid samples should be homogenized in PBS and after sedimentation the liquid phase should be diluted 1:2 with dilution buffer. There are several protocols to improve the homogenization of solid samples and the extraction of bacterial antigens. Here, a simple protocol is proposed (optional):

   Liquid sample:

   a) mix 150 µl unknown sample with 150 µl LPS extraction buffer in an microcentrifuge tube
   b) heat the tubes for 30 min in a water-bath at 60 °C.

   Solid sample:

   a) place sample (0.2 g) in a Petri dish or other appropriate container
   b) add 0.5 ml PBS
   c) slice the sample into fine pieces, homogenize and pipette the supernatant into a 1.5 ml microcentrifuge tube
   d) add 0.7 ml LPS extraction buffer
   e) incubate samples at 60 °C for 30 min
   f) vortex, and allow the sediment to settle; if the sediment does not settle, filter or spin at low speed (< 100 x g)
   g) carefully remove 200 µl of the sample and dilute with 200 µl (1:2) dilution buffer (make further dilution, if required)
   h) samples are now ready for analysis.

5. Prepare positive and negative controls in microcentrifuge tubes:
   a) mix 30 µl of positive control with 270 µl dilution buffer (equivalent $10^8$ CFU/ml)
   b) take 10 µl from $10^8$ CFU/ml and mix with 990 µl dilution buffer (equivalent $10^6$ CFU/ml)
   c) take 200 µl from $10^6$ CFU/ml and mix with 400 µl dilution buffer (equivalent 3 x $10^5$ CFU/ml = positive control (PC1)
   d) take 50 µl from PC1 and mix with 450 µl dilution buffer (equivalent 3 x $10^4$ CFU/ml = PC3)
   e) take 50 µl from PC3 and mix with 450 µl dilution buffer (equivalent 3 x $10^3$ CFU/ml = PC5)
   f) take 50 µl from $10^6$ bacteria/ml and mix with 450 µl dilution buffer (equivalent 1 x $10^5$ bacteria/ml = PC2)
   g) take 50 µl from PC2 and mix with 450 µl dilution buffer (equivalent 1x $10^4$ CFU/ml = PC4)
   h) take 50 µl from PC4 and mix with 450 µl dilution buffer (equivalent 1x $10^3$ CFU/ml = PC6)
   i) dilution buffer only = negative control (NC).
6. Wash the plate twice using 300 µl washing buffer per well.
7. Block the plate with 125 µl blocking buffer per well for 30 min at 37 °C.
8. Wash the plates again as in step 6.
9. Add 100 µl volumes of the controls and unknown samples as duplicates to the appropriate wells.
10. Incubate for 1 h at 37 °C.
11. Wash the plates four times as in step 6.
12. Add 100 µl of detector mAb-HRP-conjugate (10µg/ml) to all wells except blank wells.
13. Incubate for 1 h at 28 °C.
14. Wash the plates five times as in step 6.
15. Add the substrate TMB 100 µl per well and observe for approx. 7 min (up to 15 min) for colour reaction.
16. Stop the reaction by adding 100 µl of sulphuric acid.
17. Read the optical density (OD) of the samples at 450 nm (reference wavelength 620 nm).
18. Create a standard curve from the control samples and read the bacterial load of the unknown samples, if required.

**Plate scheme**

Unknown samples

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | Blank | | | | | | | | | | |
| B | PC1 | PC1 | | | | | | | | | | |
| C | PC2 | PC2 | | | | | | | | | | |
| D | PC3 | PC3 | | | | | | | | | | |
| E | PC4 | PC4 | | | | | | | | | | |
| F | PC5 | PC5 | | | | | | | | | | |
| G | PC6 | PC6 | | | | | | | | | | |
| H | NC | NC | | | | | | | | | | |

*Interpretation.* Negative specimens will show an OD close to that of the negative control (usual cut-off OD < 0.2). OD of positive specimens and controls ranges from 0.2–1.5. A quantification of the bacterial load can be done using the standard curve.

*Quality control.* Perform each test in parallel with appropriate controls as described above to ensure proper performance. Selected specimens may be tested in duplicate to ensure reproducibility. The description of the method is given as an example. When establishing the assay under given laboratory conditions, further adaptation, validation, and optimization could be required.

## A.4 Molecular detection

### A.4.1 Gel-based PCR for detection of the species *F. tularensis* and genus *Francisella*

The multiplex PCR is based on detection of the 16S rRNA gene specific for strains of the genus *Francisella* and the lpnA encoding a 17-kDa lipoprotein (TUL4; amplicon size 0.4 kb) specific for the species *F. tularensis*. PCR is a rapid assay that can be used for both presumptive and confirmatory detection of *F. tularensis*.

*Application.* The protocol has been developed primarily to detect *F. tularensis* in clinical samples obtained by taking swabs of ulcers (Johansson et al., 2000). DNA is recovered from the ulcer swabs and put in guanidine thiocyanate (GuSCN) buffer, designated L6. The swabs are transported in tubes containing 700 µl of the L6 buffer.

*Supplies and equipment for preparation of DNA*

1. Autoclaved 1.5 ml microcentrifuge tubes.
2. Autoclaved 0.5 ml microcentrifuge tubes.
3. Sterile pipette tips.
4. Heating block.
5. Sterile pipette tips.
6. Pipette (10 µl–1000 µl, adjustable).
7. Microcentrifuge.
8. PCR equipment – thermocycler.
9. Gel box.
10. UV light box and camera.

*Reagents for nucleic acid extraction (by the method of Boom et al., 1990)*

1. L6-buffer, composed of:
   a) guanidine thiocyanate: 30 g
   b) 0.1M Tris pH 6.4: 25 ml
   c) 0.5 MEDTA pH8.0: 5.5ml
   d) Triton X-100: 650 mg

   Dissolve the guanidine thiocyanate in the Tris buffer by heating at 60 °C in a waterbath. Shake the bottle repeatedly. Add the EDTA and the Triton X-100. The L6 buffer can be dispensed into 700 µl aliquots in 1.5 ml microcentrifuge tubes and stored in the dark for 3 months.
2. L2-buffer, composed of:
   a) guanidine thiocyanate: 60 g
   b) 0.1 M Tris pH 6.4: 50 ml

   Dissolve the guanidine thiocyanate in the Tris buffer by heating at 60 °C in a waterbath. Shake the bottle repeatedly. The L2 buffer can be stored in the dark for 3 months.
3. Ethanol (70 %).
4. Acetone.
5. Glassmilk® (MP Biomedicals, Irvine CA, USA).
6. 1x Tris EDTA (TE) buffer, pH 8.0.
7. Distilled water.
8. Agarose.
9. 1x Tris borate EDTA (TBE).
10. Ethidium bromide.

*Reagents for PCR*

1. Distilled water.
2. Ampli Taq Gold 5U/µl (Applied Biosystems, Foster City, California, USA).
3. 10x PCR-buffer II (Applied Biosystems).
4. 25 mM magnesium chloride ($MgCl_2$).
5. 10x dNTP stock solution (Amersham Biosciences, Buckinghamshire, UK).
6. Stock solution of PCR primers (10 µM).

| Target gene | Primer | Primer sequences (5' to '3) |
|---|---|---|
| 16S rRNA | F5 | cct ttt tga gtt tcg ctc c |
| 16S rRNA | F11 | tac cag ttg gaa acg act gt |
| 17-kDa lipoprotein | TUL-435 | gct gta tca tca ttt aat aac tgc tg |
| 17 kDa lipoprotein | TUL-863 | ttg gga agc ttg tat cat ggc act |

*PCR mixes*

*Master mix preparation:*

1. Thaw master mix reagents.
2. Spin down the contents of the tubes briefly.
3. Mix the components of the master mix in 1.5 ml microcentrifuge tubes.
4. Transfer 20 µl of the master mix to 0.5 ml microcentrifuge PCR tubes.
5. Add 5µl of distilled water to the control tube.

| Master mix for PCR mixes | µl |
|---|---|
| 10x dNTP | 2.5 |
| 25 mM $MgCl_2$ Solution | 3.0 |
| 10x PCR buffer II | 2.5 |
| 10 µMF5 | 1.5 |
| 10 µM F11 | 1.5 |
| 10 µM TUL-435 | 1.5 |
| 10 µM TUL-863 | 1.5 |
| Distilled water | 5.6 |
| Ampli Taq Gold 5U/µl | 0.4 |
| **Total** | **20.0** |

*Further preparation pre-PCR*

1. Switch on heating block at 56 °C.
2. Vortex the tube containing Glassmilk® until the suspension is dissolved.
3. Check that there are no crystals in the L6 buffer; if there are, heat the tube at 45 °C until they have dissolved, then cool to room temperature.

*Positive control preparation*

1. Suspend the positive control strain in distilled $H_2O$ to give an $OD_{600}$ of 1.3 (approximately $2 \times 10^9$ CFU/ml).
2. Make a dilution of this suspension to give $2 \times 10^5$ CFU/ml in distilled $H_2O$. This will result in 150 CFU per PCR reaction. This is the positive control.
3. Transfer 5µl of the positive control to 700 µl of L6 buffer and add 300 µl of distilled water.

