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Putting the Jigsaw Together - A Brief Insight Into the Tularemia

Abstract: Tularemia is a debilitating febrile and potentially fatal zoonotic disease of humans and other vertebrates caused by the Gram-negative bacterium *Francisella tularensis*. The natural reservoirs are small rodents, hares, and possibly amoebas in water. The etiological agent, *Francisella tularensis*, is a non-spore forming, encapsulated, facultative intracellular bacterium, a member of the γ-*Proteobacteria* class of Gram-negative bacteria. *Francisella tularensis* is capable of invading and replicating within phagocytic as well as non-phagocytic cells and modulate inflammatory response. Infection by the pulmonary, dermal, or oral routes, respectively, results in pneumonic, ulceroglandular, or oropharyngeal tularemia. The highest mortality rates are associated with the pneumonic form of this disease. All members of *Francisella tularensis* species cause more or less severe disease Due to their abilities to be transmitted to humans via multiple routes and to be disseminated via biological aerosol that can cause the disease after inhalation of even an extremely low infectious dose, *Francisella tularensis* has been classified as a Category A bioterrorism agent. The current standard of care for tularemia is treatment with antibiotics, as this therapy is highly effective if used soon after infection, although it is not, however, absolutely effective in all cases.

Keywords: Tularemia; *Francisella tularensis*; intracellular bacterium; bioterrorism

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1 Introduction

The history of research on tularemia as well as that of its causative agent's taxonomic classification goes back more than 100 years. The origin of the tularemia story is generally dated to the year 1911, when George W. McCoy, Director of the US Public Health Service Plague Laboratory, undertook bacteriological investigations of bubonic plague in ground squirrels and reported a plague-like disease of rodents in California [1]. In parallel, physician R. A. Pearse reported in the journal *Northwest Medicine* (March 1911) six cases of fever caused by the bites of deerflies and named this "deerfly fever" [2]. One year later, McCoy and Chapin successfully isolated a novel organism which was named *Bacterium tularense (B. tularense)* after Tulare County in central California, the site of the original discovery [3]. Edward Francis studied deerfly fever in Utah during the 1920s. Because of the pathological changes of the disease observed in animals and humans and due to isolation of the bacterium from human blood, he renamed the disease tularemia [4]. Almost concurrently, Hachiro Ohara was studying wild hare disease (Yato-byo) in Japan and recognized that disease's similarity to tularemia. His observation was confirmed by Francis, who isolated *B. tularense* from specimens he received from Ohara [5]. Soon thereafter, tularemia was recognized in the USSR, Norway, Canada, Sweden, and Austria [6–8]. Thus, the names deerfly fever, rabbit fever, Pahvant Valley plague, lemming fever, Yato-byo, Ohara's disease, water-rat trapper's disease, hare meat poisoning, and probably also other historical names are synonyms for tularemia.

From a historical point of view, tularemia was probably first described on September 19, 1907. At the Annual Meeting of the Medical Library Association, Portland, Oregon, on June 25, 1940, Dr. William Levin cited a letter of one Dr. Ancil Martin which had been sent to F. G. Novy at the University of Michigan. In this letter, Martin had stated that he had under observation and treatment five cases of an infection caused by the skinning and dressing of wild rabbits [9]. For the sake of completeness, it should be noted that there is a hypothesis that the biblical plague

of the Philistines and a virulent epidemic, similar to the bubonic plague or typhus, in Ancient Egypt (around 1715 BC) may have been tularemia [10,11].

The original name and taxonomy of the etiological agent of tularemia changed several times over the years. In the literature can be found the name *Pasteurella tularensis* and in older literature the name *Brucella tularensis* (due to serological cross-reactions with the *Brucella* sp. antigens). Finally, to honor the achievements of Edward Francis in the field of tularemia research, the bacterium was renamed to *Francisella tularensis (F. tularensis)* [12,13].

2 The etiological agent - Francisella tularensis

F. tularensis is a highly virulent, non-sporulating, pleomorphic, facultative intracellular, Gram-negative coccobacillus that is capable of causing the zoonotic disease tularemia in a large number of mammals. *F. tularensis* strains grow slowly in CO_2 supplemented air and almost all strains of this fastidious organism have specific requirements for iron and cysteine or cystine. After 24 hours of incubation, small, white and gray, smooth or shiny-surfaced colonies can be determined on an appropriate solid media.

Francisella is the only genus within the family *Francisellaceae* of the γ-subclass of *Proteobacteria* [14]. The *Francisellaceae* family is distinguished by a unique set of phenotypic characteristics such as their morphology, a capability for degrading only a limited number of carbohydrates, a growth requirement for cysteine, and a unique fatty acid composition [15]. The taxonomy of the genus *Francisella* is still subject to debate. A recent study of the phylogenetic relationship of all known *Francisella* species divided the genus *Francisella* into two genetic clades. One is represented by *F. tularensis*, *F. novicida*, *F. hispaniensis*, and the close neighbor *Wolbachia persica*; the second by *F. philomiragia* and the fish pathogen *F. noatunensis* [16]. The majority of publications divide the species *F. tularensis* into four closely related subspecies that are highly conserved in their genomic content but differ in their virulence, biochemistry, and epidemiology: *F. tularensis* subsp. *tularensis* (also known as Type A), which is endemic in North America, while in Europe there is known only one isolate that possibly represents a laboratory escape [17]; *F. tularensis* subsp. *holarctica* (also known as Type B), widespread throughout the Northern Hemisphere; *F. tularensis* subsp. *mediasiatica*, endemic in Central Asia; and *F. tularensis* subsp. *novicida*. Most human cases of tularemia are caused by the Type A and Type B strains, with Type A strains being significantly more virulent. Type A strains are now newly divided into 3 genotypes (clades) A1a, A1b, and A2, all of which have been shown to be epidemically important [18,19]. Strains of *F. tularensis* subsp. *tularensis* are considered the most virulent for humans, with an infectious dose of less than 10 colony forming units (CFU). The lethality of Type A strains is up to 24% in untreated cases depending on the Type A genotype [19]. *F. tularensis* subsp. *holarctica* also causes debilitating diseases, albeit with a milder course. The fatality rate barely reaches 1%. *F. tularensis* subsp. *mediasiatica* rarely causes human illness and is less virulent than *F. tularensis* subsp. *holarctica* [20]. *F. tularensis* subsp. *novicida* is an opportunistic pathogen for humans, and it is significantly less pathogenic than the other subspecies. It can cause the disease mainly in immunocompromised people [21,22]. Fully virulent strains must be handled in labs under containment meeting the requirements of Biosafety Level 3 [23]. The Centers for Disease Control and Prevention classify *F. tularensis* as a Category A bioterrorism agent[1].

3 Intracellular lifestyle

To establish infection, *F. tularensis*, as an intracellular pathogen, needs to enter cells, find a target place to survive, and then grow inside host cells. The ability of *F. tularensis* to survive and multiply intracellularly has been well described in both professional and non-professional phagocytes in *in vitro* and *in vivo* models [24–26]. The molecular mechanisms used by *F. tularensis* to mediate its uptake into the host cell are mostly unknown, however. In general, serum-opsonized *F. tularensis* can enter host cells using a process dependent on the presence of complement factor C3 in the serum and complement receptors on the surface of the host cell, which are engaged in the formation of pseudopod loops in the host cell's surface membrane [27]. Under this condition, class A scavenger receptors, lung surfactant protein A, nucleolin, as well as the Fcγ receptors are involved, to various degrees, in the internalization of *F. tularensis* by mammalian cells [28–30]. In the non-opsonic uptake of *F. tularensis* by macrophages, a significant role is played by the mannose receptor [28] and possibly other cell surface receptors that have not yet been defined. The mode of entry may influence the fate of *Francisella* inside the host cell by triggering different signaling pathways that control the

1 See the website: http://emergency.cdc.gov/agent/agentlist-category.asp

expression of intracellular defense mechanisms and, in parallel, influence the survival of intracellularly localized bacteria [31].

