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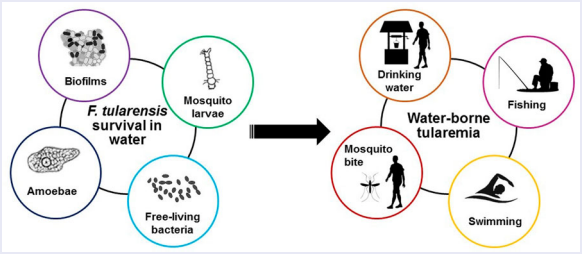
Tularemia as a waterborne disease: a review

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ABSTRACT

Francisella tularensis is a Gram-negative, intracellular bacterium causing the zoonosis tularemia. This highly infectious microorganism is considered a potential biological threat agent. Humans are usually infected through direct contact with the animal reservoir and tick bites. However, tularemia cases also occur after contact with a contaminated hydro-telluric environment. Water-borne tularemia outbreaks and sporadic cases have occurred worldwide in the last decades, with specific clinical and epidemiological traits. These infections represent a major public health and military challenge. Human contaminations have occurred through consumption or use of *F. tularensis*-contaminated water, and various aquatic activities such as swimming, canyoning and fishing. In addition, in Sweden and Finland, mosquitoes are primary vectors of tularemia due to infection of mosquito larvae in contaminated aquatic environments. The mechanisms of *F. tularensis* survival in water may include the formation of biofilms, interactions with free-living amoebae, and the transition to a 'viable but nonculturable' state, but the relative contribution of these possible mechanisms remains unknown. Many new aquatic species of *Francisella* have been characterized in recent years. *F. tularensis* likely shares with these species an ability of long-term survival in the aquatic environment, which has to be considered in terms of tularemia surveillance and control.



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Introduction

Francisella tularensis is a small, Gram-negative coccobacillus and the causative agent of the zoonosis tularemia [1]. Because it is highly infectious and can be spread by aerosols, this microorganism is classified in the category A of potential agents of biological threat by the US Centers for Disease Control and Prevention (CDC) [2]. The species *F. tularensis* is classically divided into four subspecies: *F. tularensis* subsp. *tularensis* (Type A strains), *F. tularensis* subsp. *holarctica* (Type B strains), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*, which may be considered a different species (*F. novicida*) because it is an aquatic bacterium of low virulence in humans [3,4]. Type A and type B strains of *F. tularensis* are the two etiological agents of tularemia. The former subspecies is mainly localized in North America, although it has been occasionally detected in arthropods in Slovakia and Austria [5]. The latter one is found throughout the Northern Hemisphere [1], but has recently been detected in Australia [6].

F. tularensis has multiple reservoirs. Firstly, this bacterium can infect a multitude of animal species, including lagomorphs and small rodents, which are the primary sources of human infections [1,7,8]. Secondly, *Ixodidae* ticks are vectors but also a probable reservoir of *F. tularensis* owing to the transstadial transmission of this pathogen in these arthropods [1,8]. Mosquitoes and deer flies can also transmit *F. tularensis* to humans and animals in specific areas, but are not considered long-term reservoirs of this pathogen [1,8]. Finally, a hydro-telluric reservoir of *F. tularensis* is suggested by numerous studies but has not been extensively characterized [1,8]. Water-borne

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tularemia was first described in the 1930s in the USSR [9]. Human infection with *F. tularensis* may occur from these different reservoirs, and the clinical presentation of tularemia is primarily related to the mode of infection. Six major clinical forms of tularemia are classically recognized. The most frequent route of contamination is through the skin, through contact with an infected animal (especially hares in hunters) or arthropod bites. The ulceroglandular form of tularemia combines a skin ulcer at the site of *F. tularensis* inoculation and regional lymphadenopathy. The glandular form corresponds to regional lymphadenopathy without any visible skin lesion. Infection with *F. tularensis* through the conjunctiva (e.g. hand to eye contamination) or the oral route (ingestion of contaminated water or food) correspond to the oculoglandular and oropharyngeal forms, respectively. The involvement of lungs through inhalation of infected aerosols or hematogenous spread of bacteria corresponds to the pneumonic form. Finally, whatever the portal of entry of bacteria, severe sepsis often associated with confusion and *F. tularensis* bacteremia corresponds to the typhoidal form. The present review summarizes the literature data on human tularemia cases infected from aquatic sources, detection of *F. tularensis* in aquatic environments, and potential mechanisms of *F. tularensis* survival in water environments.