*Negative control preparation.* The negative control is composed of 700 µl of L6-buffer + 300 µl of distilled water.

*Sample preparation*

1. To each patient sample (700 µl), add 300 µl of L6.
2. Add 5µl of glassmilk to each tube (samples and controls), vortex for 5 s.
3. Leave for 10 min at room temperature.
4. Vortex for 5 s.
5. Centrifuge at 10 000 x g for 15 s.
6. Discard the supernatant.
7. Wash the deposit twice with 1 ml volumes of L2 buffer, twice with 1 ml volumes of ethanol and finally once with 1.0 ml of acetone. Between each step, vortex briefly and centrifuge at 10 000 x g for 15 s and discard the supernatant.

8. Dry the pellets, with the lids of the tubes open, at 56 °C for 10 min.
9. Add 30 µl of 1 x TE-buffer, vortex briefly.
10. Incubate for 10 min at 56 °C.
11. Vortex briefly.
12. Centrifuge at 12 000 x g for 2 min.
13. Transfer the supernatant to another 1.5 ml microcentrifuge tube.
14. The tubes can be stored at 4 °C if the PCR is performed the same day, otherwise they should be stored at -18 °C overnight.
15. Add 5µl of purified DNA from each sample to the tubes containing the PCR mixes (addition of DNA to PCR mixes should be performed in a designated pre-PCR area in a laminar flow hood).
16. Vortex briefly and rapidly spin down.

*PCR programme*

1. 95 °C for 10 min
2. 94 °C for 30 s
3. 60 °C for 1 min
4. 72 °C for 1 min
5. perform 29 more cycles of steps 2–4 (30 cycles in total)
6. hold at 4 °C.

*Electrophoresis.* To analyse the PCR products, run 5µl on a 2% agarose gel with ethidium bromide. Include an appropriate molecular weight ladder on the gel. Use a UV light box to visualize the gel and take a picture to document the results.

*Interpretation.* The amplicon size for TUL4 is 386 bp. No TUL4 product is amplified for *F. philomiragia*. The amplicon size for 16S rDNA is 1104 bp.

*Quality control.* Positive (subspecies *tularensis* and subspecies *holarctica*) and negative (no template) controls should be included each time the PCR is performed. Dilutions of template DNA should be tested, as inhibition can be caused by high template DNA concentrations.

## A.5 Serology

### A.5.1 Microagglutination

Perspective A single serum/plasma specimen with a titre ≥1:128 is presumptive for *F. tularensis*. Paired serum specimens, taken 14 days apart, giving a 4-fold increase or decrease in titre with at least one titre being ≥1:128, are confirmatory for *F. tularensis*. A tube agglutination format with the same reagents also exists. The tube agglutination cut-off is ≥1:160.

*Application.* Serum samples.

*Reagents*

1. Microagglutination (MA) diluent: phosphate-buffered saline with 1% normal rabbit serum, 0.4% formaldehyde; pH 7.2.
2. Formalinized killed safranin-stained *F. tularensis* cells (CDC, Fort Collins, USA; Annex D).
3. Positive control serum, high titre (> 1:128).

4. Positive control serum, low titre (1:128) (CDC, Fort Collins, USA; Annex D).
5. Negative control serum, (< 1:16), matched by species to positive control serum.

*Equipment/supplies*

1. Microtitre plates, 96-well "U" bottom (Costar, Cambridge, MA, USA).
2. Sterile pipette tips.
3. Reagent reservoir.
4. Plate-sealing tape.
5. Multichannel pipette (10 µl–100 µl, adjustable).
6. Light box or mirror reader.
7. Water-bath at 56 °C.

*Procedure*

1. Add 25 µl of MA buffer to each of the 96 wells of a microtitre plate.
2. Add 25 µl of test serum to the first column of 8 wells (well 1): a total of 5 test samples and 3 controls (high and low positive, negative) can be done on each plate.
3. Carry out serial 2-fold dilutions of sera from well 1 to well 12, i.e. transfer 25 µl from well 1 to well 2 and so on to well 12; discard the last 25 µl.
4. Add 25 µl of antigen preparation to each well; mix gently.
5. Seal plate to prevent evaporation, and incubate at room temperature for 12–18 h.
6. Read and record the results.
7. Discard microtitre plate in an appropriate container for decontamination and disposal.

*Interpretation.* Negative specimens will have a button of cells at the bottom of the well. Positive specimens agglutinate the antigen, thus there will be a mat that looks like a diffused net of cells. There should be no cell button at the bottom of the well. Titres of ≥1:128 are considered positive. Prozone may be observed in cases of high titre serum when antibody is in excess of antigen.

*Quality control.* Perform each test in parallel with high-, low-positive and negative control sera to ensure proper performance. Selected specimens may be tested in duplicate to ensure reproducibility. If *Brucella* infection is suspected, microagglutination should be performed in parallel with *Brucella* antigen to exclude cross-reactions.

### A.5.2 Enzyme-linked immunosorbent assay (ELISA)

*Perspective.* This procedure determines the titres of anti-*Francisella* immunoglobulins, isotype G and M. A single serum/plasma specimen with a positive ELISA titre is presumptive for *F. tularensis*. Paired serum specimens, with at least one having a significant IgG or IgM titre is considered confirmatory for *F. tularensis*. In part, this procedure might be automated i.e. washing the plates.

*Application.* Human serum samples.

*Consumables*

1. 96-well microtitre plates, flat-bottomed (Nunc, Immunoplate MaxiSorp).
2. 15 ml conical tubes (Falcon).
3. Plate seal/adhesive cover (Nunc, can be replaced by parafilm).
4. Pipette tips (Sarstedt).

*Solutions (see recipes in section A.5.3)*

1. Coating buffer.
2. *Francisella tularensis* LPS.
3. Washing buffer.
4. Incubation buffer.
5. Substrate buffer.
6. Substrate tablets (Sigma 104 phosphatase substrate tablets number S0942).
7. 5M and 3M NaOH.
8. Standard and positive control (titrated patient sera, possibly pooled (see *Titration of standards and controls*, section A.5.3).
9. Anti-IgM/anti-IgG.

*Equipment*

1. ELISA reader (Tecan Sunrise).
2. Multichannel pipette.
3. Pipette charger.
4. Single channel pipette.
5. If applicable: automated plate washer (Anthos fluido).

*Procedure.* All buffers and solutions should be at room temperature.

*DAY 1*

1. Coating:
   a) Take out plates from fridge. Coat the plates according to the coating scheme below; note lot numbers of reagents, coating date and assign consecutive numbers to the plates.
   b) Dilute the antigen in coating buffer to a concentration of 3µg LPS/ml. Allow at least 4 ml per plate.
   c) Load 100 µl per well according to the coating scheme below.
   d) Cover plate with plate seal and incubate overnight at 20–26 °C.

**Coating scheme (CB = coating buffer, LPS = *F. tularensis* LPS**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB |
| B | CB | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | CB |
| C | CB | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | CB |
| D | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB |
| E | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB |
| F | CB | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | CB |
| G | CB | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | CB |
| H | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB | CB |

*DAY 2*

2. Blocking:
   a) Empty the wells, blot the inverted plate on absorbent paper to remove any residual buffer. Load 200 µl washing buffer per well in rows B-G, columns 2–11.
   b) Cover plate with plate seal. Incubate plates at 20–26 °C for at least 30 min. After this step the plates can be stored at either 4 °C or -70 °C.
3. Preparations:
   a) Design loading scheme for the plates in form of a computer protocol, Word or Excel file format. The outermost wells are left empty. Include standards on all plates (column 2 and 3) but only include a positive control on the first plate (column 3).
   b) Take out patient sera and standard/positive control and allow to thaw. Do not inactivate.
   c) Note the lot numbers of reagents, solutions and controls.
   d) Label two test tubes per patient serum: 1/100 and 1/1000.
4. Rinse:
   a) Empty the wells, blot the inverted plate on absorbent paper to remove any residual buffer. Fill the wells with washing buffer (c. 300 µl/well) and wait one minute. Repeat five times.
   b) Place the plates upside-down on moist tissue; they should not dry out!
5. Dilute samples:
   a) Dilute the serum samples 1/1000, in two steps:
      i. 25 µl serum + 2.5 ml incubation buffer, mix carefully (1)
      ii. 400 µl of (1) + 3.6 ml incubation buffer, mix.
   b) Dilute standard/control according to titration (see *Titration of standards and controls,* section A.5.3).
6. Incubate samples:
   a) Load 100 µl per well according to the loading scheme below. Place standard/control (C) in column 2, 3 and 4. Place each sample (the 1/1000 dilution) into four antigen-coated wells and into two wells with coating buffer. The latter serve as control for unspecific reactions.

**Loading scheme (IB = incubation buffer)**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB |
| B | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| C | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| D | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| E | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| F | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| G | IB | C | C | C | 01 | 02 | 03 | 04 | 05 | 06 | 07 | IB |
| A | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB |

   b) Cover plate with plate seal and incubate for 3–5 hours at 20–26 °C.