Inside the host cell, *F. tularensis* resides in an initial vacuolar compartment along the general endocytic degradative pathway, recently termed the *Francisella*-containing phagosome (FCP). The FCP sequentially acquires early and late endosomal markers, such as EEA-1, Lamp-1, and Rab-7, but not the marker cathepsin D, which is an indicator of phagosome–lysosome fusion [32,33]. The next step in the intracellular trafficking of the bacterium consists in active FCP membrane disruption followed by escape into the cytosol, where it then replicates [32–34]. It is still under debate whether the FCP is acidified before disruption of the membrane by *F. tularensis*. There are experimental data demonstrating the progressive acidification of the vacuole by acquiring vacuolar ATPase before phagosomal disruption [35,36], as well as contradictory data that exclude FCP acidification [32,37,38]. Whether this discrepancy is due to different experimental conditions or different infectious agents is not yet clear. Phagosomal escape is followed by extensive cytosolic replication and, finally, the programmed cell death of the host macrophage [39,40]. During later intervals of infection, some *F. tularensis* have been observed in the multi-membrane vacuolar compartment of the endocytic pathway that has the characteristics of an autophagosome [41,42]. Still unclear, however, are the reason why *F. tularensis* reenters the membranous compartment and the consequences for the further dissemination of infection and induction of immune response (the scheme of whole process see Fig. 1). If the re-entering of *Francisellae* into

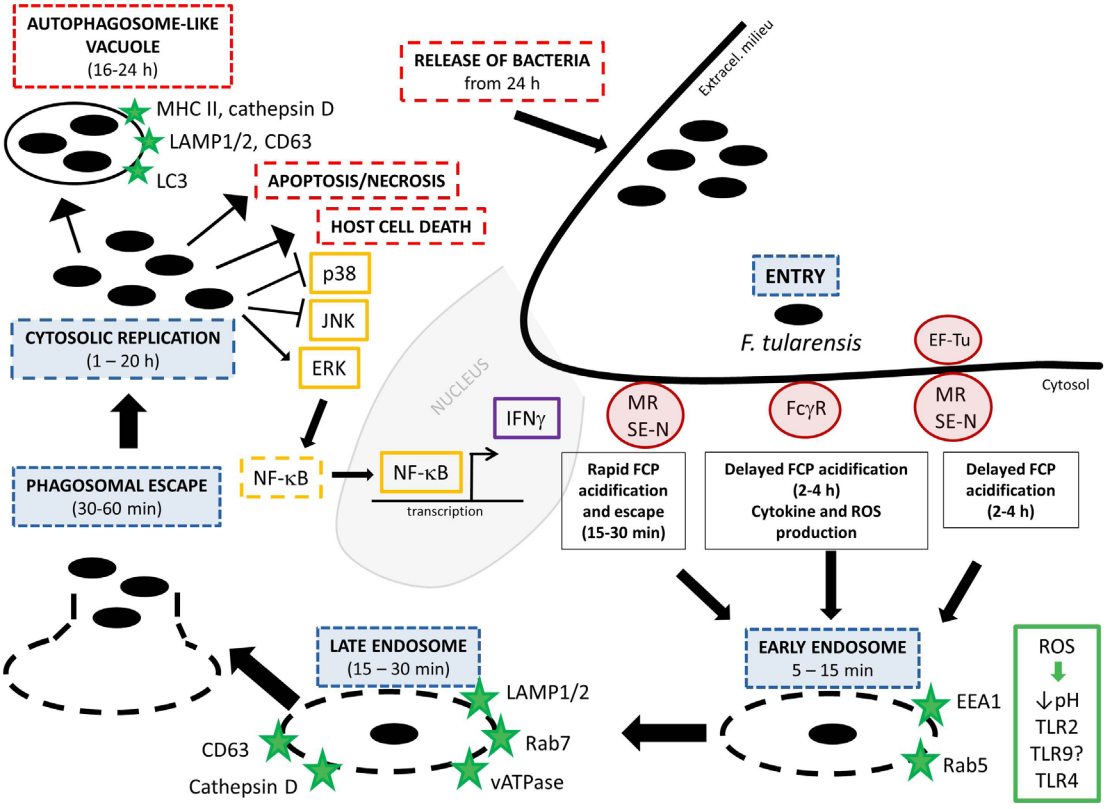

Fig. 1: **Intracellular trafficking of *F. tularensis* after uptake by different macrophage receptors**: *Francisella* uses multiple mechanisms to evade host defence. *Francisella* can be recognized by multiple macrophage receptors such as FcgR, complement receptor 3 (CR3), scavenger receptor A (SRA), mannose receptor (MR) and surface-exposed nucleolin (SE-N). *Francisella* blocks the NADPH oxidase and detoxifies reactive oxygen species (ROS). *Francisella* does also signal through TLR2 and may activate intracellular TLR9, but it does not signal through TLR4. Upon entry, *Francisella*-containing phagosome (FCP) matures into early endosome characterized by the early endosomal antigen 1 (EEA1) and Rab5 GTPAse (Rab5). The maturation progress into the late endosome characterized by the late endosomal markers Rab7 GTPase (Rab7), lysosomal-associated membrane protein 1 a 2 (LAMP1, LAMP2) together with proton ATPase pump. Subsequently, *Francisella* multiplies in cytosol within 24h after infection with engagement of a selected signaling pathway. Following replication, re-entering the endosomal-lysosomal pathway through the autophagosome-like vacuole characterized with LAMP1/2, microtubule-associated protein light chain 3 (LC3) and major histocompatibility complex class II (MHCII) can be observed. *Francisella* induces host cell death (apoptosis or necrosis) with subsequent robust infection of other cells.

multilamelar compartment is induced by *Francisella* itself or if it is the result of defence mechanism orientation to eliminate intracellular threat is still under debate.

During its history, *F. tularensis* has developed molecular tools to avoid the intracellular defense mechanisms of the host cell. More than 300 genes considered to be virulence factors have been identified so far. Among these are genes involved in adhesion to host cells [43]; genes associated with capsule biosynthesis contributing to serum resistance; and genes, including those from the *Francisella* pathogenicity island coding probably of the type VI secretion system, enabling the "neutralization" of intraphagosomal milieu [44–47], escape into the cytosol, and proliferation inside the host cell [48–50]. *F. tularensis* is also able to delay cell death to increase its survival and replication through activation of Ras by the SOS2/GrB2/PKCα/PKCβI quaternary complex, which stimulates cell survival through the downregulation of caspase-3 activation [51]. Moreover, *Francisella* spp. actively manipulate the timing of autophagy onset, interferon signaling, Toll-like receptor signaling, and phagocytosis [52,53], thus manipulating one of the early cell defense mechanisms against infection [54]. Equally important for *Francisella* is the adaptation to the nutritional limitations inside a host cell [55] Despite numerous studies dedicated to virulence factors, *F. tularensis* virulence in its complexity has not yet been sufficiently elucidated [56]. Some important factors involved in a process of *Francisella* phagosomal escape and in intracellular growth are listed in Table 1.