Search strategy and selection criteria

Data on tularemia cases related to aquatic sources, on the presence of *Francisella* species in water environments, and the mechanisms of survival of these bacteria in water environments were collected from the English literature in the PubMed database. Only articles published in the last two decades (1998–2018) were included. They were extracted using the keywords 'tularemia' or '*Francisella*' and 'case report' or 'water' or 'mosquito' or 'biofilms' or 'amoeba.' In addition, data on other aquatic *Francisella* species were added, including *F. novivida*, *F. philomiragia*, *F. halioticida*, *F. hispaniensis*, *F. noatunensis*, *F. salina, F. frigiditurris*, *Allofrancisella* (formerly *Francisella*) *gangzhouensis*, *F. marina*, *F. ulginis* and *F. endociliophora*.

Tularemia of aquatic sources

Tularemia and drinking water

In the last 20 years, tularemia cases linked to drinking water have been reported in Turkey, Kosovo, Bulgaria, Georgia, Macedonia, Norway, Sweden, Italy, and Germany (Table 1).

In Turkey, tularemia reemerged in 1988 in the form of water-borne outbreaks of oropharyngeal tularemia cases [10]. Between 1988 and 2018, 28 tularemia outbreaks and non-outbreak tularemia cases linked to consumption of contaminated water were reported in this country [11–36]. The Turkish outbreaks were frequently of large scale, involving more than one hundred people for some of them [11–15]. Patients were almost exclusively suffering from the oropharyngeal form of tularemia, in accordance with the oral route of contamination with *F. tularensis*. In addition, cases were observed in the whole population, with a male/female sex ratio close to 1 or even lower, and both children and adults involved [11–19,31,33–35]. This is in sharp contrast to countries where infections usually occur through contact with animals or tick bites, and therefore predominate in middle-aged men due to more frequent occupational or leisure exposures [8]. Less frequently, exposure to contaminated water resulted in glandular [12,15], oculoglandular [14,15,19,25,26], and pneumonic [24] forms of tularemia. In some reports, *Francisella* species or more specifically *F. tularensis* were detected by PCR or culture in the suspected water sources [12,15–20,24,32]. Moreover, the same *F. tularensis* genotypes were detected concomitantly from water sources and related tularemia patients in some studies [24,32,37], confirming the epidemiological link between drinking water and human infection. In the majority of cases, the source of contamination was spring water, water from the community water supplies or water from wells. These water sources were not or inadequately disinfected. Contamination of the water by infected animal carcasses or excreta was the most likely hypothesis [10]. Although *F. tularensis* subsp. *holarctica* was identified only for some of these outbreaks, only type B strains have been associated with human infections in Europe and Asia.

In Kosovo, two huge oropharyngeal tularemia outbreaks occurred in 1999–2000 and 2001–2002, each one involving more than two hundred patients [38,39]. It was not possible to establish if the primary source of infection was contaminated water or food, but it can be assumed that both were involved in the human transmission of *F. tularensis* [38,39]. These outbreaks were likely a collateral damage of the Kosovo war, which ended in 1999 and left the country with bad sanitary conditions. Indeed, people reported that mice and rats invaded their villages during this period, which may correlate with contamination of water wells and cellars with *F. tularensis*. This bacterium was detected in a field mouse recovered from a water well in an affected village [39].

A long-lasting outbreak occurred between 1997 and 2005 in Bulgaria, involving 285 people [40]. *F. tularensis* was isolated from water samples collected from private wells. Thus, the route of human contamination was likely water consumption. The majority of patients suffered from the oropharyngeal form of tularemia [40]. Similarly, in Georgia, a tularemia outbreak involving 26 patients occurred in 2006 [41], with a

Table 1. Tularemia outbreaks and sporadic cases related to drinking water.