7. Rinse:
   a) Repeat rinse steps as above.
8. Incubate conjugate:
   a) Every sample is tested with two conjugates, anti-IgG and anti-IgM. Allow at least 3 ml of each conjugate per plate.
   b) Immediately before use dilute the conjugates in incubation buffer (according to lot-specific instructions from the manufacturer).
   c) Load 100 µl per well according to the loading scheme below; anti-IgG in rows B-D; anti-IgM in rows E-G.

**Conjugate loading scheme (IB = incubation buffer)**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB |
| B | IB | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | IB |
| C | IB | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | IB |
| D | IB | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | antiG | IB |
| E | IB | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | IB |
| F | IB | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | IB |
| G | IB | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | antiM | IB |
| H | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB | IB |

   d) Cover plates with plate seal and incubate overnight at 20–26 °C.

*DAY 3*

9. Repeat rinse steps as above.
10. Prepare substrate:
    a) Prepare at least 6 ml substrate dilution per plate. Dissolve one substrate tablet in 5 ml substrate buffer. Incubate for 30 min at room temperature.
    b) For blanks mix 50 µl 5M NaOH with 5 ml substrate buffer. Allow 800 µl per plate.
11. Incubate substrate:
    a) Load 100 µl per well according to the loading scheme below.

**Loading scheme**
**(B = blank, SB = substrate buffer, SS = substrate solution)**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | SB | SB | SB | SB | SB | SB | SB | SB | SB | SB | SB |
| B | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| C | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| D | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| E | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| F | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| G | B | SS | SS | SS | SS | SS | SS | SS | SS | SS | SS | SB |
| H | B | SB | SB | SB | SB | SB | SB | SB | SB | SB | SB | SB |

   b) Once you have finished loading the first plate start the timer to record the incubation time.

12. Read and stop the reaction:
    a) Read absorbance at 405 nm. Stop the reaction in the first plate when the standards/calibrator (columns 2 and 3) have an absorbance of approx. 1.
    b) Stop the reaction by adding 50 µl 3M NaOH.
    c) Read absorbance at 405 nm. Save the results as an Excel file connected to your loading scheme. Use this reading for calculations.
13. Calculation:
    a) This calculation can be done automatically by setting up an Excel file. Single wells are identified by the combination of a letter from A-H and a number from 1–12, according to the rows and columns of the plate.
    b) Calculate the titre of anti-*F. tularensis* LPS IgG:
        i. mean of the controls:
        (B2 + C2)/2 = $mean^1$
        (B3 + C3)/2 = $mean^2$
        ii. background correction:
        $mean^1$ – D2 = background corrected mean 1 (bcm)
        $mean^2$ – D3 = $bcm^2$
        calibrator = ($bcm^1$ +$bcm^2$)/2
        correction factor = 1/calibrator

        Due to previous titration of the standards, the expected absorbance (A405) in wells B2, C2, B3 and C3 is OD 1. This calculation is done to adjust the expected value to the real value of this reading.
    c) Calculate the IgG titre in patient sera. Repeat the following steps for each sample. For example:

        mean = (B5 + C5)/2
        background correction: mean - D5 = background corrected value
        relative IgG titre = background corrected value x correction factor x dilution of the serum.
    d) Calculate the anti-*F. tularensis* LPS IgM titres in patient sera. Repeat the previous steps as described for IgG titres but use absorbance values for wells F2, G2, F3 and G3 for the correction factor, row E for background correction and sample absorbance values in rows F and G for the relative IgM titre.

*Interpretation.* Negative blood samples from humans who had no record of tularaemia or vaccination against tularaemia typically result in IgG and IgM titres of 0.2–0.4.

*Quality control.* Perform each test in parallel with positive and negative control sera to ensure proper performance. Document all test results.

### A.5.3 Buffer recipes and storage conditions

#### Coating buffer

*Ingredients*

a) Sodium hydrogen carbonate, $NaHCO_3$: 3.47 g.
b) Sodium carbonate, $NaCO_3$: 1.18 g.
c) Sodium azide, $NaN_3$: 0.20 g.
d) Ultra-pure water to total volume: 1000 ml.

*Preparation*

a) Dissolve the substances in water to approximately three-quarters of the total volume.

b) Adjust the pH to 9.5–9.7.

c) Dilute with water to 1000 ml.

d) Autoclave at 121 °C for 15 min.

*Storage.* 2–8 °C.

*Best before.* 3 months.

*Environmental and safety instructions*

a) Sodium azide is a highly toxic substance that can be absorbed through the skin. If sodium azide gets in contact with acids a toxic gas develops. If sodium azide gets in contact with lead or copper explosive metal azides develop.

b) Work in a fume hood and wear gloves when handling sodium azide. Excess sodium azide solutions must not be poured down the sink but should be disposed of in accordance with national regulations.

### Washing buffer (PBS with 0.5% BSA)

*Ingredients*

a) Bovine serum albumin (BSA): 5.00 g.

b) Sodium azide, $NaN_3$: 0.20 g.

c) PBS: 1000 ml.

*Preparation*

a) Dissolve the substances in PBS.

b) Adjust the pH to 7.2–7.4.

c) Filter the solution through a 0.2 µm membrane filter.

*Storage.* Room temperature, dark.

*Best before.* 2 months.

*Environmental and safety instructions.* As for sodium azide (see coating buffer above).

### Incubation buffer (PBS with 0.05% Tween 20)

*Ingredients*

a) Sodium chloride, NaCl: 8.00 g.

b) Disodiumhydrogenphosphate $Na_2HPO_4$ x $2H_2O$:1.43g.

c) Potassium chloride, KCl: 0.20g.

d) Potassium dihydrogenphosphate $KH_2PO_4$ x $2H_2O_2$: 0.20g.

e) Sodium azide, $NaN_3$: 0.20 g.

f) Tween-20: 0.5 ml.

g) Ultra-pure water to a total volume: 1000 ml.

*Preparation*

a) Dissolve the substances in water to approximately three-quarters of the total volume.

b) Adjust the pH to 7.3–7.4.

c) Dilute with water to 1000 ml.

d) Autoclave at 105 °C for 15 min.

*Storage:* Room temperature, dark.

*Best before:* 1 month.

*Environmental and safety instructions:* As for sodium azide (see above; coating buffer).

### Substrate buffer

*Ingredients*

a) Diethanolamine: 97 ml.

b) Magnesium chloride MgCl2 x 6H2O2: 0.101 g.

c) Sodium azide, NaN3: 0.20 g.

d) Ultra-pure water to a total volume: 1000 ml.

*Preparation*

a) Dissolve the substances in water to approximately three-quarters of the total volume.

b) Adjust the pH to 9.8 with HCl.

c) Add water to give a total volume of 1000 ml.

*Storage:* Room temperature, dark.

*Best before:* 2 months.

*Environmental and safety instructions*

a) For sodium azide, see above (coating buffer).

b) Diethanolamine can be harmful to the eyes.

*Titration of standards and controls*

a) Test patient serum or alternatively serum from vaccinees for anti-*F. tularensis* IgG and IgM according to the ELISA protocol.

b) Select a serum that is positive for both immunoglobulin isotypes and gives absorbance of > 1 at 450 nm.

c) Perform serial two-fold dilutions of the selected serum and perform the ELISA protocol again.

d) An appropriate standard/control should reach an absorbance of 1 within 20–40 min. The dilution of the selected serum which fulfils this criterion is used as standard in future experiments.

## A.6 References

Boom et al (1990). Reagents for nucleic acid extraction by the Boom method. *Journal of Clinical Microbiology*, 28:495–503.

Grunow et al (2000). Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clinical and Diagnostic Laboratory Immunology*, 7:86–90.

Johansson et al. (2000). Comparative analysis of PCR versus culture for diagnosis of ulcero-glandular tularaemia. *European Journal of Clinical Microbiology*, 38:22–26.

# B. Tests for supplemental characterization

The following protocols have been provided by the University of Umeå, Umeå, Sweden and the Centers for Disease Control and Prevention, Fort Collins, CO, USA.

Once an isolate has been confirmed as *F. tularensis*, additional methods can be used for supplemental characterization. The following protocols should be considered only supplementary and not diagnostic for *F. tularensis*.

## B.1 Antimicrobial susceptibility

### B.1.1 E-test

*Perspective.* The E-test (AB Biodisk, Solna, Sweden) method is based on the use of plastic strips containing a predefined antibiotic gradient. The primary reason for performing this supplementary test is to determine the antibiotic susceptibility of the recovered isolate.

*Application.* Isolates confirmed as *F. tularensis*.

*Procedure.* Consult the E-test® Technical Manual (AB Biodisk) for more detailed information.

*Agar medium*

1. Cysteine heart agar + 10% blood (Tomaso et al., 2005) or + 2% haemoglobin (Ikäheimo et al., 2000).
2. Glucose cysteine blood agar (Scheel et al.,1993).
3. GCII agar with 1% haemoglobin and 1% IsoVitaleX™ (Johansson et al., 2000) (See section C.2 ).

*Inoculum*

Suspend 48-h colonies from chocolate agar in broth to give an opacity equivalent to 1 McFarland standard.

*Incubation*

37 °C in 5% $CO_2$ or ambient atmosphere for 48 h.