Altogether, the ability of intracellular pathogens to evade clearance inside host cells and disseminate to other areas of the body is essential to the pathogen's virulence and pathogenicity. It remains unclear, however, as to how *Francisella* spp. actually kills their hosts. Deactivation of immune cells, uncontrolled cytokine response, and toxin production has all been implicated as mechanisms by which bacterial pathogens induce death during systemic infection. For obligatory and facultative intracellular pathogens, factors essential for their *in vitro* and/or *in vivo* survival and growth should be considered as virulence determinants. Nevertheless, inasmuch as each intracellular pathogen has developed its own strategy for survival inside macrophages, the application of knowledge from one pathogen to another should be made very carefully.

Francisellae are able to evade defence mechanisms of the host. Then, there is still a question if the host is able to clear the infection in full or if there are some *Francisellae*, inside unactivated macrophages, that are able, under the specific conditions of the host, to reactivate infectious process. The clear evidence is still missing.

4 Epidemiology

Tularemia is a zoonotic infection caused by *F. tularensis* that occurs endemically in most countries of the Northern Hemisphere. It is widespread throughout the Old World and North America [79]. On the Eurasian continent, tularemia is traditionally reported from Scandinavian countries, particularly from Sweden [80,81]; from countries of Central Europe, including Germany [82,83], Austria [84,85], the Czech Republic and Slovakia [84,86]; from countries of the former Soviet Union [87–89]; from Central Asia [90–92], including Mongolia [93]; and from the Japanese islands [94,95]. Tularemia has been occurring in Turkey [96–98] and Spain [99,100] since the 1990s. Several dozen cases have been reported from Italy [101,102] and France [103]. The British Islands seem to be free from the disease. It is important to note that in Europe, Asia, and Japan the frequency of tularemia cases has generally grown in situations when the socioeconomic and environmental conditions of the population have been disrupted. During the Second World War, epidemics comprising from 10,000 to 100,000 cases each year and large outbreaks of waterborne tularemia were recorded in Eastern European countries [104–106]. An increased frequency of tularemia cases during World War II was also registered in Japan [107]. More recently, 327 cases of tularemia were reported during the postwar period in Kosovo (1999–2000) [108].

In the New World, the disease, or the presence of *F. tularensis* in wildlife, is reported from Canada, the USA, and Mexico. Tularemia has occurred in all Canadian jurisdictions except the Yukon and Nunavut [109]. In the USA, human cases have been reported from all states except Hawaii [110,111]. An isolated small endemic area is Martha's Vineyard in Massachusetts, with diverse *Francisella* spp. in an environment where human cases of pneumonic tularemia occurred during recent decades [112–114]. A serological survey of wildlife carried out during 1988 and 1989 in Mexico documented the exposure of two animal species to *F. tularensis* [115].

While it is generally known that there are zones where tularemia has been occurring for decades, the overall ecology of *F. tularensis* is not well understood, and particularly the transmission cycle, ecological requirements of the different subspecies, and true natural reservoir hosts [116,117]. More than 250 species of mammals, birds, amphibians, invertebrates, and protozoans have already been identified as hosts for *F. tularensis*, which complicates understanding the transmission cycle. In general, human cases of tularemia are most often associated with exposure to lagomorphs, rodents, and blood-feeding arthropods; the

Table 1: Some important factors involved in a process of a Francisella phagosomal escape and in intracellular growth

Protein/gene	Name of protein	Role/function[1]	FT strain[2]	Host cells[3]	Ref.
AcpA	Acid phosphatase	PE	*F. novicida* U112	THP-1/BMMs	[57]
		IG	*F. novicida* U112	THP-1/BMMs	[57]
CarA	Carbamoyl-phosphate synthase small chain	PE	LVS	BMMs	[50]
		IG	LVS	BMMs	[50]
CarB	Carbamoyl-phosphate synthase large chain	IG	LVS	BMMs	[50]
DsbA	DSBA-like thioredoxin domain protein	PE	SchuS4	J774	[58]
			FSC200	BMMs	[59]
		IG	SchuS4	HepG2	[60]
			SchuS4	J774	[58]
			LVS	J774	[61]
DsbB	Disulfide bond formation protein	PE	SchuS4	HepG2	[62]
FevR/PigR	*Francisella* effector of virulence regulation/	PE	LVS	BMMs	[63]
	Uncharacterized protein	IG	*F. novicida* U112	BMMs	[63]
			LVS	BMMs	[64]
IglA	Intracellular growth locus A	PE	LVS	J774	[65]
		IG	*F. novicida* U112	J774	[66]
			LVS	J774	[67]
IglB	Intracellular growth locus B	PE	LVS	J774	[67]
		IG	LVS	J774	[67]
IglC	Intracellular growth locus C	PE	LVS	J774	[37]
			F. novicida U112	U937/hMDMs	[68]
		IG	LVS	J774	[69]
			F. novicida U112	U937/hMDMs	[68]
IglD	Intracellular growth locus D	PE	LVS	J774	[37]
		IG	LVS	J774	[37]
			F. novicida U112	U937/hMDMs	[37]
IglG	Intracellular growth locus G	PE	LVS	J774	[65]
IglH	Intracellular growth locus H	PE	FSC200	BMMs	[59]
		IG	FSC200	BMMs	[59]
IglI	Intracellular growth locus I	PE	LVS	J774	[65]
			F. novicida U112	BMMs	[70]
		IG	*F. novicida* U112	J774	[70]
MglA	Macrophage growth locus, subunit A	PE	*F. novicida* U112	J774	[71]
			F. novicida U112	U937/hMDMs	[68]
			LVS	J774A.1	[37]
		IG	*F. novicida* U112	J774	[71]
			F. novicida U112	U937/hMDMs	[68]
			LVS	J774A.1	[37]
MglB	Macrophage growth locus, subunit B	IG	*F. novicida* U112	J774	[71]
MigR	Macrophage intracellular growth regulator	PE	LVS	BMMs	[63]
		IG	LVS	BMMs	[63]
			LVS	dMDMs	[72]
PdpA	Pathogenicity deteminant protein pdpA	PE	*F. novicida* U112	BMMs/J774	[73]
		IG	*F. novicida* U112	BMMs/J774	[48]
PdpB	Pathogenicity deteminant protein pdpB	IG	*F. novicida* U112	BMMs/J774	[73]
PmrA	Orphan response regulator	IG	*F. novicida* U112	THP-1/J774	[74]
PurA	Adenylosuccinate synthetase	IG	*F. novicida* U112	J774	[75]

table modified from [264]

[1] PE – phagosomal escape; IG – intracellular growth

[2] *FT – Francisella tularensis;* LVS - *F. tularensis* subsp. *holarctica* live vaccine strain; SchuS4 – *F. tularensis* subsp. *tularensis* SchuS4; FSC200 - *F. tularensis* subsp. *holarctica* FSC200

[3] THP-1 - human monocytic cell line derived from an acute monocytic leukemia patient; BMMs - bone marrow-derived macrophages; J774 - murine macrophages cell line; HepG2 - human hepatocellular liver carcinoma cell line; U937 - human leukemic monocyte lymphoma cell line; hMDMs - human monocyte-derived macrophage

continued **Table 1:** Some important factors involved in a process of a Francisella phagosomal escape and in intracellular growth

