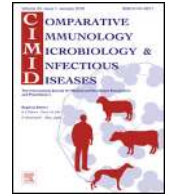Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Review

Tularaemia: A challenging zoonosis

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A R T I C L E   I N F O

A B S T R A C T

In recent years, several emerging zoonotic vector-borne infections with potential impact on human health have been identified in Europe, including tularaemia, caused by *Francisella tularensis*. This remarkable pathogen, one of the most virulent microorganisms currently known, has been detected in increasingly new settings and in a wide range of wild species, including lagomorphs, rodents, carnivores, fish and invertebrate arthropods. Also, a renewed concern has arisen with regard to *F. tularensis*: its potential use by bioterrorists. Based on the information published concerning the latest outbreaks, the aim of this paper is to review the main features of the agent, its biology, immunology and epidemiology. Moreover, special focus will be given to zoonotic aspects of the disease, as tularaemia outbreaks in human populations have been frequently associated with disease in animals.

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Contents

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1. Introduction

Seventy-five per cent of emerging infectious diseases are zoonotic [1]. Some wildlife species have been recognised as being major reservoirs for infectious diseases and the proximity of wildlife habitats and the existence of arthropod vectors with a wide geographical spread have rendered epidemiological cycles more complex [1].

Tularaemia is a zoonosis caused by the *Francisella tularensis* bacterium, which was first isolated in 1912 in Tulare County, California, by George McCoy and Charles Chapin [2–4]. Initially termed *Bacterium tularense*, it was allocated to a new genus and named *F. tularensis* in honour of the pioneer of research on the organism, Edward Francis [2,4]. Arthropod-borne transmission of tularaemia was first demonstrated by Francis in 1919 when he isolated the etiologic agent in a patient with "deer fly fever" [2,5,6].

Tularaemia was recognised as an important disease in the last century and since then there has been a growth in enthusiasm for research on this pathogen [7,8]. Interest has arisen with regard to *F. tularensis* as it has emerged in new locations, populations and settings, and increasingly figured in scientific research gauging its potential use in bioterrorism [7,9]. The European Centre for Disease Control and Prevention (ECDC) 2012 surveillance report refers a total of 891 confirmed cases of tularaemia in a number of European countries in 2010, with Sweden reporting the highest confirmed case rate, followed by Finland and Hungary [10]. Tularaemia is considered an unusual disease and the confirmed case rate in Europe has remained stable from 2006 to 2010. Recent outbreaks of tularaemia have occurred in several European countries, presented in Table 1, including the Czech Republic, Kosovo, Bulgaria, Germany, Sweden, Finland, Spain, Turkey, France and Norway [11–20]. Besides these outbreaks, sporadic case notifications have occurred in Austria, Estonia, Italy, Lithuania, Poland, Romania, Slovakia and the United Kingdom [10]. Although there are no reports of tularaemia for Denmark during this period, a confirmed case of the disease in a human was recorded there in 2003 [21]. In Portugal, the bacterium has been detected in the blood of an asymptomatic man and in a *Dermacentor reticulatus* tick by molecular methods [9].

2. Microbiology and phylogeography of *F. tularensis*

F. tularensis is one of the most virulent microorganisms currently known, while as few as ten microorganisms can cause potentially fatal disease in man and animals [7,22]. This high rate of infectivity has led the Centre for Disease Control and Prevention (CDC) to classify *F. tularensis* as a Category A biowarfare agent [23].

F. tularensis is a gram-negative, catalase-positive, pleomorphic and non-motile cocobacillus, characterised as a facultative intracellular pathogen that can grow within different types of cells including macrophages, hepatocytes and epithelial cells [2,22,24,25]. The cell wall of *F. tularensis* has an unusually high level of fatty acids with a unique profile for the genus, and wild strains have a lipid-rich capsule, with neither toxic nor immunogenic properties [2,5,6]. Capsule loss has been related to a decrease in virulence, although the viability or survival of the bacterium within neutrophils may remain unaltered.