*Caution*

1. Testing should only be performed if appropriate biosafety measures are available (see section 6.6).
2. Public health officials should be notified regarding all isolates presumptively identified as *F. tularensis*.

### B.1.2 Quality control

*Quality control (MIC mg/l).* The values given for the reference strains below are based on the CLSI broth microdilution method using supplemented cation-adjusted Mueller-Hinton broth (CAMHB) and 48 h incubation at 37 °C (Clinical and Laboratory Standards Institute, 2005).

| | ***Staphylococcus aureus*** ATCC 29213 | ***Pseudomonas aeruginosa*** ATCC 27853 |
|---|---|---|
| Ciprofloxacin | 0.25–1 | 0.25–1 |
| Chloramphenicol | 4–32 | not tested |
| Doxycycline | 0.25–2 | 4–32 |
| Gentamicin | 0.25–1 | 0.5–4 |
| Levofloxacin | 0.12–0.5 | 0.5–4 |
| Streptomycin | 8–64 | 32–256 |
| Tetracycline | 0.5–4 | 8–64 |

**Interpretation:** CLSI MIC criteria (mg/l) for *F. tularensis*. Values are based upon broth microdilution using supplemented CAMHB and 48 h incubation at 37 °C (Clinical and Laboratory Standards Institute, 2005).

| | **MIC criteria (mg/l)** | | |
|---|---|---|---|
| **Antibiotic** | **Susceptible** | **Intermediate[a]** | **Resistant** |
| Ciprofloxacin | ≤ 0.5 | – | – |
| Chloramphenicol | ≤ 8.0 | – | – |
| Doxycycline | ≤ 4.0 | – | – |
| Gentamicin | ≤ 4.0 | – | – |
| Levofloxacin | ≤ 0.5 | – | – |
| Streptomycin | ≤ 8.0 | – | – |
| Tetracycline | ≤ 4.0 | – | – |

[a] The absence of resistant strains precludes defining any result categories other than 'susceptible'. For strains yielding results suggestive of a 'nonsusceptible' category, organism identification and antimicrobial susceptibility test results should be confirmed.

*Precautions when reading results*

1. For bactericidal drugs, read at complete inhibition of growth (microcolonies, hazes and isolated colonies).
2. For bacteriostatic drugs, read at 80% inhibition when trailing is seen.
3. Please consult the E-test Technical Manual for more information, downloadable at www.abbiodisk.com.

## B.2 Molecular biotyping

The methods below allow the discrimination of different subspecies of *F. tularensis*.

### B.2.1 Gel-based PCR for identification of *F. tularensis* subspecies *holarctica* (type B) strains (Byström et al., 2005)

The targeted *F. tularensis* DNA marker has been denoted (Ft-M19) and is present in all four *F. tularensis* subspecies: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*. The genomes of subspecies *holarctica* strains universally exhibit a specific 30-bp deletion at the targeted genomic locus (Johansson et al., 2004; Byström et al., 2005). The PCR assay relies on amplification of Ft-M19 that is located between the genes deaD and ppiC using a pair of amplification primers that are located inside the flanking genes.

*Application.* The protocol has been developed for rapid subtyping of *F. tularensis* cultures. The Ft-M19 assay can be used after identification to the species level using diagnostic methods outlined in Annex A.

*Supplies and equipment.* See protocol A.4.1 for listing of supplies and equipment needed.

*Preparation of DNA*

1. Grow bacteria for 24–48 h on an appropriate solid agar medium at 37 °C.
2. Collect 5–10 bacterial colonies on a sterile bacteriological loop and prepare a suspension of bacteria in 1 ml of water.
3. Kill the bacteria by incubating the suspension at 65 °C for 2 h.
4. Transfer 100 µl of the heat-killed bacterial suspension to 900 µl of buffer L6 (see *Reagents for nucleic acid extraction*, section A.4.1).
5. Add 5 µl of Glassmilk® to each tube with samples or controls, vortex for 5 s.
6. Leave for 10 min in room temperature then vortex 5 s.
7. Centrifuge at 10 000 x g for 15 s and then discard the supernatant.
8. Wash the deposit twice with 1 ml of L2 buffer (see *Reagents for nucleic acid extraction*, section A.4.1), twice with 1 ml of ethanol and finally once with 1 ml of acetone. Between each step, vortex briefly, centrifuge at 10 000 x g for 15 s and discard the supernatant.
9. Dry the pellets with the lids of the tubes open, at 56 °C for 10 min.
10. Add 30 µl of 1x TE-buffer, vortex briefly.
11. Incubate for 10 min at 56 °C, vortex briefly then centrifuge at 12 000 x g for 2 min.
12. Transfer the supernatant to another 1.5 ml micro centrifuge tube. The tubes can be stored at 4 °C if the PCR is performed the same day, otherwise they should be stored at -18 °C overnight.

### B.2.2 PCR

1. For PCR reagents, see *Reagents for PCR*, section A.4.1.
2. PCR primers:

   Forward: 5′-aggcggagatctaggaaccttt-3′

   Reverse: 5′-agcccaagctgactaaaatcttt-3′

*PCR mixes.* For PCR mix set-up and positive and negative control preparation, see section A.4.1.

*Master mix preparation*

| **Master mix for PCR** | **µl** |
|---|---|
| dNTP (2.5 mM) | 2.0 |
| $MgCl_2$ solution (25 mM) | 1.2 |
| 10xPCR buffer II | 2.5 |
| Forward primer (5 pmol/µl) | 2.0 |
| Reverse primer (5 pmol/µl) | 2.0 |
| Distilled water | 14.1 |
| Ampli Taq Gold 5 U/µ | 24.0 |
| **Total** | **25.0** |

*Sample preparation*

1. Add 1 µl of purified DNA sample to each PCR mix to a final volume of 25 µl. This should be performed in a designated pre-PCR area in a laminar flow hood.
2. Vortex briefly and spin down the contents of the tubes without delay.
3. Immediately place the PCR tubes in the cycling equipment and start the PCR.

*PCR programme*

1. 95 °C for 10 min
2. 94 °C for 30 s
3. 62 °C for 30 s
4. 72 °C for 30 s
5. repeat steps 2–4 for 29 cycles
6. hold at 4 °C.

*Electrophoresis*

1. To analyse the PCR products, load 3 µl sample mixed with loading dye on a 3 % NuSieve 3:1 agarose gel (Cambrex Bioscience Rockland, Inc., Maine) with ethidium bromide.
2. Include an appropriate molecular weight ladder on the gel and run in 1x TBE buffer at 5 V/cm for 3 h.
3. Use a UV light box to visualize the DNA and photograph to document the results.

*Interpretation.* The PCR fragment size of Ft-M19 is 220 bp for all strains of subspecies *holarctica* (type B). PCR amplifications of DNA from strains of subspecies *tularensis* (type A), *mediasiatica*, and *novicida* each result in a 250 bp product.

*Quality control.* Positive (subspecies *tularensis* and subspecies *holarctica*) and negative (no template) controls should be included each time the PCR is performed. Dilutions of template DNA should be tested, as inhibition can be caused by high-template DNA concentrations.

### B.2.3 Subspecies-specific real-time PCR assays for subspecies *tularensis* and subspecies *holarctica* (Kugeler et al., 2006)

*Perspective.* The subspecies *tularensis* (type A) specific assay is targeted against the pdpD gene and the subspecies *holarctica* (type B) specific assay detects a unique location of the ISFtu2 insertion-like element. The type A and type B assays are TaqMan® assays utilizing two primers and a fluorogenic probe for target detection. These assays were developed and evaluated on the LightCycler® 1.2 (Roche Applied Science, Indianapolis, IN) real-time PCR instrument.

*Application.* The protocol has been developed for rapid subtyping of bacterial *F. tularensis* cultures. These assays can be used after identification to the species level using diagnostic methods outlined in Annex A.

*Note.* Due to the sensitive nature of real-time PCR, care should be taken to ensure that false positive results do not occur as a result of cross-contamination.

*Supplies and equipment*

1. Lightcycler capillaries.
2. Lightcycler carousel.
3. Lightcycler centrifuge adapters.
4. Lightcycler carousel centrifuge (optional).

5. Sterile microcentrifuge tubes (1.7 ml).
6. Sterile PCR tubes (0.5 ml).
7. Pipettors.
8. Pipette tips (aerosol barrier).
9. Microcentrifuge.