Protein/gene	Name of protein	Role/function[1]	FT strain[2]	Host cells[3]	Ref.
PurF	Amidophosphoribosyltransferase	IG	*F. novicida* U112	J774	[75]
PurMCD	Phosphoribosylformylglycinamidine cyclo-ligase/Fusion protein PurC/PurD	IG	LVS	J774	[76]
			LVS	PEC	[77]
PyrB	Aspartate carbamoyltransferase	IG	LVS	BMMs	[50]
VgrG	VgrG protein	PE	*F. novicida* U112	BMMs	[70]
		IG	*F. novicida* U112	J774	[70]
FTT0369	Uncharacterized protein	IG	SchuS4	BMMs	[78]
FTT0383	Uncharacterized protein	PE	SchuS4	BMMs	[78]
		IG	SchuS4	BMMs	[78]
FTT1676	Hypothetical membrane protein	PE	SchuS4	BMMs	[78]
		IG	SchuS4	BMMs	[78]

table modified from [264]

[1] PE – phagosomal escape; IG – intracellular growth

[2] *FT – Francisella tularensis;* LVS - *F. tularensis* subsp. *holarctica* live vaccine strain; SchuS4 – *F. tularensis* subsp. *tularensis* SchuS4; FSC200 - *F. tularensis* subsp. *holarctica* FSC200

[3] THP-1 - human monocytic cell line derived from an acute monocytic leukemia patient; BMMs - bone marrow-derived macrophages; J774 - murine macrophages cell line; HepG2 - human hepatocellular liver carcinoma cell line; U937 - human leukemic monocyte lymphoma cell line; hMDMs - human monocyte-derived macrophage

inhalation of contaminated dust particles; or the drinking of contaminated water. Two cycles, terrestrial and aquatic, have been described for the disease caused by *F. tularensis*. Hares and rabbits are prototypical hosts for the terrestrial cycle while ticks, mites, and biting flies represent arthropod vectors. Beavers, muskrats, and voles are mammalian hosts that can contaminate water within the aquatic cycle [118,119]. The aquatic cycle is associated with rivers, streams, and flooded landscapes, and it can be promoted by the persistence of the bacterium within protozoans [116,120–122].

Outbreaks of tularemia in humans are typically associated with outbreaks of tularemia in animal populations. Thus, rural populations, and especially those individuals who spend some time in endemic areas, such as farmers, hunters, forest workers, and tourists, are most at risk of tularemia [123–125]. For example, two outbreaks of pneumonic tularemia on Martha's Vineyard, Massachusetts, were associated with the use of lawn mowers or brush cutters while people were working around the houses [112,126]. Some animal species such as skunks and raccoons moving within the environment of this island are seropositive and may constitute the source of infecting agent [127]. Moreover, the ability of *F. tularensis* subsp. *tularensis* to survive in salt-influenced soil or moisture on this island has been already documented and this probably contributed to the epidemiological situation in this territory [128]. Another example of pneumonic tularemia outbreaks associated with farming can be seen in outbreaks during work campaigns at sugar factories within the former Czechoslovakia in the 1950s and 1960s [129]. Based on previously accepted techniques, heaps of sugar beets were being washed using jets of water that obliterated the corpses of *Francisella*-infected small rodents inside the heap and subsequently created an aerosol that was inhaled by the workers.

Significant vectors of tularemia in the countryside are blood-sucking arthropods, including ticks, flies, and mosquitos. In the United States four tick vectors consisting of *Amblyomma americanum, Ixodes scapularis* [130,131], and *Dermacentor variabilis* in the southeastern and south–central states and *D. andersoni* in the west were identified to be the most important for the transmission of tularemia to humans. All developmental stages, larva, nymph, as well as imago, can carry the disease-causing agent. Larvae of *D. variabilis* in the US as well as larvae of *Ixodes ricinus* in Europe have been shown able to acquire, maintain, and transstadially transmit *F. tularensis* [132–134]. The percentage of ticks infected with *F. tularensis* in endemic areas is nevertheless relatively low. Over a period of 3 years, out of 4,246 *D. variabilis* ticks assayed at Martha's Vineyard, only 0.7% were positive in the specific PCR assay for *F. tularensis* [135]. Similarly, studies carried out in Central Europe demonstrated a minimal infection rate of around 2.0% for *D. reticulatus* and less than 0.5% for *Ixodes ricinus* [136–138]. Transmission, especially by the deerfly, *Chrysops discalis*, and by the Tabanidae (horseflies), has been documented in western regions of the USA. While in the western USA both biting flies and ticks are considered important vectors, in the eastern USA only ticks seem to be significant vectors [139].

In northern European countries, such as Sweden, Finland, and the northern part of Russia, mosquitoes are the dominant vector transmitting tularemia to humans [139,140]. By contrast, mosquitoes in Central Europe probably do not carry *F. tularensis* in natural foci of tularemia; contact with infected animals, ingestion of contaminated food or water, along with possible infection caused by tick vectors are the dominant modes of transmission in this region [129,138]. The seasonality of reported tularemia cases corresponds well with these transmission modes.

5 The disease – signs and symptoms

Signs and symptoms of tularemia in wild animals are not well documented and unfortunately are based mostly on postmortem examinations of carcasses. Some data can be found describing naturally infected animals such as rabbits, hares, cats, and prairie dogs [141–144]. The majority of information on tularemia symptoms in animals has originated from laboratory experiments using mice, rabbits, guinea pigs, and monkeys as animal models of natural infection. In these cases, however, most studies utilized the *F. tularensis* live vaccine strain or *F. novicida*, which in murine models induce symptoms similar to the infection in humans caused by wild virulent strains [145]. The clinical manifestations of tularemia depend on the route of infection and the susceptibility of any particular animal species to tularemia.

Similarly to other bacterial zoonoses, tularemia is transmitted to humans by direct contact with infected animals, tissues or fluids from infectious animals or by bites from infected arthropod vectors. Inhalation of aerosol or ingestion of contaminated food and water are other sources of infection. Thus, the gateways for *F. tularensis* into the body include the skin, mucosal membranes, lungs, and gastrointestinal tract. In general, infection is characterized by common symptoms as are fever, sweats, headache, body ache, nausea, vomiting, and diarrhea. Pulse-temperature dissociation is seen in less than half of the patients. The incubation time, which delineates the period of delay between infection and the outbreak of symptoms, varies around 6 days. Moreover, the delay between the onset of symptoms and the seeking of treatment ranges around 7 days [146,147]. Disease onset is abrupt, usually within 3 to 5 days, but it can be as rapid as 1 day or as prolonged as 14 days post-exposure.