F. tularensis is a gamma (γ)-*Proteobacteria* of the *Francisellaceae* family [2,4,22]. *F. tularensis* is the most common and pathogenic species and is formally divided into three subspecies with different pathogenicities and geographic distributions: *tularensis*, *holarctica* and *mediasiatica*. The species *Francisella novicida* is currently widely accepted as a fourth subspecies of *F. tularensis* [3,4,26–31], as it shares with *F. tularensis* an average of 99.2% nucleotide identity over a 1.1 Mbp of genome sequence [4,26,27,30]. However, some objections to the transfer of *F. novicida* to the subspecies rank of *F. tularensis* have been recorded, based on recent multiple genome sequencing results, which show divergent evolutions for *F. tularensis* and *F. novicida* populations. Therefore, separate species may be retained [32].
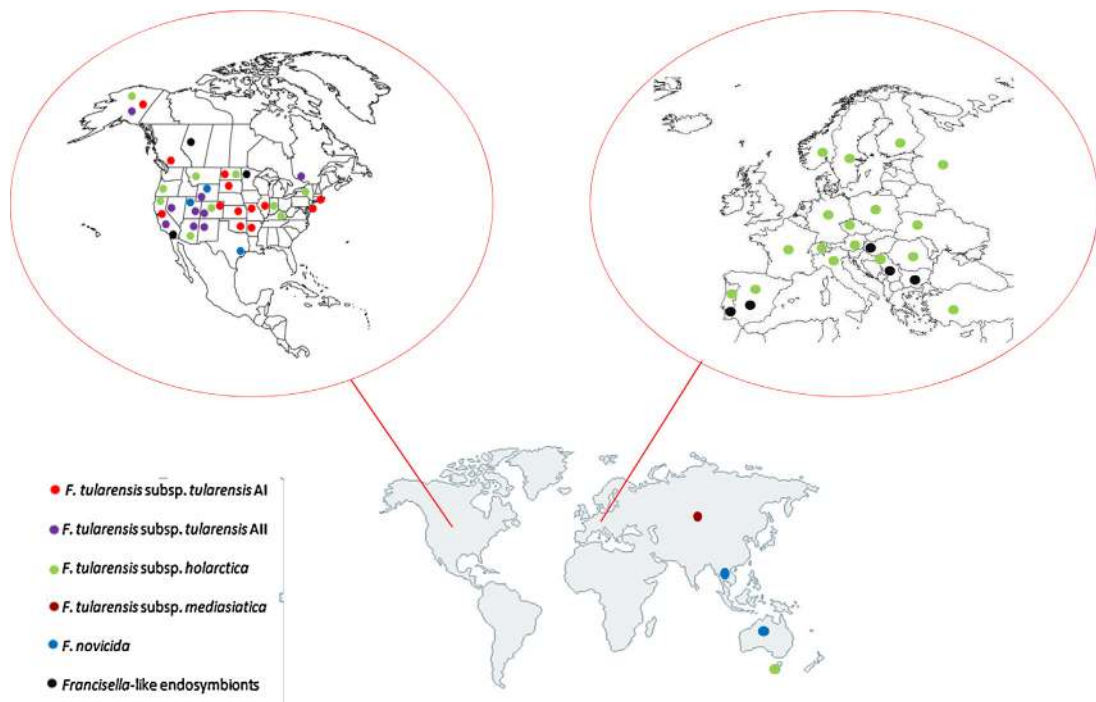
The *F. tularensis* subspecies *tularensis*, regarded as the most virulent subspecies and classified as Type A, occurs predominantly in North America [3,4,6,22,33]. Two distinct genetic sub-populations have been identified, AI and AII, which have different geographic distributions, hosts and vectors [3,4,6,26,30,34]. Sub-population AI has been additionally sub-divided into groups AIa and AIb [3,6,30,35]. The subspecies *holarctica*, related to milder forms of the disease and classified as Type B, occurs throughout the Northern Hemisphere [3,22,30,33]. Human infection with AIb strains usually have a fulminant clinical progression and are associated with high mortality rates, in contrast with infections by AIa and AII strains or Type B tularaemia [25,30,35]. Recently, this subspecies has also been detected in Tasmania, Australia [36]. Subspecies *mediasiatica* presents a similar virulence to subspecies *holarctica*, but its geographic distribution is restricted so far to Central Asia [26,33]. *F. novicida* is less virulent and has been isolated in North America, Australia and Thailand [3,26,29–33,37].

Based on a high degree of similarity between 16S rRNA gene sequences, other microorganisms have been classified as probable members of the *Francisellaceae* family; these include the *Francisella*-like endosymbionts or FLEs [6,8,38]. FLEs belong to a distinct phylogenetic clade from *F. tularensis* species [39]. The effect of FLEs, if any, on vector competency and in the transmission of *F. tularensis* by ticks is still unknown [6]. FLEs have a worldwide distribution and are vertically transmitted by hard and soft ticks of the genera *Amblyomma*, *Dermacentor, Ixodes* and *Ornithodoros* [39–42]. FLEs have been detected in ticks in North America

Table 1
Cases of tularaemia recorded in Europe.

European country	First report	Latest report	Suspected animal host	Tularaemia transmission to humans	Number of cases (year(s))	References
Czech Republic	1936 (humans and hares)	2000	Small mammals, particularly wild hares, rodents	Contact with tissues of infected animals, aerosols, contaminated food and water, tick bite	48 (2000)	[11,87–91]
Kosovo	1999–2000	2001/2002–2010 [a]	Ingestion of contaminated food or water	Ranging from 25 to 327 (2001–2010)	[12]
Bulgaria	1962 (muskrat)	1997, 2003–2005	Wild hares	Contaminated food and water, tick bite	285 (1997–2005) 24 (2003–2004)	[13,92]
Germany	1949	2004–2005	Wild hares, rodents	Contact with tissues of infected animals	39 (2004–2005)	[14,93,94]
Sweden	1931	2000–2005	[a]	Contaminated water, aerosols (farming), Mosquitoes	270 (2000) 698 (2003) 90 (2005)	[15,95–97]
Finland	[a]	2000, 2003, 2007	[a]	Mosquitoes, aerosols (farming), tick bite	50 (2007)	[16,21,76,97–99]
Spain	1997 (Human, wild hares)	2007	Small mammals (especially hares and rodents)	Aerosols, wild lagomorphs, canids, rodents, sheep, haematophagus vectors, crayfish	507 (2007)	[17,51,100,101]
Turkey	1936	2000, 2004–2010		Contaminated water	12 (2000) 61 (2004–2005) 12 (2005–2006) 40 (2010)	[18,102–107]
France	[a]	1997–2011	Wild hares	Contact with tissues of infected animals, aerosols, tick bite	144 (2007–2008) 51 (2011)	[19,108–110]
Norway	[a]	2003, 2005, 2007, 2011	Rodents (lemmings) and hares	Contaminated water or food	5 (2005) 9 (2007) 39 (2011)	[20,111,112]

[a] Information unavailable.

Fig. 1. Phylogeography of *F. tularensis* and *Francisella*-like endosymbionts.

(Texas, California, Minnesota), Canada (Alberta) and European countries such as Spain, Portugal, Hungary, Serbia and Bulgaria [38–45]. Their pathogenicity to humans is undetermined. They have recently been detected in free-living small mammals in Europe, suggesting the possible transmission of some FLE types from ticks to small mammals, although, to date, attempts to demonstrate it have failed [39,41,42,45,46]. The phylogeographic distribution of *F. tularensis* is given in Fig. 1; the geographic locations where FLEs have been detected in ticks are also indicated.