*Reagents*

1. Qiagen QIAamp® DNA Mini Kit (Valencia, CA).
2. LightCycler® Fast Start DNA Master Hybridization Probes (Roche).

   *Components:*

   a) PCR grade water (#3)
   b) $MgCl_2$ (#2)
   c) 10x reaction mix (#1)
3. Uracil DNA glycosylase (2U/µl) (Roche).
4. TaqMan® primers and probes

   *Type A assay:*

   a) Forward primer (stock = 50 µM) (final= 750 nM per reaction)
      5′ gagacatcaattaaaagaagcaatacctt 3′
   b) Reverse primer (stock = 50 µM) (final= 750 nM per reaction)
      5′ ccaagagtactatttccggttggt 3′
   c) Probe (stock = 10 µM) (final= 200 nM per reaction)
      5′ aaaattctgc*t*cagcaggattttgatttggtt 3′

   *Type B assay:*

   a) Forward primer (stock = 50 µM) (final= 750 nM per reaction)
      5′ cttgtacttttatttggctactgagaaact 3′
   b) Reverse primer (stock = 50 µM) (final= 750 nM per reaction)
      5′ cttgcttggtttgtaaatatagtggaa 3′
   c) Probe (stock = 10 µM) (final= 200 nM per reaction)
      5′ acctagttcaacc*t*caagacttttagtaatgggaatgtca 3′

   Probes are synthesized with 5′ FAM (6-carboxyl-fluorescein) and internal dark quenchers (either BHQ1 or QSY-7) at the nucleotide positions indicated by the *.

*DNA template preparation*

1. Grow bacteria for 24–48 h at 37 °C on an appropriate agar medium.
2. Collect 1 µl of cells using a sterile bacteriological loop and add to 180 µl of Buffer ATL (QIAamp DNA Mini Kit).
3. Purify DNA according to the QIAamp® tissue protocol.
4. As a final step, elute DNA from the QIAamp® column in 200 µl of Qiagen elution buffer.

*Negative control preparation*

1. The negative control should be prepared using sterile water distributed in aliquots into sterile tubes in a clean DNA-free area.
2. Aliquots may be prepared and stored at -20 °C until use.

*Positive control preparation*

1. Both a type A and a type B strain are needed for positive controls.
2. The control strains should be diluted to approximately 1000–10 000 genomic equivalents per µl (between 2–20 pg/µl) to minimize risk for cross-contamination.
3. Aliquots may be prepared and stored at -20 °C until use.

*Procedure.* In a DNA-free area (clean room with hood, with dedicated gloves, pipettors, tips, lab coat, etc.).

1. Calculate how many reactions are going to be performed for each assay (type A and type B) including an appropriate number of positive and negative controls. In calculating volumes for master-mix preparation, add extra reactions (10%) for pipetting errors. For example, if you have 10 samples, 1 positive and 1 negative control (12 capillaries total) to run with the type A assay, base the master-mix calculation on 15 capillaries.

| Reagent | Volume per reaction (µl) |
|---|---|
| Water (#3) | 3.3 |
| MgCl2 (#2) | 3.2 |
| 10X reaction mix (#1) | 2.0 |
| Uracil DNA glycosylase (2 U/µl) | 0.5 |
| Forward primer (50 µM) | 0.3 |
| Reverse Primer (50 µM) | 0.3 |
| Probe (10 µM) | 0.4 |
| **Total** | **10.0** |

2. Prepare the master mix for each assay separately. Prepare one sterile tube with the master mix for the type A assay, and one tube with the master mix for the type B assay.
3. Move master mixes to a separate area for loading and addition of DNA.
4. Working in a separate area or hood for DNA work (with dedicated gloves, pipettors, tips, lab coat, etc.):
   a) Add capillaries either directly to a LightCycler® carousel or LightCycler® centrifuge adapters with clean gloves, taking extra care to not touch the opening of the capillaries.
   b) The final reaction volume for each capillary is 20 µl.
   c) Add 10 µl of master mix to each capillary.
   d) Prepare negative control capillaries first. Add 10 µl of sterile water to the negative control capillary and cap.
   e) Prepare sample capillaries next. Add 9 µl of sterile water and 1 µl of samples to be tested and then cap. If necessary, more sample DNA can be added (up to 9 µl) and water volume adjusted accordingly for the final reaction volume of 20 µl.
   f) Prepare positive control capillaries last. Add 9 µl of sterile water and 1 µl of DNA and then cap.
   g) Gently centrifuge (either in carousel with LightCycler® carousel centrifuge or in an all-purpose microcentrifuge using LightCycler® centrifuge adapters) to collect contents at the bottom of the capillaries.
   h) Load carousel into LightCycler® and run programme.

*PCR programme*

1. 50 °C for 2 min
2. 95 °C for 10 min
3. 95 °C for 10 s
4. 65 °C for 30 s
5. repeat steps 3–4 for 44 cycles
6. 45 °C for 5 min.

*Results and interpretation.* After the programme has finished, proceed to analysis by adjusting the y-axis to channel F1/F3. Baseline adjustment should be selected as arithmetic. The threshold-crossing values are then automatically calculated using the second derivative maximum method. A sample is considered positive if it crosses the threshold during the 45 cycles of the programme AND displays logarithmic amplification.

*Quality control.* Any PCR run should be repeated if the negative and positive controls do not perform as intended. PCR inhibition can be caused by numerous factors. Negative samples can be spiked to check for PCR inhibition. It is important to ensure weak positive results (high Ct values) are not due to cross-contamination from positive control samples.

## B.3 References

AB Biodisk, Solna, Sweden: Package insert, product labels, E-test Technical Guide, Media/Inoculum/Incubation, Reading and Trouble Shooting charts, CIS 007; also see www.abbiodisk.com.

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Tomaso et al. (2005). Antimicrobial susceptibilities of Austrian *Francisella tularensis holarctica* biovar II strains. *International Journal of Antimicrobial Agents*, 26:279–284.

# C. Protocols for preparation of selected *F. tularensis* culture media

The following protocols have been provided by the University of Umeå, Sweden and the Centers for Disease Control and Prevention, Fort Collins, CO, USA.

## C.1 Cysteine heart agar base with 9% chocolatized sheep blood

Cystine heart agar (CHA) with blood (CHAB) is a nonselective medium used for primary isolation and cultivation of *F. tularensis*. Additionally, growth on CHAB provides a presumptive identification of *F. tularensis* as the organism shows characteristic growth on this medium (green, opalescent, raised, shiny colonies at 24–48 h). The recipe can be supplemented with antibiotics for the isolation of *F. tularensis* from materials that may be contaminated with other organisms.

### C.1.1 Materials (for 1 litre of medium)

1. Cysteine heart agar powder (Becton Dickinson Diagnostic Systems).
2. Defibrinated sheep blood.
3. For preparation of antibiotic supplemented CHA (CHAB-A) (per litre of medium):
   a) 7.5 mg colistin
   b) 2.5 mg amphotericin
   c) 0.5 mg lincomycin
   d) 4 mg trimethoprim
   e) 10 mg ampicillin.

### C.1.2 Procedure

1. Suspend 51 g of CHA powder in 1 litre of purified water.
2. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder.
3. Autoclave at 121 °C for 15 min.
4. Cool to 72 °C.
5. Aseptically add 90 ml of defibrinated sheep blood (9% final) and mix on heated stirrer until blood becomes chocolatized.
6. If preparing CHAB-A, cool to 55 °C and add antibiotics.
7. Mix gently but carefully using a magnetic stirrer and avoid the formation of air bubbles (i.e. foam) in the agar.
8. Dispense 20 ml volumes of medium into sterile Petri dishes.
9. Replace the lids on the Petri dishes, and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4 °C.

### C.1.3 Quality control

1. Incubate several uninoculated plates at 37 °C for 24 h and check for contamination.
2. Inoculated CHAB should support the growth of *F. tularensis* (e.g. LVS) after incubation at 37 °C in ambient air or in a 5% $CO_2$ humid atmosphere for 48 h.
3. *F. tularensis* should appear as white to greenish colonies, usually too small to be seen as individual colonies at 24 h. After incubation for 48 h, colonies are about 2–4 mm in diameter, opaque and exhibit greenish-lavender colour. An opalescent sheen is apparent on the surface of the colonies if incubated 48–72 h.

## C.2 GCII agar with 1% haemoglobin and 1% IsoVitaleX

GCII agar with haemoglobin and IsoVitaleX is a nonselective medium used for primary isolation and cultivation of fastidious microorganisms; especially *Francisella*, *Neisseria* and *Haemophilus* species. To obtain a suitable level of standardization for qualitative microbiological procedures, haemoglobin powder has replaced whole blood products in this 'chocolate agar' medium. GCII agar with haemoglobin was originally developed for standardized culture of *Neisseria gonorrhoeae* and can be used with various supplements that enhance the growth of fastidious bacteria (e.g. IsoVitaleX) and/or to suppress contaminant bacteria (e. g. various antimicrobial agents).

The following methods allow for the production of 200 ml of medium (five plates, 90 mm diameter); adjust quantities proportionately for the production of larger volumes of medium.

### C.2.1 Haemoglobin solution

1. Add 2 g of soluble bovine haemoglobin powder (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) to 100 ml of ultrapure water and mix using a magnetic stirrer. Store overnight at 4 °C.
2. Mix thoroughly the following day using the magnetic stirrer until a smooth suspension is achieved.
3. Autoclave the haemoglobin solution at 121 °C for 15 min. Cool in a water-bath set to 50–60 °C.

### C.2.2 GCII agar

1. Suspend 7.2 g of GCII agar base (Becton Dickinson Diagnostic Systems) in a small amount of ultrapure water and add water to a final volume of 100 ml.
2. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder (approximately 1 min).
3. Autoclave the medium at 121 °C for 15 min. Cool in a water-bath set to 52 °C.