According to a study by Dr. Francis, tularemia was delineated into four major clinical manifestations (ulceroglandular, glandular, oculoglandular, and typhoidal) while there also exist additional manifestations, including oropharyngeal, gastrointestinal, pneumonic, and other rare forms [148]. The most common form of human tularemia is the ulceroglandular form that results from contact with infectious material or from vector-borne transmission. Typically, a papule develops into a pustule surrounded by a zone of inflammation at the site of infection, subsequently manifesting by enlargement of the regional lymph node. An ulcer can persist for months. A similar form is taken by glandular tularemia, which is characterized by similar symptoms but without appearance of the primary lesion in the form of an ulcer. During the incubation period (typically 3 to 6 days), bacteria disseminate from the site of infection via lymphatic vessels to the regional lymph node [149]. The enlargement of the draining lymph nodes often resembles the buboes of bubonic plague. Finally, the bacteria disseminate to such other tissues as the spleen, liver, lung, or peritoneum. It is likely the bacteria are carried there by phagocytes of the bloodstream, although bacteremia occurs transiently and relatively early after infection. The mortality rate for this form of tularemia is less than 3% [150]. The oculoglandular form occurs as a result of infection though the eye by touching the eye with a contaminated finger or by the ingress of infected dust particles into the eye. The conjunctiva is the primary site of infection. The appearance of ulcers and nodules on the conjunctiva is a characteristic feature. Without treatment, the bacteria disseminate to the draining lymph node and to other organs. This form is not so frequent and according to data in the older literature comprises less than 1% of all human cases of tularemia [151,152]. Typhoidal tularemia, an acute form of the disease caused mainly by *F. tularensis* subsp. *tularensis*, is characterized by various clinical symptoms typical for septicemia, without the formation of a primary lesion or lymphadenopathy. Occasionally, the patients are delirious and this stage may be followed by shock. Nevertheless, clinical classification and acceptance of a typhoidal designation is generally applied only for cases when no route of infection is diagnosed. Typhoidal tularemia has a substantial mortality rate of 30–60% without antibiotic treatment [153–155]. Outbreaks of pneumonic tularemia resulting from the inhalation of infected aerosols are commonly associated with activities which may aerosolize *F. tularensis* from environmental and animal reservoirs [149]. This is probably the most acute form of the disease. A substantial complication is the problem of diagnosis, because the clinical and roentgenological picture is not specific for tularemia. Respiratory tularemia may present

symptoms of pneumonia, including a cough, chest pain, increased respiratory rate, and high fever, as well as other unspecific symptoms such as nausea and vomiting. Pneumonia may occur as a primary manifestation of the respiratory form, but secondary pneumonia may also appear as a complication in any form of tularemia. This is a consequence of the bacteria's dissemination throughout the body, including the lungs.

Why is the pneumonic tularemia the most acute and the most severe form of the disease? The answer to this question was searched using different experimental models.

One of the options can be a spectrum of cells in the lung that are infected by *Francisellae*. Lung macrophages and dendritic cells, as the mobile phagocytes and effective APCs, but also lung endothelial cells and structural alveolar type II epithelial cells all are targets of *F. tularensis* LVS as well as *F. tularensis* Schu S4 invasion. The promiscuity of *Francisellae* in relation to the cell types that are infected in the lung can have a substantial impact on the course of infection through modulated functions of infected cell [156]. There are evidences that pulmonary macrophages and DCs infected with virulent *Francisellae* produce a significant amount of immunosuppressive cytokine TGF-β [157] that, through the feedback loop, can differentially modulate secretory and phagocytic functions of the lung APCs [158,159], can promote the development and activation of tolerogenic DCs. Produced IL-10 and TGF-β stimulate proliferation of T(regs) that may restrain Th1-type of protective immune response [160]. Moreover, migrating DC may serve as an effective carrier of *F. tularensis* during the early stages of infection and can play a role in pathogen dissemination [161,162]. Infected endothelial cells may attract polymorphonuclear leukocytes to transmigrate across the endothelium, but, concurrently, downregulate responsiveness of the PMN to subsequent activation [163]. Invasion of *F. tularensis* into and proliferation within nonphagocytic lung epithelial cells [164,165] may modulate their multiple biological functions, some of them are associated with immune responsiveness of the host [166].

Thus, collectively with our own results demonstrating questionable function of neutrophils and B cells in the early stages of inhalation infection with *F. tularensis* subsp. *holarctica* strain 15L and 130 [129,167], all the effects on secretory and functional profile of infected cells within lung may resulted into poorly controlled protective response and severity of pneumonic tularemia.

Important differences can be observed during the course of infection by the Type A versus Type B subspecies [15]. Tularemia infection associated with Type A shows a high mortality rate for infected hosts in general. In humans the onset of Type A infection is often sudden and is characterized by chills, high fever, dyspnea, cough, pharyngitis, chest pain, headache, profuse sweating, and general weakness. If this is the case, the patient's condition is extremely severe, symptoms and signs may mimic those of typhoid fever, and the changes are highly variable [168–170]. Infections induced by Type B differ from infections caused by the Type A subspecies. Type B infected patients in general suffered high fever, chest pains, and flu-like symptoms [171].

To summarize, the clinical forms of tularemia can be divided into various syndromes that complicate diagnosis. The incubation period is usually 3 to 5 days after inoculation. Clinical manifestation of illness begins with the rapid onset of fever, chills, headache, malaise, fatigue, and myalgia. Some patients suffer from coughing, nausea, and vomiting. Other findings may include skin ulcers, sore throat, pleural effusion, primary or secondary pneumonia, acute respiratory distress syndrome, and pericarditis. General signs and symptoms of different clinical types of tularemia are included in Table 2.

6 Host immune response

Immunity against *F. tularensis* has been studied for decades, but unanswered questions remain. Some kinds of bacteria have evolved mechanisms for survival inside host cells, and *Francisella* spp. is among these. These pose complications for the immune system and its ability effectively to respond. The immune system is classically divided into innate and adaptive branches, and the innate branch is the most evolutionarily conserved part of the host defense. A substantial part of innate responses are based on such phagocytic cells as macrophages and neutrophils that represent the cellular component while the complement system constitutes the most important humoral component of the innate system. Just macrophages are considered to be primary host cells for *F. tularensis*, but several other cell types within the organism, such as neutrophils, dendritic cells, hepatocytes, and alveolar epithelial cells, also serve as host cells for *Francisella* spp. [172,173]. Recently, B cells also have been shown to be infected by *F. tularensis* [174]. The complement system is the second major key of the innate immune response, and it plays an essential role in defense against foreign pathogens. Generally, the complement system's crucial activity is in inducing immune responses via the optimal contact of target antigens with macrophages, dendritic cells, and both T and B cells. However, the process of

Table 2: Common clinical signs and manifestation of various type of tularemia

	Characteristics	Means of spread	Portal of entry
Ulceroglandular type	Skin papule followed by persistent ulcer Enlargement of regional lymph node Chronic granulomatous inflammation Fever	Vector-borne transmission Direct contact Indirect contact (tools)	Skin
Glandular type	Tender lymphadenopathy (usually axillary/epitrochlear) Absence of visible skin lesions Fever	Vector-borne transmission Direct contact Indirect contact (tools)	Unknown (probably skin)
Oropharyngeal type	Severe pharyngitis Tonsillitis Regional neck lymphadentis Cervical adenitis Persistent fever	Ingest of contamined food/water	Oropharyngeal mucosa
Oculoglandular type	Unilateral conjunctivis Swelling of eyelids Photophobia Mucopurulent discharge Enlargement of regional lymph node Parinaud´s syndrome	Touch eye with contamined fingers Infective dust	Conjunctiva
Typhoidal type	Myalgia Headache Fever of unknown origin Skin or mucous membrane lesions Lymph node enlargement	Unknown (probably oral/ respiratory)	Oropharyngeal mucosa Respiratory tract
Pneumonic /respiratory Type A	Sudden onset of symptoms Pneumonia Bronchopneumonia Chest pain Dry or productive cough Dyspnoea Fever Profuse sweating Mental deterioration Septicemia Erythema nodosum	Inhaling contaminated dust Laboratory-acquired infection	Respiratory tract
Pneumonic /respiratory Type B	Hilar adenopathy Pneumonic infiltration Erythema nodosum Pneumonia (rarely)	Inhaling contaminated dust Laboratory-acquired infection	Respiratory tract