3. Epidemiology of *F. tularensis*

In nature, *F. tularensis* has been detected in a high number of wild species including lagomorphs, rodents, insectivores, carnivores, ungulates, marsupials, birds, amphibians, fish, and invertebrates [6,22,27,39,46–48].

Lagomorphs and rodents are considered as the main reservoirs of *F. tularensis* [6,22,46]. Wild lagomorphs, such as the European brown hare (*Lepus europaeus*), are thought to be suitable sentinels for *F. tularensis* and disease surveillance [46,47]. Recently, there have been serological evidences that foxes and raccoon dogs could also act as biological indicators for tularaemia [48].

Natural infections with *F. tularensis* have also been documented in different arthropods, although only a subset of these have been identified as important in *F. tularensis* transmission to humans. Still, few pathogens show the adaptability of *F. tularensis* to such a wide range of arthropod vectors capable of infection dissemination [6]. Arthropod found infected in nature include ticks of the genera *Amblyomma, Dermacentor*, *Ixodes* and *Ornithodoros*, mosquitoes of the genera *Aedes*, *Culex*, *Anopheles* and *Ochlerotatus excrucians*, and flies from the *Tabanidae* family (*Tabanus* spp., *Chrisozona* spp. and *Chrisops* spp.) [6,22,27,49]. Nevertheless, vector competence has only been demonstrated in ticks of the genera *Dermacentor* [35]. Tick-borne transmission of *F. tularensis* usually results in sporadic cases, although occasional outbreaks have also been reported [6]. Although regarded as merely mechanical vectors, mosquitoes have been associated with widespread epidemics of tularaemia and are capable of transient disease transmission [6,50]. Both ticks and mosquitoes may be infected in the larval phase. Transtadial transmission has been demonstrated in ticks although in mosquitoes evidences for transtadial transmission are only based in molecular methods [35,50]. Although transovarial transmission of *F. tularensis* in ticks was reported [2,6,51], a recent study in *Dermacentor variabilis* has proved otherwise [52]. Despite dissemination to ovaries and then to the oocytes, the pathogen was not recovered from the subsequently hatched larvae. Tabanid flies are regarded as mechanical vectors for *F. tularensis* and the long-term survival of this bacterium does not occur in these arthropods [6].

The epidemiologic characteristics of vector-borne tularaemia vary throughout the northern hemisphere and also within a given geographic location. This is thought to be related to the abundance of different vectors and host species. This could explain why, in the USA, Sweden, Finland and Russia, the arthropod bite is a common mode of transmission to humans, whilst in Western and Central Europe, contact with infected animals and the ingestion of contaminated food or water have been reported as more common transmission modes. Differences in transmission patterns have also been recorded within the USA: in western states, both ticks and deer flies are considered to be important vectors of tularémia, while in the east only ticks are considered relevant. In Sweden and Finland, mosquitoes have been identified as the primary vectors [6].
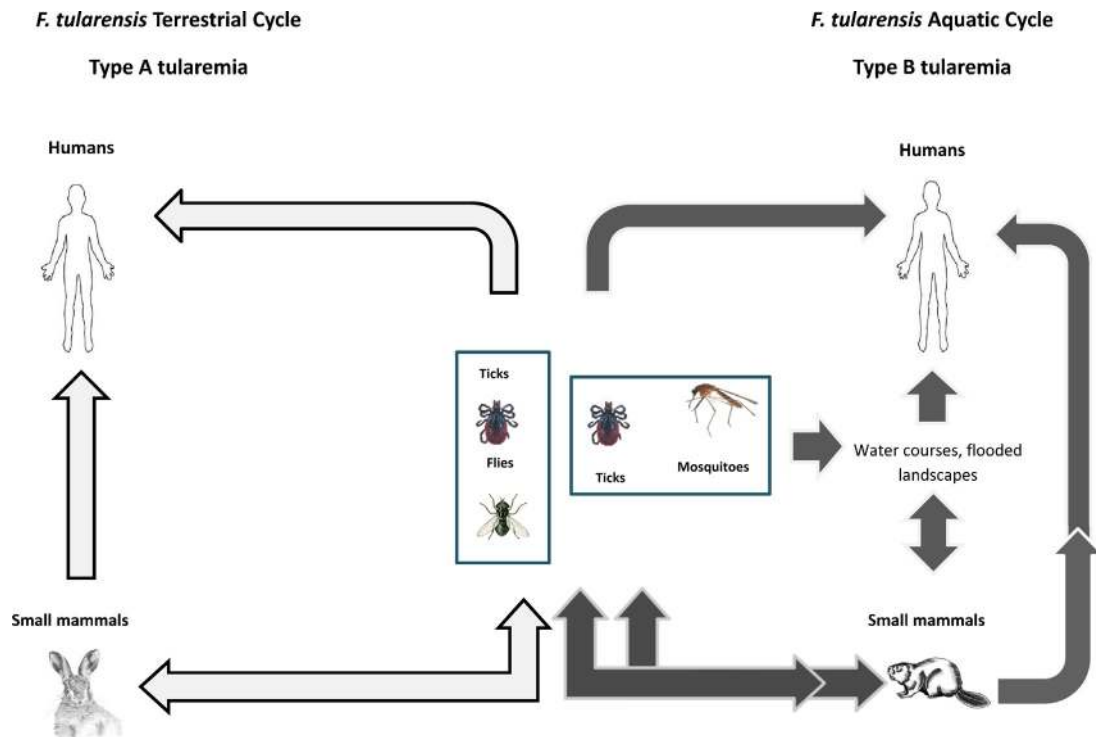
In Portugal, the role of ticks and small mammals in the transmission of tularaemia is still the subject of research. A collection of 4949 mosquitoes belonging to the genus *Culex* (63.97%), *Ochlerotatus* (35.34%), *Anopheles* (0.42%), *Culiseta* (0.14%) and a small number of *Aedes aegypti* females from the island of Madeira (0.12%) have been analysed, although all the results were found to be negative [53]. So far, this is in accordance with previous findings regarding the epidemiology characteristics of vector-borne tularaemia, suggesting that, in Portugal, mosquitoes have no role in the transmission of this disease. Ticks are thought to be the most important vectors of tularaemia in the majority of countries where tularaemia is endemic [53]. Nevertheless, major on-going research on tularaemia, aiming at gauging the overall impact of the disease in Portugal, is expected to throw further light on the main *F. tularensis* sources.