### C.2.3 Enrichment supplement (IsoVitaleX)

IsoVitaleX, a registered trademark of Becton Dickinson, is a chemically defined mixture of supplements that enhances the growth of fastidious bacteria. IsoVitaleX™ (Becton Dickinson) comes in vials of lyophilized substance.

1. Aseptically open the vial containing the lyophilized growth supplement.
2. Use a sterile needle and syringe to aseptically transfer 10 ml of the accompanying diluent to the vial.
3. Shake to ensure complete solution. After reconstitution use the growth supplement immediately, or store at 4 °C and use within 2 weeks.
4. The composition of an alternative supplement is detailed in Table C.1.

### C.2.4 Preparation of plates

1. Aseptically add 100 ml sterile haemoglobin solution and 2 ml of reconstituted growth supplement to 100 ml of GCII agar base medium.
2. Mix gently but carefully using a magnetic stirrer and avoid the formation of air bubbles (i.e. foam) in the agar.
3. Dispense 20 ml volumes of medium into each sterile Petri dish to achieve a uniform agar depth of 3–4 mm.
4. Replace the lids on the Petri dishes and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4 °C.

### C.2.5 Quality control

Inoculated GCII agar with haemoglobin and IsoVitaleX should support the growth of *F. tularensis* (e.g. the avirulent and fastidious strain ATCC 6223) after incubation at 37 °C in ambient air or in a 5 % $CO_2$ humid atmosphere for 48 h.

**Table C.1**
Chemically-defined enrichment supplement for *F. tularensis* (Department of Clinical Bacteriology, Umeå University Hospital, Sweden)

| Ingredient | Weight (g) per litre ultrapure water |
|---|---|
| Vitamin B12 | 0.01 |
| L-Glutamine | 10.0 |
| Glucose | 100.0 |
| Nicotinamide adenine dinucleotide | 0.25 |
| Co-carboxylase | 0.10 |
| Thiamine hydrochloride | 0.003 |
| Ferric nitrate | 0.02 |
| L-Cysteine hydrochloride | 25.9 |
| Adenine sulphate | 1.5 |
| Guanine hydrochloride | 0.03 |
| L-Cystine | 1.10 |
| p-Aminobenzoic acid | 0.013 |

# D. Reagent list

**Table D.1**
Diagnostic reagents for *F. tularensis*

| **Reagents: commercially available** | |
|---|---|
| *Francisella tularensis* antiserum (for slide and tube agglutination) | Becton Dickinson Diagnostic Systems<br>Loveton Circle Sparks MD USA 21152<br>Phone: 001 410 316 4000;<br>www.bd.com/ds/index.asp: Catalogue number: 240939 |
| Anti-*Francisella tularensis* LPS (clone FB11)<br>Anti-*Francisella tularensis* LPS (clone T14) | Advanced ImmunoChemical Inc<br>105 Claremont Ave Long Beach CA USA 90803<br>Phone: 001 562 434 4676;<br>www.advimmuno.com; Catalogue number: G3-T4E1014 |
| Hybridoma against *F. tularensis* subsp. *holarctica* (LVS) | American Type Culture Collection (ATCC)<br>P.O.Box 1549 Manassas VA 20108 USA<br>Phone: 001 703 365 2700;<br>www. atcc. org; Catalogue number: HB-10830 |
| Monoclonal antibody anti-*F. tularensis*<br>Monoclonal antibody anti-*F. tularensis*-HRP conjugate | Senova GmbH<br>Winzerlaer Strasse 2 07745 Jena Germany<br>Phone: +49 (0) 3641 508 508;<br>www. senova. de |
| Diagnostic Kit for Tularemia (Souprava k diagnostice tularemie) including *F. tularensis* antigen for agglutination and *F. tularensis* control antiserum | Bioveta a.s.<br>Komenskeho 212 CZ-68323 Ivanovice na Hane<br>Czech Republic<br>Phone: +420 517 318 599; www.bioveta.cz |
| **Reagents: available from reference laboratories** | |
| FITC-conjugated rabbit-anti *F. tularensis* polyclonal serum (DFA); formalin-fixed *F. tularensis* cells (microagglutination); positive control anti-*F. tularensis* serum (microagglutination) | Centers for Disease Control and Prevention (CDC)<br>Division of Vector-Borne Infectious Diseases, Bacterial Zoonoses Branch, Diagnostic and Reference Laboratory<br>Fort Collins CO USA<br>Phone: 001 970 221 6400 |
| *F. tularensis* clones (positive controls) fopA, iglC, pdpD | Dr. Fran Nano University of Victoria, Victoria, BC V8W 3P6 Canada<br>Phone: 001 250 721 7074 |
| **Strains** | **Source** |
| ATCC 6223 *Francisella tularensis* subsp. *tularensis* (B-38); avirulent strain<br>ATCC 25015; ATCC 25016; ATCC 25017;<br>ATCC 25018 *Francisella philomiragia* | American Type Culture Collection (ATCC)<br>P.O. Box 1549 Manassas VA 2010 |

# E Transport of specimens and cultures of *F. tularensis*

The transport of infectious substances, including *F. tularensis*, is highly regulated. Implementation of regulations is mandatory. To help understand the current regulatory framework and support compliance with current international regulations for the transport of infectious substances and patient specimens by all modes of transport, WHO has developed the document *Guidance on regulations for the Transport of Infectious Substances 2007–2008*, World Health Organization, 2007, applicable as from 1 January 2007. We recommend the reader to consult the above document for details regarding the transport of infectious substances.

Infectious substances are divided into two categories, A and B. A flowchart for the classification of infectious substances and patient specimens is given in Figure E.1.

According to applicable transport regulations, cultures (as defined in the transport regulations) of *F. tularensis* are classified as Category A, while specimens of *F. tularensis* not in the form of cultures are assigned to Category B.

## E.1 Shipping F. tularensis

It is the responsibility of the shipper to ensure the correct classification, packaging, labelling and documentation of all samples containing *F. tularensis* destined for transport.

All specimens to be transported must be packaged according to applicable regulations, as described in *Guidance on regulations for the Transport of Infectious Substances 2007–2008*, World Health Organization, 2007, applicable as from 1 January 2007.

Infectious substances in Category A may only be transported in packaging that meets the United Nations class 6.2 specifications and complies with UN Packing Instruction P620 (PI602 air mode). This ensures that strict performance criteria are met. There is no comprehensive list of suppliers for packaging that complies with these packing instructions; however, an Internet search using search terms such as 'UN Packaging' and 'UN infectious substance packaging' provides the appropriate information.

Maximum net quantities for packages vary depending on the transport mode. For surface transport, there is no maximum quantity per package. For air transport, the limits per package are 50 ml or 50 g for passenger aircraft and 4l or 4 kg for cargo aircraft. Note: hand carriage of Category A and Category B infectious substances and transport of these materials in diplomatic pouches is strictly prohibited by international air carriers!

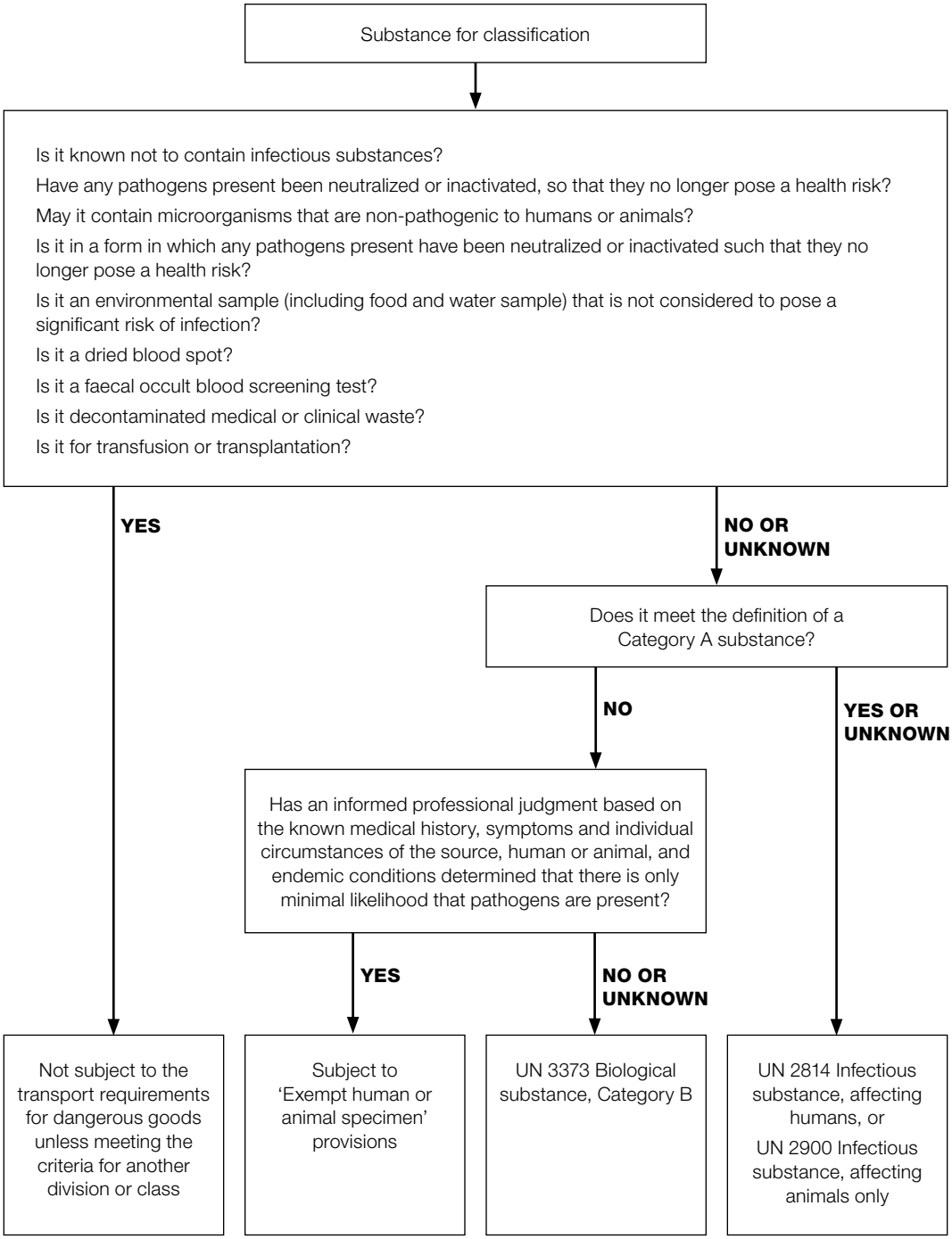
For substances in Category B, it may be possible to source packaging locally rather than finding an authorized supplier, provided that the packaging manufacturer and the shipper can comply fully with the requirements of P650. Testing documents are not required, however. Dangerous goods documentation (including a shipper's declaration) is not required.