eliminating *Francisella* spp. from the body – similar to that for other intracellular pathogens – is controlled primarily by adaptive immunity and depends on the function of T cell subsets which finalize the expression of protective immunity [175,176]. Regarding intracellular bacteria, T-cell-mediated immune responses are paramount for the control of both primary and secondary infections. The crucial role of T cells in both the control and eradication of *F. tularensis* has been predominantly demonstrated in experiments carried out on experimental animals [145]. In humans, an immunospecific T cell response can be demonstrated after the first 2 weeks from disease onset [21]. Notwithstanding this fact, during the initial stages of tularemia the immune response is almost entirely independent of T cells. Meanwhile, the final resolution and clearance of bacteria from the cells and tissues is completely dependent on αβ T cells which need to be activated [175,176]. Furthermore, in contrast to the well-known role of $CD4^+$ and $CD8^+$ T cell subpopulations in the immune response against *F. tularensis*, the role of other T cell subpopulations is not well understood. For example, it seems likely that $CD4^-CD8^-$ double negative T cells play

a substantial role during pulmonary *F. tularensis* infection by producing IL-17A and IFN-γ cytokines that additively contribute to the control of the infection [177,178].

Considerable attention has also been devoted to understanding effector mechanisms provided by the γδ T cells. Although these cells appear to play a minimal role during primary infection of mice, they seem to have a larger role in the case of *F. tularensis* infection in humans, where their numbers remain elevated for as long as one year after infection [179,180]. Their contribution to the protective response is still rather unclear.

In recent decades, research on anti-infection immunity has been focused on understanding the effects of important cytokines and chemokines during tularemia infection. Studies on murine models have clearly demonstrated that, as early as the initial stage of infection, cells of the innate immune system can produce IFN-γ that activates mononuclear phagocytes and thus controls the retardation of bacterial replication. It is known that CD 4^+ T helper (Th) 1 cells produce interleukin 2 and IFN-γ and mediate macrophage activation. Th2 cells, on the other hand, are able to produce interleukin 4 and interleukin 5 and provide B cell help. Macrophages secrete TNF-α and IL-12 that stimulate natural killer (NK) cells to produce IFN-γ as well [181,182]. Thus one major mechanism of cytokine-mediated early host response is operated through the activation of immunocompetent cells, namely NK cells and T cells. Both activated cell types produce IFN-γ and TNF-α that subsequently activate mononuclear phagocytes to escalate their bactericidal effect and eliminate bacteria from cells and tissues.

Other studies have also described the major role of IL-12 and IL-23. Both IL-12 and IL-23 have been found able to positively regulate IFN-γ production despite the fact that IL-23 still has an unidentified role in the clearance of bacteria during intradermal sublethal *F. tularensis* LVS infection [183,184, 265]. Moreover, IL-23 is also an important contributor to promotion of Th17 response that is critical for host immunity to type A *F. tularensis* infection during primary immune response but not required during secondary immune response [185,186].

In contrast, Th2 cell cytokines production has not been studied in similar detail as have Th1 cytokines. This is despite the fact that data have been accumulated confirming the role of B cells that, in classic model need the Th2 assistance. Moreover, in looking for the role of Th-2 cytokines one comes upon conflicting data from experiments utilizing the vaccine and the virulent *F. tularensis* strains. Recent studies have demonstrated, for example, that IL-6, one of the Th-2 cytokines, is essential for primary resistance to *F. tularensis* LVS [187], but it fails to exert any effect on the progression of virulent strain infections [188].

Protective immunity against *F. tularensis* infection is usually attributed to an effective T cell response. Indeed, there is evidence that B cells are necessary for mice to develop fully protective immunity to primary and secondary LVS infections [189,190]. Nevertheless, the role of antibodies in protecting against intracellular pathogens remains poorly understood. In general, antibodies can offer only a minimal protective advantage during intracellular infection due to the fact that pathogens can be sheltered from antibodies inside the cells. *F. tularensis* has a significant extracellular phase in the host, however, and that makes it vulnerable to humoral immune responses [191].

While many laboratories have demonstrated that serum antibodies are mainly directed against *F. tularensis* lipopolysaccharide, serum antibodies with reactivity to bacterial proteins have also been detected. Among these are antibodies oriented against some outer membrane proteins such as FopA, OmpA [192,193], and Tul4 [194]; against other intracellular proteins such as GroEL, KatG [192], and DnaK; and against several putative virulence markers such as nucleoside diphosphate kinase, isocitrate dehydrogenase, the RNA-binding protein Hfq, and the molecular chaperone ClpB [194,195]. Thus, antibodies can potentially contribute to the protective response by eliminating *Francisella* virulence factors and, together with the antibody-independent functions of B cells, can demonstrate the potential of B cells to collaborate with T cells in the induction, regulation, and expression of protective immunity against *F. tularensis* infection.

The construction of B-cell-deficient mice renders studying the role of B cells in protective immune mechanisms against *F. tularensis* more feasible [190,196]. They have no B cells and no detectable antibody levels, but a fully functional T cell compartment. Nevertheless, today's knowledge of B cell functions still does not allow us to draw clear and unambiguous conclusions. It is generally accepted, however, that only the concerted action of both cell-mediated immunity and humoral immunity can ensure effective protection against this intracellular bacterial pathogen.

7 Diagnosis, detection, and laboratory confirmation

Diagnosis and detection of tularemia is still most challenging point of "tularemiology". Due to its various acquisition routes and entrance sites, tularemia presents

different types of clinical pictures. The ulceroglandular, oropharyngeal, glandular, pneumonic, typhoid, and ocular forms all have characteristic symptoms that make the diagnosis of tularemia rather difficult. Moreover, respiratory-acquired tularemia does not have specific signs or symptoms. In endemic areas, therefore, where farming and hunting are risk factors for acquiring tularemia, a patient who has been exposed to wild small rodents, rabbits, hares, or ticks and biting flies should be suspected of being infected by *F. tularensis*. Serological confirmation of the disease is possible only until the second week after infection, which presents another complication in diagnosing the disease. The pneumonic form and secondary pneumonia are associated with abnormal chest radiographic findings. Oval opacities, hilar adenopathy, and pleural effusions are more likely associated with tularemia. If this is the case, then epidemiological circumstances should be considered because pneumonia can be the consequence of a variety of zoonotic and environmental agents [15].

Laboratory confirmation of the tularemia diagnosis relies mainly on serology or on any of the polymerase chain reaction (PCR) techniques. However, cultivation of *F. tularensis* requires specifically enriched media. Recently, solid as well as liquid media have been made commercially available (see Supplementary). Nevertheless, some modified agglutination tests or enzyme-linked immunosorbent assay (ELISA) show sufficient sensitivity and specificity and still dominate in common immunological labs [15]. Cross-reactions are possible only with serum obtained from patients with brucellosis or yersiniosis, but sera with a titer lower than 1:320 do not agglutinate *Brucellae* at all [129,197]. The agglutination test can be followed by the Rose Bengal plate test, which is often used as a rapid screening test in the diagnosis of brucellosis [198], or, according to our experience, with immunoproteomic techniques to exclude cross-reactions [194].