In endemic areas, tularaemia is a seasonal disease, with higher incidence in late spring, summer and autumn, occurring annually over a 5-year period or unreported for more than a decade. Often, the number of cases varies widely from 1 year to another, which is thought to be due to temperature or precipitation variability. However, the association between climactic conditions and tularaemia outbreaks has yet to be demonstrated [49]. *F. tularensis* has been found to be extremely resistant to environmental stress, surviving for weeks in soil, water and animal carcasses, at low temperatures [22].

Human tularaemia outbreaks are often preceded by animal outbreaks, particularly in wild lagomorphs and rodents. This is usually related to an increase in the numbers of these species, increasing the probability of exposure to infected animals [4,22,27,49].

The transmission of tularaemia to humans can occur either by direct contact with infected animals or indirectly due to arthropod vector bites, the ingestion of contaminated water, food or aerosols inhalation. Aerosols can be dispersed by ventilators, farming, and the deposition of contaminated hay, either intentionally or unintentionally [22]. Domestic dogs and cats can also transmit tularaemia to humans after contact with an infected animal, environment or infected ticks [54–56]. Person-to-person transmission has not been described so far [2,22,49,54].

Tularaemia has been reported to occur in any age group. Men tend to present a higher prevalence than women [2,49]. Professions that are prone to contact with reservoirs or arthropod vectors have been associated with a higher infection risk: these include laboratory technicians, hunters, farmers, veterinary surgeons, and anyone handling the flesh of infected animals [22,27].

Fig. 2. Two life cycles of tularemia are recognised: the terrestrial cycle and the aquatic cycle. The terrestrial cycle is more commonly associated with Type A tularemias and the aquatic cycle with Type B.
Adapted from Akimana and Kway (2011) [113].

4. *F. tularensis* life cycle

Few pathogens show the adaptability of *F. tularensis* to varying vector, host and environmental conditions. Variations occur in local transmission cycles in association with differing ecologies. Both *F. tularensis* type A and type B are associated with different life cycles in which different animal hosts and arthropod vectors intervene [6]. Type A tularaemia is more commonly associated with the terrestrial cycle of the disease, with wild lagomorphs such as rabbits and hares acting as vertebrate hosts in which amplification of the agent occurs and where arthropods are disease-disseminating vectors [6,22,54,57]. Type B tularaemia is more frequently associated with the aquatic cycle, although outbreaks of tick-borne tularaemia involving subspecies *holarctica* have been reported [2,6,57]. In this life cycle, *F. tularensis* circulates in rodents such as beavers, muskrats and voles, and can be introduced in water courses from animal carcasses [6,22,27,54]. There is also evidence that *F. tularensis* can persist in water courses in association with amoebas [27,49,58]. Contaminated water can be the source of infection to humans, flies and mosquitoes [49]. An unusual waterborne outbreak of human tularaemia has been described in Spain associated with crayfish (*Procambarus clarkii*) caught in a contaminated freshwater stream. The crayfish acted as mechanical vectors, through mud- or water-contaminated carapaces, although the presence of *F. tularensis* in crayfish stomach and hepatopancreas could indicate their eventual role as hosts [51]. A diagrammatic representation of the terrestrial and aquatic cycles of tularaemia is shown in Fig. 2.

5. Immunopathogenesis

F. tularensis is a remarkable bacterial pathogen that can invade and multiply in a wide range of cell types [4,22,24,25,59]. Antigen-presenting cells (APC) such as macrophages or dendritic cells, appear to be the primary cell types targeted by the bacterium at the outset of infection [59]. The virulence of the bacterium is directly related to its capacity to replicate within the cytosol of infected cells [60]. *F. tularensis* clearly possesses several mechanisms by which it manipulates immunity. The bacterium evades detection at the point of entry in the host in three ways: (a) it has modified cell-surface structures that enable it to avoid interaction with host receptors that are associated with the induction of inflammation; (b) it targets cells that lack co-receptors which facilitate binding to receptors that might alert the host cell to invasion; (c) it utilises receptors that fail to initiate the production of pro-inflammatory cytokines [60].

6. Innate immune response

The entry of *F. tularensis* in macrophages occurs by means of a specific mechanism inherent to *Francisella* spp. [24]. The bacterium induces the macrophage to produce asymmetric spacious pseudopod loops in a "looping phagocytosis" process [4,61]. Uptake of *F. tularensis* is markedly

enhanced by serum opsonisation, which depends on serum intact complement factor C3 and host cell receptors (CR3), involving bacterial surface polysaccharides [4,62].

Utilisation of CR3 (and of mannose receptors of dendritic cells (MR) under non-opsonising conditions) is considered to be a fairly innocuous route for entry of *F. tularensis*, since it is not associated with the induction of signalling cascades that result in pro-inflammatory cytokines production. When opsonised by serum, *F. tularensis* binds iC3b and gains entry to host cells via the CR3 receptor [59].

The lipopolysaccharide (LPS) of subspecies *tularensis* is only moderately inflammatory and acts as an extremely weak toll-like receptor (TLR) 4 agonist stimulating a reduced production of pro-inflammatory cytokines [59,63]. These is attributed to the presence of only four acyl groups on the LPS that do not bind to the "LPS-binding proteins", subverting TLR4 recognition [4,25,59]. In addition to LPS, *F. tularensis* possesses two other TLR agonists [59]: Tul4 and FTT1103 lipoproteins. These interact with TLR2 and may alert the host cell for the presence of the bacterium prior to phagocytosis [4,25,59]. TLR2/myeloid differentiation primary response gene (88) (MyD88) signalling is essential for the production of pro-inflammatory cytokines and is critical for host defence against *Francisella* infection [24,61,63,64].