For surface transport, there is no maximum quantity per package. For air transport, no primary receptacle shall exceed 1l (for liquids) or 1 kg (for solids) and the volume shipped per package shall not exceed 4l or 4 kg. Note: hand carriage of Category A and Category B infectious substances and transport of these materials in diplomatic pouches is strictly prohibited by international air carriers!

## E.2 Training

Persons engaged in the transport of dangerous goods, including infectious substances, shall have received training commensurate with their responsibilities. For transport of Category A infectious substances, personnel must undergo training in accordance with the modal requirements. This can include attendance at approved courses and passing examinations. Further information should be obtained from the local transport authorities.

**Figure E.1 Flowchart for the classification of infectious substances and patient specimens**




Reproduced from the *Guidance on regulations for the transport of infectious substances* 2007–2008, WHO, 2007.

## E.3 References

*Guidance on regulations for the Transport of Infectious Substances 2007–2008*, Geneva, World Health Organization, 2007 (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html).

# F. Checklist for outbreak investigation

## F.1 Preparations

### F.1.1 Conditions under which a tularaemia outbreak investigation may be useful

1. Clinical manifestations support tularaemia.
2. Single cases have been confirmed by laboratory methods.

### F.1.2 Outbreak investigation team

An outbreak investigation team could be composed of the following members covering different tasks:

1. Team leader: coordinates team activities, communicates (information, agreements) with the national/international health authorities.
2. Epidemiologist: designs epidemiological study design, ensures that the right type of data is collected in a manner that is suitable for epidemiological investigation, analyses the epidemiological data, prepares a report to convey the information.
3. Microbiologist: supports appropriate collection, storage and shipment of clinical and environmental specimens; undertakes microbiological analyses.
4. Ecologist and/or veterinarian and/or entomologist: assesses environmental risks for *Francisella* infection and evaluates the involvement of animal species and vectors in the tularaemia outbreak.
5. Physician: supports the treatment of patients and preventive measures to reduce the risk of exposure to infection sources, collects clinical specimens for further analyses.
6. Laboratory staff: support first field analyses, preparation of specimens, etc.
7. Local health authorities and personnel: of high priority for inclusion as they usually know the local situation and specific circumstances.
8. Translator, if needed.

### F.1.3 Coordination with local health authorities

1. Existence of a request for support made by local authorities.
2. Consideration of local specificities, e.g. political and social environment, safety considerations in crisis-afflicted areas, laboratory and clinical capacities.
3. Agreements by local authorities to undertake an outbreak investigation in compliance with regulations for investigations of humans and collection of environmental samples, including animal carcasses.
4. Local conditions for supporting the outbreak investigation including personnel to undertake field investigation, cars, laboratory facilities (with appropriate safety level), established laboratory methods for diagnosis of tularaemia and detection of *Francisella*, possibilities for storage of samples, accommodation for the investigation team.

The investigation must be adapted to local prerequisites and regulations. Instruments and materials need to be transported into the affected region. Administrative requirements need to be initiated, including regulations for transportation of specimens to specialized laboratories.

### F.1.4 Action to be taken by the investigation team before going to the field

1. Communicate with local authorities (objectives of the planned investigation, timeframe, type of samples to be collected, number of samples to be collected, expected results, ownership of samples etc.).
2. According to the identified aims, set up the investigation team (see above).
3. Vaccinate the investigation team against tularaemia, if possible.
4. If no biosafety level 3 laboratory facility is available in the affected area, only preliminary investigations or investigations with inactivated specimens can be done. For further investigation, including isolation of the pathogen by cultivation, the specimens have to be shipped to specialized laboratories.
5. Full protection is not needed for epidemiological investigations of, for example, households or patients; for the latter, normal hygienic measures should be sufficient.
6. Respect visa and vaccination regulations when the outbreak investigation is international.

### F.1.5 Equipment

1. Personal computer (including power adaptor, storage media (Floppy-disks, CD-ROM, USB-key).
2. Systems for drawing blood samples.
3. Swabs and appropriate transportation materials.
4. Instruments to handle carcasses and environmental samples.
5. Sufficient freezing capacity (dry ice) to store and ship samples, in appropriate containers (in compliance with regulations for shipment of dangerous goods).
6. Materials and instruments for laboratory investigations when these are to be performed locally. It should be recognized that in certain regions technical support is completely unavailable.

## F.2 Outbreak investigation in the field

Data available from epidemiological and laboratory investigations should be analysed promptly to obtain, as a minimum, information on the size and duration of the outbreak, on the source of infection and suspected transmission routes of the pathogen.

Once it has been decided that an outbreak investigation for tularaemia is to be carried out, the following steps may be undertaken.

1. Contact local authorities: the team leader communicates with the local authorities regarding the coordination of work with local staff, procedures for reporting and the communication of results.
2. Enquire about the epidemiological situation: contact patients and local physicians (see sample questionnaire for case-control study), analyse laboratory data (for evidence of their plausibility).
3. Formulate a hypothesis on source and cause of the outbreak, e.g. who was affected; where and when did the suspected outbreak occur?
4. Construct a time schedule for the investigation.
5. Make preliminary recommendations for treatment and prevention of the disease.
6. Set up facilities for diagnosis and/or for shipping specimens to a different laboratory for confirmation/further analyses (see chapter 6).

7. Take samples (clinical and environmental). All samples should be frozen as soon as possible if not analysed within 24 hours. An appropriate system for labelling and identification of the samples should be used, e.g. barcodes, numbering.
8. Analyse samples.
9. Report the results according to the plan of action agreed upon with the local authorities.
10. Write up recommendations for: the limitation of the outbreak; avoiding new cases; adequate treatment of patients; prevention of further outbreaks; and in the longer term for improvement of the surveillance and diagnosis of tularaemia.
11. Agree on further analysis of specimens and epidemiological data.
12. Educate local health workers and authorities if requested. Especially in poorly developed areas, long-term support could be important to prevent or recognize early new outbreaks of tularaemia. This could include training of physicians for recognition of the disease, training of health-care workers in epidemiological analysis and of laboratory staff in diagnostic methods for tularaemia.
13. Publish the results to a wider audience (public media, scientific literature).

## F.3 Example for a tularaemia outbreak, Kosovo (Serbia)

An outbreak of tularaemia in UN Administered Province of Kosovo (Serbia) in 2000 was investigated by an international team brought together by the WHO Regional Office for Europe (Reintjes et al., 2002). The following information formed part of the report of the outbreak investigation which gathered together useful practical experiences. The main part was a case-control study to assess the risk factors for infection with *F. tularensis*.

### F.3.1 Protocol for case-control study of tularaemia infection used by the investigation team

1. After arrival at a case household:
   a) Ask whether the case was living in this house in the month before the illness started. If yes, please continue the questionnaire and ask for control households (see definition below). If no, please continue, but do not select controls for the case household.
   b) Ask whether either the person with suspected tularaemia infection or the person preparing the food is available to give a blood specimen; if not available, visit again another time/day.
   c) In the household of a case, a blood specimen should be taken from all family members who have experienced fever and cervical lymphadenitis after 1 November 1999 (the probable date of onset of the outbreak).
   d) After taking blood samples and completing the questionnaire, enquire about the two closest neighbours. If these neighbours use the same water supply, do not visit them but ask for other neighbours.
2. After arrival at a control household:

   Ask whether anybody in the family has had fever plus lymphadenitis colli since the first identification of index case. If no, continue with questionnaire and take blood from person preparing food. If yes, use as new case – take blood from all family members with symptoms. Select new control for both cases (you now have two cases and need four controls).