Identification of tularemia antigens in clinical or environmental specimens using ELISA or RNA hybridization is also possible [199,200]. Recently, after sequencing of the genomes of individual *F. tularensis* subspecies, mass spectrometric identification of *F. tularensis* and its typing into subspecies and even into individual strains has become possible [201–204]. Another possibility for precisely identifying *F. tularensis* isolates consists in automated genotyping assay based on the analysis of variable number tandem repeat (VNTR) or multiple loci VNTR analysis (MLVA) markers [205,206].

Thus, advanced laboratory techniques have recently come into existence that are highly sensitive and selective while having the capacity to characterize individual isolates of *F. tularensis* and predict their geographical relationships. Some of these, however, are costly and require specific samples preparation and skilled laboratory personnel.

8 Prophylaxis

Research on a tularemia vaccine was initiated soon after identification of the bacterial agent. The first attempts to prepare a vaccine, carried out in the 1930s, were directed toward killing *F. tularensis* with nitric acid. This corpuscular vaccine was then completed by adding 0.5% of phenol (the so-called Foshay vaccine) [207,208]. The efficacy of this type of vaccine was subsequently tested on humans. The Foshay vaccine sufficiently protected against small doses of intradermal challenge with the virulent *F. tularensis* Schu S4 strain, but it failed to protect against infection by inhalation [209,210]. Similarly, a vaccine based upon ether extract from bacteria demonstrated marginal protection of experimental animals against infection by virulent strains [211–213]. The protective effect of this chemo-vaccine was tested on human volunteers, but also with limited success [214].

It is generally accepted that cell-free extracts or lysates of *F. tularensis* prepared under specific conditions contain immunogenic substances. To look for these, attempts were made to utilize genetically modified microorganisms that combined the "live bases" of the modified microorganism and an immunogenic component coded by the introduced gene. For example, experiments with oral administration of the *Salmonella enterica* serovar *Typhimurium* strain expressing the *F. tularensis* 17 kDa membrane protein (Tul4) showed that the 17-kDa protein mediated a limited protective response against *F. tularensis* that was not as high as the LVS-mediated protection in the mouse model [215]. This 17-kDa protein was also incorporated into the immunostimulatory complexes (ISCOMs), but again the induced immunity was lower than that induced by LVS [216].

In spite of limited success with simple subunit vaccines, the sequencing of the *F. tularensis* strain genomes will facilitate the identification of protective antigens through bioinformatics. Moreover, it is likely that a subunit vaccine will be composed of a number of immunogens to provide protection against virulent strains. New adjuvants promoting general immune response will be needed for constructing an effective subunit vaccine. A recent report demonstrates that a mucosal subunit vaccine composed of the *F. tularensis* heat shock protein

DnaK, Tul4, and quillaja saponin derivate GPI as an adjuvant had substantial protective effect against lethal *F. tularensis* LVS infection in the murine model [217]. To date, however, and despite intensive research on virulence factors, immunogenic proteins, and other membrane-associated components, decisive immunogens suitable for constructing a subunit vaccine have not been identified.

The first attempts at the construction of a vaccine based on attenuated strains were made in the 1930s by Francis, Kudo, and Gotschlich, but without satisfactory results [129,218]. In 1942, however, Elbert and Gaiskii successfully attenuated the "Moscow" strain, obtained on the basis of a natural isolate of *F. tularensis* at the Irkutsk Anti-Plague Institute in the USSR [219,220]. Later, Elbert and Gaiskii prepared other attenuated strains, among which especially strain 155 and strain 15 were extensively tested and recommended for the preparation of a commercially safe and effective vaccine. Such a vaccine was introduced in 1946 for mass vaccination within the USSR [221–224].

In 1956, a vial of the Soviet commercial live vaccine was transferred from the Gamaleia Institute in Moscow to the US Army Medical Research Institute of Infectious Diseases, at Fort Detrick, Maryland. The isolation of one selected *F. tularensis* colony from this ampoule gave rise to the *F. tularensis* Live Vaccine Strain (LVS). After protective efficacy testing on animal models, LVS was tested for safety and efficacy in humans and used for vaccination of at-risk personnel [209,210,225]. Retrospective analysis of laboratory-acquired *F. tularensis* infections among civilian employees at Fort Detrick vaccinated with the Foshay vaccine (data analyzed from the years 1950 to 1959) and LVS (from 1960 to 1969), respectively, revealed that vaccination with the LVS was more effective than vaccination with the chemo-vaccine. Nevertheless, LVS could not eliminate ulceroglandular tularemia, even though the signs and symptoms become less pronounced [226]. Despite the undisputed protective effect of the live vaccine strain, however, the vaccine was not licensed for human use due to difficulties with its standardization. To eliminate this problem researchers prepared targeted mutants of the LVS, the *holarctica* strain FSC 200, and the Schu S4 strain that are highly attenuated and are protective against challenge with virulent strains of *F. tularensis* [227–229]. Some of these may constitute a promising basis for the construction of a new live vaccine in the future.

To complete the information on the prophylaxis of tularemia, we should also mention attempts at passive transfer of immunity by immune sera or antibodies against *F. tularensis*. Classical experiments on animals have already demonstrated that injection of immune serum before virulent challenge only prolongs survival but cannot ensure protection against even low doses of virulent bacteria [230,231]. These results nevertheless clearly demonstrated that immune sera contain protective antibodies and their protective effect correlated well with precipitating antibody content [232]. More recently, studies on the passive transfer of immunity against tularemia in prophylactic mode utilizing both (hyper)immune sera as well as monoclonal antibodies have shown that specific antibodies limited manifestation of the disease, thereby facilitating a sterilizing T cell response to resolve the infection [233–238]. Moreover, antibodies can also be used in therapeutic mode, especially when given early after infection [193,239]. In combination with the information that immune sera can protect irradiated mice against otherwise lethal LVS infection [240], immune sera or monoclonal antibodies represent one of the promising tools for immediate prophylaxis of tularemia during threats of modern warfare [241,242]. In our view, however, the use of antibodies in medical practice for protection or therapy against tularemia will require much more information than we have at the present time.

9 Treatment

Since the description of *Francisella* spp. as an emerging pathogen in 2001, many molecular tools for diagnosis have been developed to rapidly confirm tularemia-positive patients and type the strain in order to recommend therapeutic treatment and predict patient prognosis. Tularemia Type A, as well as the less virulent Type B, is often associated with various complications that involve substantial periods of convalescence. Due to the long incubation period of tularemia it is imperative to treat the patient immediately after the onset of the symptoms. However, still, only antibiotics we have at disposal and from this reason we need new therapeutic strategies for tularemia including the development of new antibiotics or new ways of using existing (summarized in [243]). It is worth recollecting, too, that while the discovery of antibiotics had once led people to the idea that infectious diseases would soon be eradicated, it very quickly became clear that this notion had been only a poor assumption. Intracellular parasitism further complicated the situation; only xenobiotics that could cross cell membranes could effectively exert their effect on bacteria inside the cellular compartments.