F. novicida has been used as a model organism to study immunity to *F. tularensis*. Nevertheless, *F. novicida* expresses a structurally distinct chemotype of LPS that is more pro-inflammatory in mice than the dominant LPS chemotype, and is expected to result in different inflammasome activations [25]. *F. novicida* escapes the phagossome and replicate in the cell cytosol where it is recognised by the inflammasome signalling system [24,25,60,64]. Inflammasome *stimuli* activate the protease cysteine aspartate-specific Caspase-1, promoting the release of potent pro-inflammatory cytokines responsible for cell apoptosis [24,60]. This results in *F. novicida* release from infected cells and enables the infection of new ones [24,60].

F. tularensis survival and replication within macrophages is enabled by a large set of virulence genes that include the "macrophage growth *locus*" (*mgl*) A and B and the "*Francisella Pathogenicity Island*", FPI [24]. FPI encodes for a putative type VI secretion system [4,8] and contains 19 genes that have been demonstrated as essential for intra-cellular growth and virulence [24]. Less virulent *F. novicida* presents only one copy of FPI in contrast with *F. tularensis* subspecies *tularensis* and *holarctica* that present two copies [4,24]. Genes within the FPI are regulated by *mgl*A [4]. Although current knowledge of the gene's functions is far from complete, this is one of the most active areas of *Francisella* research [8].

Following phagocytosis of opsonised *F. tularensis* by polymorphonuclear cells (PMN), the bacterium actively inhibits superoxide anion generation (ROS) via NADPH oxidase. This allows *F. tularensis* to evade the phagosome and persist in the cell cytosol. The contribution of polymorphonuclear cells seems to be related to the secretion of cytokines and chemokines that recruit effector cells to the infection site [25]. However, an excessive recruitment of

neutrophils, modulated by an increase in metaloprotease-9 from the matrix, plays an important role in modulating leucocyte recruitment and seems to be directly related to *F. tularensis* pathogenesis [24,25].

Natural killer (NK) cells from the liver, spleen and lung also play an important role in the innate immune response, in particular by producing INF-α following primary infection by *F. tularensis* [25].

7. Acquired immune response

As *F. tularensis* is an intracellular pathogen, cellular immune response is believed to be the main defence mechanism. Memory effector T cells CD4+ and CD8+ are clearly important for the primary control of infection. These cells produce Type Th1 cytokines like INF-γ, TNF-α and IL-2 that are critical for the initial response to *F. tularensis* infection [25].

Although the role of humoral immunity in *F. tularensis* infection is believed to be less important, some studies have demonstrated the enhanced recovery of infected humans that have received hyper-immune serum [59]. Also, infection-specific IgM, IgA and IgG antibodies produced are good exposition indicators and may interfere with the ability of bacteria to infect host cells [25,49,59]. The contribution of B cells in defence is thought to be dependent on strain virulence [8,25]. Research on anti-*Francisella* antibodies targets is expected to allow for the identification of new diagnostic or reactive antigens and the development of vaccines [8].

Furthermore, *F. tularensis* is capable of influencing multiple pathways, and continued research into the specific mechanisms by which *F. tularensis* evades, modulates and suppresses the host immune response will improve our understanding of tularaemia pathogenesis and the regulation of host immunity [59].

8. Clinical manifestations of tularaemia

8.1. Humans

Relevant clinical disease has been reported with *F. tularensis* subsp. *tularensis* and *holarctica*. Clinical manifestations of tularaemia depend on strain virulence, infective dose and infection route, the extent of systemic involvement and host immune status [2,4,49]. The incubation period averages 3–5 days but ranges from 1 to 20 days. The disease has an acute onset, with the occurrence of fever (38–40 °C), chills, fatigue, generalised myalgia and headaches, resembling a flu-like syndrome [2,22,49]. The subspecies *tularensis* (Type A) causes severe disease, potentially fatal if untreated. The subspecies *holarctica* (Type B) causes less severe disease and fatalities are rare [49]. Depending on the route of infection, the following forms of the disease are described: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septic [22,49].

Ulceroglandular and glandular forms of the disease are the most common and frequently result from an arthropod bite or animal contact [2,4,49]. In ulceroglandular tularémia, a soft, painless ulcer develops at the inoculation

site and evolves to a scar [6,22]. This presentation is associated with fever, lymphadenopathy and, in Type A tularaemia, pneumonia and pleural effusion can occur [49]. In glandular tularaemia, the primary ulcer is unrecognisable [2,6,22,49].

Direct contamination of the eye through contaminated fingers, splashes or aerosols, may be followed by oculoglandular tularaemia. Unilateral conjunctivitis, with ulcers or papules in some patients, photophobia and epiphora are the main signs of this form of the disease [2,49].

Oropharyngeal tularaemia is acquired by means of contaminated food or water intake and aerosol inhalation [22]. It develops with ulcerative and exudative stomatitis and pharyngitis [49].

Pneumonic tularaemia occurs by means of contaminated aerosol inhalation but can also arise as a complication of any of the other disease forms by haematogenous generalisation [2,22,49]. Initial disease development is characterised by fever, cough, pleuritic chest pain and dyspnoea, along with other unspecific symptoms. Type A tularaemia is associated with significantly severer and more fulminant forms of pneumonia [2,49].

Typhoidal tularaemia refers to a systemic and febrile form of the disease in which no route of infection acquisition can be established [2,49].