### Case definition

Any person who has had lymphadenitis colli after 1 December 1999, with laboratory confirmation of tularaemia (see section 6.1).

### Definition of a case household

A household with one or more family members with laboratory confirmation of tularaemia infection and with lymphadenitis colli after confirmation of tularaemia.

### Definition of a control household

Closest household to a case household during one month before onset of illness in the case, but with different water supply, with all family members free of high fever (at least 38 °C) and lymphadenitis colli, or, if any family member with lymphadenitis colli, with laboratory confirmation that all family members are negative for tularaemia infection.

## Questionnaire for case-control study: risk factors for tularaemia in households

CONTACT DETAILS:

Name: _____

Date of birth (day; month; year): _____

Village: _____

Case household (number): _____

FOOD CONSUMPTION:

In the month before case became ill; did you eat any of the following?

☐ Uncooked vegetables
☐ Meat of hares
☐ Pershut (dried ham)
☐ Fruits (e.g. apples)
☐ Milk/cream

WATER SUPPLY

In the month before illness what water did you use for drinking? (boiled;chlorinated):

☐ Central water (piped)
☐ Bottled water
☐ Personal well-water
☐ Water from collective well
☐ Water from tanks
☐ Streams

In the month before illness what water did you use for food preparation? (boiled; chlorinated)

☐ Central water (piped)
☐ Bottled water
☐ Personal well-water
☐ Water from collective well
☐ Water from tanks
☐ Streams

ANIMALS

In the past year have you seen large numbers of rodents? If yes, when?

☐ Inside house: _____

☐ Outside house: _____

What kind of rodent did you see?

☐ Mouse in the field

☐ Mouse in the house

☐ Rat in the field

☐ Rat in the house or yard

In the past year have you seen sick or dead rodents? If yes

☐ Inside the house?

☐ Outside the house in the private yard?

☐ In your water supply?

☐ Do you have any domestic animals (pigs, cow, hens)?

☐ Did you use flour from a humanitarian organization?

☐ If yes, which organization? _____

☐ What country of origin is this flour?

☐ Do you have dogs or cats in your household?

☐ Did you have contact with wild animals or carcasses?

☐ Did you get bites by ticks, fleas, bugs, mosquitoes, flies, etc.?

☐ If yes; did you get inflammation or an ulcer at the site of the bite?

OBSERVATIONS OF WATER SUPPLY (ASK TO BE SHOWN THE WATER SUPPLY)

☐ Well?

☐ Piped water?

☐ If well; is it protected against rodents?

☐ If well; is it protected against water leakage?

FOOD STORAGE (PLEASE ASK TO BE SHOWN THE FOOD STORAGE ROOM)

☐ Is the food storage room protected against rodents?

☐ Do you see faeces of rodents near the food or food touched/partly eaten by rodents?

# G. Protocols for trapping and sampling small mammals

Field investigations involve capturing the small mammal hosts of tularaemia for collection of blood, ectoparasites (ticks and fleas), and occasionally tissue samples. The primary goal is to collect sufficient numbers of animals representative of small mammal communities at study sites to determine which species of animals are infected with *F. tularensis*. The surveys include collection of live rabbits and rodents, and ectoparasites from these animals.

Much of the information below is derived from Sudia et al., (1970), Mills et al., (1995) and Dennis et al., (1999). National regulations on the ethical prerequisites for animal research need to be considered.

## G.1 Trapping of small mammals

1. Set a combination of Tomahawk and Sherman live traps in areas of special interest. Check the proper functioning of the traps before setting.
2. Place upholstery cotton in each trap during cold weather to reduce the potential for hypothermia. Cover traps with canvas on top and sides to provide additional protection during adverse weather.
3. Bait traps with rolled oats, peanut butter, or other appropriate items that are attractive to the animals.
4. Check traps at 1–2-hour intervals to ensure that animals do not suffer from prolonged exposure to heat or other threatening conditions. Check the traps as early in the morning as possible.
5. Wear rubber gloves while handling the traps with captured animals.
6. Label traps with captured animals with information about the location of trap on a strip of tape attached to the top of the trap.
7. Place the traps with captured animals in double plastic collecting bags. Place collecting bags with the captured rodents in a cool area out of the sun until all traps have been checked.
8. Replace collected traps with spare baited traps.
9. Transport the captured rodents to the field station for processing as soon as possible.
10. Surviving animals should be released as soon as possible, at or near the original point of capture (usually within 1–2 hours after the traps have been brought to the processing site).
11. Traps should be decontaminated after each trapping session.

## G.2 Protocols for collecting diagnostic samples from small mammals

### G.2.1 Anaesthesia

1. Captured animals are anaesthetized with Isoflurane or Ketamine depending on the animal species. All handling of animals and other possibly contaminated materials should be done with caution and using personal protection such as gloves, full face protection (FFP3) masks and other measures according to the risk assessment. All manipulations with sharp tools such as dissection of carcasses or taking blood samples with needles must be done by trained personnel and with special caution.

2. Small rodents captured in Sherman traps can be easily removed to 12″ x 12″ ziplock bags containing a cotton or gauze with Isoflurane. Animals in Tomahawk traps may be anaesthetized by placing the entire trap into a large plastic bag containing anaesthetic-soaked gauze or in a tool box. Alternatively, insert a large pair of forceps through the mesh of the trap, grasp the skin of the animal, and inject it with ketamine/xylazine (10:1) with a needle and syringe.
3. If an animal begins to recover during collection of ectoparasites or during collection of blood samples, it should be re-anaesthetized immediately. If an animal escapes while attempting to anaesthetize it, personnel should not try to recapture it with their hands because of the high risk of being bitten.

### G.2.2 Collecting ectoparasites

1. The anaesthetized animals are placed in a pan and processed for fleas and ticks.
2. Fleas are removed from anaesthetized animals by brushing with a fine toothed comb. Attached ticks are removed with fine forceps.

### G.2.3 Taking blood samples

1. Label the cryovials for blood samples before collecting blood. Only screw-cap plastic cryovials with external thread should be used.
2. Blood samples are collected from anaesthetized animals by bleeding from the retroorbital plexus or by cardiac puncture. Use a sterile heparinized tube or Pasteur pipette for retroorbital bleeding. Use only gentle pressure to avoid breaking the tube. Use small hematocrit bulbs to eject the blood that may remain in the capillary tube into a cryovial.
3. If the animal has died only recently, blood can be obtained directly from the heart by cardiac puncture. This procedure should be also done when retroorbital bleeding is impractical. For example, large animals can be difficult to bleed by the retroorbital technique, and cardiac puncture is likely to be a more satisfactory procedure.

   The cardiac puncture procedure is as follows:

   a) Choose an appropriately sized syringe and needle based on the size of the animal. For mice, a 1 cc syringe with a 25G x 5/8 inch needle (or similar small gauge needle) is preferable. Larger syringes are likely to create too much vacuum and collapse the heart chambers of these small animals. For larger animals, such as squirrels and rabbits, a 3 cc syringe can be used, although a 1 cc syringe fitted with a 23G needle that is 1–1.5 inches in length is satisfactory.

   b) Lay the animal out flat in a white enamel pan.

   c) Insert the needle just below the base of the sternum and slightly to the left of centre. As you insert the needle, gently pull back on the plunger until blood appears at the base of the needle. Once the needle is correctly positioned and blood is entering the syringe, gently pull back on the plunger, being careful not to withdraw the needle from the heart. Pulling back too quickly on the plunger will create excessive vacuum and collapse the heart, interfering with the collection of blood and increasing the chance that a blood clot will form in the needle. Continue to remove blood until it no longer flows freely into the syringe.

   d) Carefully squirt the blood into a cryovial or onto a Nobuto strip.

### G.2.4 Dissection for organ tissue recovery

1. Flame a pair of scissors and forceps over an alcohol burner.
2. Place the animal ventral side up in a clean pan.
3. Squirt the ventral surface with 70% ethanol from a squeeze bottle and wipe with a gauze square.
4. Pinch the skin of the lower part of the abdomen with forceps and lift it. Place the scissors below the forceps and cut through the skin and abdominal musculature. Insert one blade of the scissors into the incision and make cuts on each side of the abdominal wall, and then pull the cut skin and musculature back above the diaphragm to completely expose the abdominal cavity.
5. Using blunt-ended, nontoothed, sterile forceps, lift the stomach to expose the spleen. Grasp the spleen with the forceps and gently pull to remove it and place it in a cryovial. Using the same forceps (flame sterilized between each organ; ensure that the forceps are cooled between each usage) grasp the kidneys, one at a time, and place them into a second cryovial. With the forceps, grasp a portion of liver of appropriate size to fit into the third cryovial. Additional samples such as enlarged lymph nodes or liver lesions may be collected as needed.
6. After the processing of rodents has been completed, the processing area should be carefully decontaminated and carcasses must be collected for later incineration or other appropriate disposal.

## G.3 References

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