Many studies characterizing antimicrobial compounds effective against *Francisella* spp. have already been published. The key targets of current antibacterial

treatment are the inhibition of DNA replication and translation and inhibition of cell wall synthesis. The aforementioned biochemical pathways are generally essential for all bacteria to survive and cause infection. Streptomycin became established early on as the drug of choice against tularemia infection, and especially tularemia meningitis [244]. But parental administration of streptomycin is not generally preferred these days. The aminoglycoside family of antibiotics is currently used and is effective against most cases of tularemia, whereas beta-lactams such as penicillin are ineffective. Prompt treatment with streptomycin, gentamicin, doxycycline, or ciprofloxacin is recommended because the first signs of tularemia (due to the incubation time) typically occur 3 to 5 days after exposure. Streptomycin and gentamicin are the preferred antibiotics for the treatment of tularemia due to their bactericidal activity, which clears the host of bacteria and thus significantly reduces the relapse rate [149,245]. In comparison with the murine model of tularemia, where streptomycin has shown a better therapeutic effect, gentamicin has become a useful alternative for parenteral treatment of humans. Gentamycin is the antibiotic of choice for reasons of its better tolerability. In spite of the fact, that gentamicin has limited cell penetration, it is still capable of successfully treating tularemia [245–247]. Recently, new antibiotics, including tigecycline, ketolides, and fluoroquinolones have been evaluated for treatment of tularemia [248]. The explanation may lie in pinocytosis that allows gentamycin to reach the cell interior or in the effect on *F. tularensis* during its extracellular phase. Oxytetracyclines or chlortetracyclines were used for treating tularemia in the 1950s [249,250]. Because a higher rate of recurrence has been shown during the application of tetracyclines, however, tetracyclines are not considered first-line therapeutics in this case [149]. Although considered more risky, tetracyclines can be alternatively used, in unavoinable case, for the oral treatment of tularemia. The risk of relapse can be minimized by using a prolonged treatment period [245,251].

At least in part, however, the choice of antibiotic type for tularemia treatment is dependent on the clinical manifestation of the illness, the subspecies of the infectious agent, and the immune status of the host. For example, chloramphenicol, which is a bacteriostatic, penetrates into the cerebrospinal fluid relatively easily and may be used for treating tularemia meningitis [252]. Prophylactic use of doxycycline may be useful in the early post-exposure period. In addition to aminoglycosides, fluoroquinolone ciprofloxacin has been shown to have bactericidal activity against *Francisella* spp. both *in vitro* and in animals, and it is considered to be a frontline therapeutic that can offer new options for the treatment of tularemia, especially for children. Moreover, quinolones can be seamlessly used for treating both Type A and Type B tularemia [253].

Newer and newer antibiotics are being tested for the treatment of tularemia. A "consensus" summary of preferred and alternative choices of antibiotic treatments for tularemia is presented at the Centers for Disease Control and Prevention website[2]. Streptomycin and gentamycin are the preferred choices for all categories of patients (adults, children, and pregnant woman) while doxycycline, chloramphenicol, and ciprofloxacin are alternative choices for adults and children. Chloramphenicol is not recommended for pregnant women. Practically the same recommendations for tularemia treatment can be found in the WHO Guidelines on Tularemia published in 2007[3].

In addition to testing new antibiotics, attempts are being made to use chemotherapy and immunotherapy against tularemia. The specific enzymes that *F. tularensis* needs for its survival and proliferation are the targets of enzyme inhibitors in the form of proteins or small molecules. These include, for example, recombinant cystatin 9 [254] and small compounds prepared by ligand- and structure-based drug design [255]. Currently, promising moderate therapy against tularemia comprises the immunosuppressive and/or immunomodulatory effects of antimicrobial peptides, such as human cathelicidin LL-37 peptide, novel synthetic hybrids designed from cecropin A, magainin II, granulysin peptides, or specific fly antimicrobial peptides as are attacin, cecropin, drosocin and drosomycin from *Drosophila melanogaster* [256–259]. These positively charged antimicrobial peptides are capable to disrupt the negatively charged bacterial membrane and limit the proliferation of microbes. Such antimicrobial peptides and enzyme inhibitors are effective in cell-based *in vitro* and in animal *in vivo* systems, display significant growth inhibition of *F. tularensis* or reduce organ bacterial burden, and improve survival of experimental mice. In the future, alternatives to antibiotic therapy may be offered in cases of natural or intentionally generated *F. tularensis* (multi)resistance.

2 The conclusions of the paper by Dennis are summarized on the Centers for Disease Control and Prevention website at: http://www.bt.cdc.gov/agent/tularemia/tularemia-biological-weapon-abstract.asp#4

3 See the website: http://apps.who.int/iris/handle/10665/43793

10 Decontamination/disinfection

The etiological agent of tularemia, *F. tularensis* subsp. *tularensis*, is classified as a biological agent that poses a military and terrorist threat due to its ability to be weaponized. The CDC's list of bioterrorism agents includes this bacterium in Category A, meaning that it is a bacterium that can "result in high mortality rates and [has] the potential for major public health impacts, might cause public panic and social disruption, and require[s] special action for public health preparedness" [4]. In the case of a biological contamination incident, therefore, it is imperative to limit the spreading of the agent and to have an effective decontamination and disinfection strategy for environmental surfaces, hospitals, and households. *Francisella* spp., as non-spore forming bacteria, exist only in vegetative form and therefore their ability to survive outside the host is considerably less than is that of spore-forming bacteria. Thus, the majority of general decontamination tools can be used for the decontamination and cleaning of *Francisella*-contaminated surfaces. Generally, it can be said that chlorine dioxide in all chemical states is the disinfecting agent of first choice. Among other disinfectants, chlorine dioxide was tested using a spray-based application method on several environmental surfaces (aluminum, carpet, concrete, glass, and wood coupons) [260], its gaseous form was tested for the decontamination of hospital rooms [261], and a chlorine dioxide solution in potable water was tested for the efficacy to inactivate bacterial threat agents [262].

Of course, there are other commercially available preparations; their spectra and names, however, keep changing over time. According to our experience, a very simple preparation can be prepared as a 0.5% water solution of peracetic acid. This has been utilized in our labs for decades, and it can be recommended as an alternative for decontamination in laboratory practice. One cannot be certain that the decontamination procedures recommended in the literature and in homeland security leaflets will have 100% efficacy. Experimental results have documented that decontamination efficacy is dependent upon the ambient temperature and porosity of the surfaces to be decontaminated. Woody surfaces and carpets are particularly difficult to decontaminate [260]. Moreover, the number of bacteria in the area to be decontaminated, the solution from which the agent was dispersed (proteinaceous coating can stabilize bacteria against the effect of external influences including chemical treatment), and the possible unexpected expansion of the agent can complicate decontamination. Thus, in all cases it is necessary to have a sensitive and selective checking system to identify residual bacterial contamination.

4 See the website: http://www.bt.cdc.gov/agent/agentlist-category.asp#a

11 Conclusion

The etiological agent of tularemia, *F. tularensis*, is an enigmatic bacterium. During informal discussions at conferences, it is sometimes referred to as a microbe from another world. In some publications, it has been characterized as a stealth pathogen [162,263]. It is a close neighbor to the endosymbionts, and its behavior inside the host cell resembles a search for a suitable niche for long-term persistence. It is frequently used as a model for the study of intracellular parasitism. Various immune mechanisms have been shown to be important for protection against infection, but the identification and sequence of cellular and molecular factors (entities) involved in creating protective immunity are still mostly unknown. The same can be said about the determinants of *F. tularensis* virulence. To date, we still lack an effective vaccine for prophylaxis of human tularemia as well as no satisfactory tools for therapy. In spite of its more than 100-year history of study, tularemia remains a continuing scientific challenge for the future.

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