Septic tularaemia is a severe and often fatal form of the disease that can occur as a complication of the ulceroglandular form in Type A tularaemia [22,49]. Patients can present unspecific and neurologic symptoms, and septic shock, SIRS (systemic inflammatory response syndrome), DIC (disseminated intravascular coagulation), haemorrhages, SARS (severe acute respiratory syndrome) and multiple organ failure [22,49]. In Type B tularaemia, complications of meningitis and septicaemia have only occasionally been described [49].

8.2. Animals

Clinical manifestations largely depend on the susceptibility of animal species to *F. tularensis* [49]. In wild animals, clinical signs of tularaemia are not well documented, and *post-mortem* findings are highly unspecific and include splenomegaly and punctual necrotic lesions in the liver and spleen [49,54].

In one experimental study in European brown hares (*Lepus europaeus*), clinical signs developed 1-day post-inoculation with a *F. tularensis* subspecies *holarctica* strain. These included fever, lethargy and anorexia. Two of the five hares in the study succumbed to the infection on days 5 and 9 following inoculation. Pathological findings included splenomegaly, diffuse spleen necrosis and focal liver necrosis with hepatocytes vacuolisation. The remaining three hares were euthanised and revealed no pathological lesions. Both bacterial culture and mouse inoculation test failed to produce *F. tularensis* isolation [46]. In a natural outbreak of tularaemia in brown hares in France, all eight hares involved presented splenomegaly, congestion and haemorrhagic lesions of several organs, tracheitis and bronchitis [65]. A similar study carried out in Hungary on European brown hares naturally infected with

F. tularensis subspecies *holarctica* also showed very similar results [47].

In another study, 20 female New Zealand white rabbits (*Oryctolagus cuniculus*) were exposed to Type A tularaemia aerosols, with three different doses. Seven of them died while the others developed fever, anorexia and weight loss, with all infecting doses. Haematological findings in six rabbits included lymphopenia, monocytopenia and thrombocytopenia. A bibasilar pneumonia and gastrointestinal tract gas distension were the only radiological findings. Necropsy findings demonstrated hepatosplenomegaly with extensive spleen necrosis and small white nodules. Some of the rabbits presented nodular lesions in the lungs while others showed haemorrhagic lesions [66].

A situation of particular public health significance, given the risk of pet-to-human transmission, is associated with infected prairie dogs (*Cynomys ludovicianus*) sold as pets in the USA and exported internationally [67,68]. A ban was put in place in the European Union and other countries regarding the import of prairie dogs and other rodent species after the USA monkeypox outbreak in 2003 [68,69]. Wild-caught prairie dogs are particularly susceptible to environmental stress, such as capture, transit and crowding, which can enhance disease manifestations. Clinical signs include lethargy, dehydration and grossly enlarged cervical lymph nodes. Prairie dogs can produce specific antibodies against *F. tularensis* and survive tularaemia infection, suggesting their potential role as *F. tularensis* reservoirs in nature. Moreover, one study found that all seropositive animals harboured live infectious bacteria, suggesting persistent infection [67].

Tularaemia has also been described in domestic dogs and cats [49,55], which may be infected by means of arthropod bites, direct contact with infected animals, their ingestion, or contaminated aerosols [70,71].

Cats usually develop severe illness with unspecific clinical signs like fever, lethargy, prostration, vomiting and anorexia, dehydration, regional or generalised lymphadenopathy, splenomegaly, tongue and oropharyngeal ulceration and jaundice [49,72,73]. Pathological findings include multiple necrotic foci on the lymph nodes, spleen, liver and lungs. Frequently, panleukopenia with toxic degeneration of the neutrophils and hyperbilirubinaemia with bilirubinuria are present [73].

Dogs are less susceptible and rarely manifest signs of the disease [55,56]. Nevertheless, they can act as carrier hosts [70] and transmit the bacterium by means their fur after contact with contaminated dead animals or soil [74]. In most cases, infection is self-limiting and recovery is spontaneous. However, only few cases of natural infection in dogs have been reported [55,56].

9. Laboratory diagnosis

9.1. Samples

In humans, samples should preferably be collected before the onset of antibiotherapy and depend on the clinical form of the disease. Samples may include non-heparinised whole blood, serum, respiratory tract

secretions and washes, swabs from visible lesions, lymph node aspirates or biopsies, urine, and autopsy materials [49].

In animals, serum is the preferential sample for all disease forms, but plasma and dry blood on paper filters can also be used. Blood samples should be collected at least 14 days after the onset of the symptoms. Lymph nodes or bone marrow aspirates, organs (lung, liver, spleen) and cerebrospinal fluid can also be used [49].

In the context of an outbreak or epidemiologic studies, samples should include arthropod vectors as well as environmental samples like water, soil and rodent faeces [49,54].

9.2. Culture

Culture is the gold standard for *F. tularensis* and must be carried out in biosecurity level 3 facilities (BSL-3) [2,22,49]. *F. tularensis* is a fastidious microorganism. Optimal growth conditions occur at 37 °C and pH 6.9 [5,24]. Cysteine-enriched media, such as enriched chocolate agar (CA) or 9% cysteine heart agar with blood medium (CHAB) must be used for this purpose [22,49,54]. Growth in a CHAB medium enables the presumptive identification of *F. tularensis* by characteristic growth at 24–48 h of round and smooth green opalescent shiny colonies, 2–4 mm in diameter [4,22,27,49,54]. Antibiotic supplementation of CHAB is possible in order to optimise growth and inhibit contaminants [22,49,54]. For cultures made from blood, the use of the BACTEC™ (BD) system or equivalent, BacT/Alert™ (Biomérieux) is recommended [49,54]. Liquid media is not suitable for *F. tularensis* growth, even when supplemented with cysteine [4,27,54].

10. Microbiologic identification of *F. tularensis*

Basic biochemical tests provide a presumptive identification of isolates and may be further complemented by immunological and molecular methods. Some additional biochemical tests, such as the ability to ferment glucose or glycerol, or the presence of the citrulline ureidase pathway are useful for subtyping purposes [54].