Country	Year of occurrence	Number of cases	Clinical forms (n)	Source of infection (water detection of *F. tularensis*)	*F. tularensis* subsp. involved	Reference
Bulgaria	1997–2005	285	OP (275), UG (6), OG (4)	Drinking water mainly from private wells (culture, pos)	*holarctica*	[40]
Georgia	2006	26	OP (21), GL (5)	Using water from the community water supply (culture, pos)	NS	[41]
Germany	2007	1	OP	Drinking surface water (NS)	*holarctica*	[49]
Italy	1988	24	OP (12), OG (1), AS (11)	Drinking water (NS)	NS	[48]
Kosovo	1999–2000	> 200	OP	Drinking water or eating food (Ag, neg)	NS	[39]
	2001–2002	> 200	OP	Drinking water or eating food (Ag, neg)	NS	[38]
Macedonia	2015	13	NS	Drinking water (NS)	NS	[42]
Norway	1997	8	OP or UG	Drinking water from wells (one well with a lemming carcass PCR-positive for *F. tularensis*) (PCR, pos)	NS	[43]
	2006	9	OP (5), GL (3), UN (1)	Drinking water from private wells or eating snow (PCR, pos)	NS	[44]
	2011	39	OP (21), UG or GL (10), PN (2), TY (2), AS (3), UN (1)	Drinking water from private wells or stream (PCR, pos)	NS	[45]
Sweden	NS	1	OP	Drinking water from a well (NS)	*holarctica*	[46]
	2013	6	OP	Drinking water from a private well (culture, pos)	NS	[47]
Turkey	1988–1998	205	OP (83%), OG (8%), UN (9%)	Drinking water (NS)	NS	[14]
	NS	1	OP	Drinking water from a well (culture, neg)	NS	[29]
	2001	14	OP	Drinking village pipe water (culture, neg)	NS	[35]
	2005	5	OP	Drinking natural spring water (PCR, neg)	NS	[30]
	2005	10	OP (7), AS (3)	Drinking spring water (culture, neg and PCR, pos)	NS	[20]
	2004–2005	54	OP	Drinking water (PCR, neg)	NS	[34]
	2004–2005	39	OP	Drinking water (NS)	NS	[36]
	NS	2	OP	Drinking water (NS)	NS	[28]
	2005	11	OP (8), OG (3)	Drinking village fountain water (PCR, pos)	NS	[19]
	2004–2005	145	OP	Drinking spring water (NS)	*holarctica*	[13]
	2005	70	OP mostly	Drinking natural spring water (culture, neg)	NS	[33]
	2004	86	OP mostly	Drinking water from a rivulet (PCR, pos)	NS	[17]
	2004–2005	135	OP	Drinking natural spring water (PCR, neg)	NS	[11]
	2000	22	OP (19), UG (3)	Drinking spring water (PCR positive for *Francisella* spp.)	NS	[18]
	2005–2006	58	OP	Drinking natural spring water (PCR, pos)	*holarctica*	[16]
	NS	1	OG	Drinking and washing face with spring water (NS)	NS	[26]
	NS	3	OP	Drinking spring water (NS)	*holarctica*	[27]
	NS	4	OP	Drinking water (NS)	NS	[23]
	2011	2	PN (with bacteremia)	Drinking water (culture, pos)	*holarctica*	[24]
	2010	4	OP (3), OP and OG (1)	Drinking (3) or exposure (1) to natural spring water (NS)	NS	[25]
	2009–2011	139	OP (74%), GL (15.8%), OG (5%)	Drinking spring water (PCR, pos)	NS	[15]
	NS	2	OP (with abdominal lymphadenopathy)	Drinking natural spring water (NS)	NS	[21]
	NS	3	OP	Drinking natural spring water (NS)	NS	[22]
	2010–2012	110	OP or GL	Drinking water (PCR, pos)	NS	[12]
	2013	55	OP	Drinking tap water contaminated by surface water (culture, neg)	NS	[31]
	2008, 2009 and 2012	89, 54 and 35	OP	Drinking water (culture, pos)	NS	[32]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), oculoglandular (OG), pneumonic (PN), typhoidal (TY), asymptomatic (AS), and unknown form (UN); (Ag) *F. tularensis* antigen detection; (pos) positive, (neg) negative.

predominance of oropharyngeal cases. The water source was the community water supply from which *F. tularensis* was isolated [41]. Finally, a small outbreak involving 13 patients was described in 2015 in Macedonia [42].

Water-borne tularemia cases are also reported in Scandinavia. In Norway, three small outbreaks, involving eight to 39 people, linked to the consumption of contaminated water were reported over 20 years [43–45]. Most of the patients suffered from oropharyngeal tularemia [43–45], although ulceroglandular, glandular, pneumonic and typhoidal forms were also observed [43–45]. The source of these infections was water collected from private water wells, as confirmed by PCR-detection of *F. tularensis* DNA [43–45]. *F. tularensis*-infected rodent carcasses probably contaminated these water wells. In one report, a lemming carcass retrieved from the contaminated water well was PCR-positive for *F. tularensis* [43]. The involved *F. tularensis* subspecies was not identified, but only type B strains are found in this country. In Sweden, a small outbreak and sporadic cases of oropharyngeal tularemia were also associated with the consumption of contaminated water from private water wells [46,47].