The commercial Microlog Microstation™ System (Biolog Inc., Hayward, CA) based on the ability to ferment glucose has been successfully used for differentiating between subspecies *tularensis* and *holarctica* [54,67]. Also, the commercially available Microbial Identification System (MIS) and Library Generation System (LGS) (MIDI, Inc, Newark, NJ) enables cell-wall fatty-acid analysis and can be used for the identification of *Francisella* at the genus level. It has also enabled the identification of atypical *F. tularensis* strains lacking cysteine requirements [54,75].

Immune based techniques have also been employed for identification: immunoblot analysis and immunofluorescence microscopy, either from grown cultures or clinical samples [54].

11. Serology

Antibodies against *F. tularensis* reach detectable levels 10–20 days post-infection [49]. A fourfold increase in the titre between acute and convalescent sera or a titre of 1:160 or greater of agglutinating antibodies is considered for diagnostic purposes [2,27,54,76]. Titres peak at a level of 320–1280 and decline slowly [76]. Serologic methods include the whole-cell agglutination test (Widal's reaction), the tube agglutination test, microagglutination assays, haemagglutination, ELISA (*Enzyme-linked immunosorbent assay*) and immunoblot [2,22]. ELISA has repeatedly been more sensitive than agglutination assays, with the additional advantage of determining separately different antibody classes (IgM, IgG and IgA) [54].

A combination of a first ELISA screening test complemented by an immunoblot confirmatory test, with higher specificity, is the current recommended two-step approach for the serological diagnosis of tularaemia [54].

The same approach can be used for animals. Serology has a limited use in highly susceptible species since death usually precedes the development of specific antibodies [47]. However, in endemic areas, antibodies for *F. tularensis* are frequently detected in wild animals that have developed immunity, including foxes and coyotes. This seroconversion is suspected as being related to subspecies *holarctica* infection since infection by the subspecies *tularensis* is expected to be fatal [27,49].

12. Molecular methods

Molecular methods are valuable diagnostic tools whenever culture is either not possible or is negative [2,22,49]. Moreover, they reduce the high risk of laboratory-acquired infections over conventional biochemical typing [2,21,77].

During recent years, polymerase chain reaction (PCR)-based methods have been successfully used for the rapid identification and classification of *Francisella* isolates, with increased sensitivity and specificity [54,78]. However, false positive results related to non-pathogenic closely related *Francisella* subspecies, occurring naturally in the environment, may hamper species and subspecies identification [78].

Conventional PCR targets are *tul*4 and *fop*A genes, which encode for *F. tularensis* superficial membrane lipoproteins. Both protocols show a good level of sensitivity and reasonable specificity in *F. tularensis* detection and may be used in blood, tissue or aerosol samples [4,49,54]. PCR product specificity is confirmed by sequencing, reverse-line blotting (RLB) or restriction fragment-length polymorphism (RFLP) [54].

Real time PCR for *F. tularensis* detection has been developed, in particular, TaqMan™ (Applied Biosystems) real time PCR multiple assay shows high specificity and sensitivity using four target genes: *ISFtu*2, 23 *kDA*, *tul*4 and *fop*A [49,54]. Real-time PCR for the differentiation between the subspecies *tularensis* and *holarctica* is also now available [79].

Further discrimination has been achieved using high-resolution genotyping methods including pulse-field gel electrophoresis (PFGE), amplified fragment-length polymorphism (AFLP), ribotyping, 16S rDNA gene sequencing, canonic insertion deletions and paired-end sequence mapping [26,27,34,80]. Still, as *F. tularensis* exhibits highly

conserved genomic sequences among strains of diverse origin, genetic polymorphisms allowing for individual strain typing have been difficult to find [77]. As for other bacteria, more recent PCR-based techniques such as variable-number tandem repeats (VNTR), multiple-locus VNTR analysis (MLVA) and short-tandem repeats (STR) typing have been successfully used for identification at the subspecies level and for molecular epidemiology purposes [54,77,80]. One of the most discriminatory methods for the molecular subtyping of *F. tularensis* is MLVA, which consists of a series of VNTR *loci* that are PCR amplified via flanking primer sites and examined for size variation [79]. One MLVA system designed for *F. tularensis* is based on polymorphisms of 25 VNTR *loci*, Ft-M1 to Ft-M25. This MLVA typing system has a greater discriminatory power when applied to a worldwide set of *F. tularensis* isolates and provides accurate classification at the subspecies level [77]. This MLVA system has recently been improved by redesigning the subset of the 25 previously identified VNTRs to produce a new optimised, multiplexed MLVA system with a similar level of discrimination but with fewer time and cost requirements [79]. Ten of the previously described VNTR *loci* were selected based on their discrimination ability within the subspecies: Ft-M02, Ft-M03, Ft-M04, Ft-M05, Ft-M06, Ft-M010, FT-M20, FT-M22, Ft-M23 and Ft-M24. Locus Ft-M20 was split into two loci, Ft-M20A (which contains the originally described 12 bp repeat and is polymorphic across subspecies) and Ft-M20B (which contains the insertion with its 15 bp repeat and varies only among type A.II and *F. novicida* isolates) [79].

While providing discrimination among strains, VNTRs are unsuited for determining deeper phylogenetic relationships due to mutational saturation. In this case, more accurate and alternative markers should be used, such as whole-genome sequence single nucleotide polymorphism (SNPs) [79]. Additional studies have shown a remarkable degree of discrimination of the *F. tularensis* phylogenetic structure, using a combined analysis with canonical whole-genome SNPs for major clade typing, and MLVA for high-resolution typing [26,79]. In a different study, the combined analysis of insertion-deletion markers, for subspecies and major clade typing, along with MLVA, was used [80].

Microarrays have also allowed for the differentiation of the four *F. tularensis* subspecies and have been proven useful for pathogenicity and virulence marker identification [54].

13. Treatment

Tularaemia usually responds to antibiotic therapy. Historically, aminoglycosides have been the drugs of choice for humans. Although clinically effective, they are rarely used now due their ototoxicity and nephrotoxicity. Nevertheless, gentamicin has been used for treatment of pneumonic tularaemia and aminoglycosides are now generally used in the most serious cases. Chloramphenicol is effective but seldom the first choice due to its possible irreversible effects on haematopoiesis. Tetracyclines have been associated with high relapse rates on withdrawal.

Fluoroquinolones, such as ciprofloxacin, have been shown to be highly effective in *per os* and are the best choice for uncomplicated tularaemia. Also, ciprofloxacin has proved suitable and effective in the treatment of tularaemia in children and pregnant women [4,49]. In domestic animals, gentamicin, enrofloxacin, doxycycline and chloramphenicol are referred to as therapeutic options for dogs [55,70]. In cats, there are reports of the use of doxycycline or enrofloxacin and amoxicilin-clavulanic acid as being beneficial in the early stages of the disease [81].

14. Vaccination

Currently, there is no available licensed vaccine against *F. tularensis* although an attenuated Type B strain, known as the Live Vaccine Strain (LVS) was developed in the United States during the 1950s and used to vaccinate military personnel and laboratory workers [4,49,82–84]. LVS failed to uniformly protect against pneumonic tularaemia and when delivered in high titres caused mild tularaemia as an undesirable side-effect [85].

One focus of current research work in the USA and in Europe is to develop a vaccine for protection against *F. tularensis* intentional release [49]. The restricted efficacy of the LVS has fostered extensive research with a view to providing alternative vaccine formulations, including the exploration of different live and killed attenuated strains and immunogenic components to produce subunit vaccines [4,82]. In view of its immunogenic antigens, an effort has been made to develop attenuated strains of SchuS4, a representative strain of Type A tularaemia, for vaccine production. In fact, between LVS and SchuS4 strains there are about 35 genes that encode for different protein sequences, whose functions are not well defined, and may represent important immunogenic antigens. Still, given the increased virulence of the SchuS4 strain, only a small number of bacteria should be required to generate effective protection against wild type *F. tularensis* [85]. A recently published study demonstrated that inoculation with low doses of specific attenuated mutants of the *F. tularensis* strain SchuS4 provided protection against parenteral and intranasal challenge with a fully virulent wild type SchuS4 strain [86]. This favours the role of T-cell memory response as a critical determinant of *F. tularensis* immunity, additionally to the humoral response. This feature is the basis of the challenges foreseen for vaccine development, aiming at identifying antigen determinants that elicit an effective cellular-mediated immune response [4,82,84,85]. Cell-mediated immunity was found to persist three decades after tularaemia vaccination. A recent study sought to identify the T-cell responses present in immune individuals in order to characterise *F. tularensis*-specific immune response [84,86]. The findings showed that the production of INF-γ, macrophage inflammatory protein (MIP)-1β and CD107a (lysosome-associated membrane protein 1 or LAMP-1) by peripheral blood mononuclear cells appeared to be a characteristic of protective immune responses and that a correlation exists between these parameters and immunity [84].

15. Conclusions

Several factors such as human demographics and behaviour, international travel and commerce, including the animal trade, climactic changes and microorganism adaptation, have a potential impact on disease ecology and the emergence of zoonosis. The same factors are thought to be related to the emergence of tularaemia. Special concerns regarding this bacterium exist in relation to its high infectivity, and easy dispersion through aerosols and contaminated water, which make it a potential bioterrorism weapon. Also, tularaemia presents a wide geographic distribution and has recently emerged in new settings, particularly in Europe. In Portugal, an on-going research project on tularaemia aims to increase our knowledge about the disease, particularly its impact in this country, which is still poorly understood, in view of the fact that there is little information available to risk population and health professionals, with the result that there is a possible underestimation of prevalence in man and animals. To this regard, efforts have been made by the National Institute of Health to increase awareness of the disease among risk populations, particularly hunters and health professionals. In accordance with the preliminary results, on-going research will further identify and characterise *F. tularensis* circulating strains and develop molecular and typing methods with increased sensitivity, specificity and discriminatory power. The role of autochthon wild lagomorphs in the *F. tularensis* life cycle, their involvement in animal-to-human transmission and their suitability as tularaemia sentinels will be accessed. Moreover, considering the economic and social relevance of hunting-related activities in this country, with very few studies having acknowledged its relation to zoonotic disease transmission risks, research into infection in game species is of major importance.

F. tularensis is also associated with a considerably wider range of hosts and vectors than most zoonotic pathogens, although there is little information on bacterium mechanisms for adaptation to such a wide diversity of arthropod vectors. Despite our increasing knowledge of tularaemia and its etiological agent, many aspects of *F. tularensis* biology and epidemiology need to be further examined, particularly its pathogenicity and virulence, vaccine development, and the specific mechanisms by which *F. tularensis* evades, modulates and suppresses the host immune response. As with any zoonotic emergent disease, the role of wild and domestic animals in *F. tularensis* epidemiology needs to be further evaluated, in particular, those which may act as reservoirs. Other epidemiologic data such as the population dynamics of susceptible animals, particularly lagomorphs and rodents in Europe, should be part of surveillance programmes, as they are thought to be directly associated with disease transmission patterns. From a public health perspective, disease surveillance in animals is crucial in order to prevent and monitor human outbreaks, particularly in endemic areas, where contact between humans and wildlife reservoirs or vectors is likely. Although tularaemia is not regarded as a common disease, and there is little awareness of the disease among health authorities and practitioners, its eventual future impact as an emergent zoonosis should not be neglected.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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