Tularemia cases related to *F. tularensis*-contaminated water consumption have been occasionally observed in other countries. In Italy, in 1988, a tularemia outbreak linked to the use of water collected from

an aqueduct involved 24 people living in the same village, most of which developed oropharyngeal tularemia [48]. More recently, a German oropharyngeal case of tularemia was suspected to be associated with drinking of non-purified surface water during a holiday in Bavaria [49].

Tularemia and mosquitoes

In Sweden and Finland, tularemia is mainly transmitted through mosquito bites. Large tularemia outbreaks, frequently involving more than one hundred people, have occurred almost annually in Sweden during the last two decades [46,50–54]. These outbreaks mainly occurred in central Sweden (counties of Örebro, Stockholm, Södermanland, Västmanland, Värmland, and Dalarna), in wet and woody areas of the Boreal Forest, during the late summer period, because of the mosquito vector habitat and seasonality [46,50–54]. A large scale epidemiological study in Sweden over 29 years identified that tularemia incidence was positively correlated with the presence of lakes and rivers [55]. Similarly, outbreak modeling demonstrated that tularemia in Sweden is concentrated in a few high-risk regions, with a high incidence in summer likely related to the population dynamics of the mosquito vector [56]. Due to this specific mode of transmission, the ulceroglandular form of tularemia is predominant in Sweden. In addition, cases occur in the exposed population regardless of age, but more frequently in men than women probably because of more outdoor activities in men [46,50,51,53]. Swedish mosquitoes collected in Örebro were PCR-positive for *F. tularensis* subsp. *holarctica*, still arguing their role in tularemia transmission in this country [57].

Tularemia is also predominantly a mosquito-borne disease in Finland, where several tularemia outbreaks [58] or sporadic cases [59,60] have been linked with this mode of transmission. Here again, tularemia is a seasonal disease occurring during late summer, and ulceroglandular tularemia is the primary clinical presentation [58,61].

The potential role of mosquitoes in the transmission of *F. tularensis* to humans has been occasionally reported in other countries, such as Germany [62]. All these reports involved *F. tularensis* subsp. *holarctica* as they occurred in Europe. Table 2 summarizes the literature reports of mosquito-borne tularemia cases. Interestingly, *F. tularensis* DNA was detected in Alaskan mosquitoes, suggesting that tularemia could be a mosquito-borne disease in this U.S. state [63].

Tularemia of other aquatic sources

All around the world, human contamination with *F. tularensis* has occasionally occurred through other types of water exposure (Table 3).

Tularemia cases were reported after near-drowning accidents in France [64] and Finland [65]. For these two cases, patients developed a pneumonic form of the disease after inhalation of contaminated water. For the French case, contamination occurred after inhalation of freshwater and was caused by *F. tularensis* subsp. *holarctica.* The involved subspecies and water salinity were not specified for the Finnish case. Four tularemia cases reported in Turkey were related to swimming activities [66,67]. Three of these cases were oropharyngeal forms that developed after swimming in the same lake [67]. The fourth patient suffered from a glandular form after swimming in a natural aquatic environment in a valley [66]. Otomastoiditis caused by *F. tularensis* subsp. *holarctica* were reported in three patients (two from France, one from Germany) after canyoning in the same river in France, between 2009 and 2014 [68]. Otitis likely occurred after exposure to *F. tularensis* contaminated water, although the source was not specifically identified [68].

Tularemia cases also occurred after handling *F. tularensis*-contaminated aquatic animals [69–71]. A small tularemia outbreak involving 19 patients occurred in Spain after crayfish fishing [69]. The patients developed a glandular or ulceroglandular form of the disease through skin injuries while catching or cleaning red swamp crayfishes sinned in the same river. Cases were grouped over three weeks after which fishing was prohibited [69]. The source of contamination was confirmed by PCR-detection of *F. tularensis* DNA in one crayfish and two water samples from the river [69]. In Canada, a patient

Table 2. Tularemia outbreaks and sporadic cases related to mosquito bites.

Country	Year of occurrence	Number of cases	Clinical forms	*F. tularensis* subsp. involved	Reference
Finland	2000	1	UG	*NS*	[59]
	2007	50	UG mostly	*holarctica*	[58]
	2010	1	UG	*NS*	[60]
Germany	NS	1	UG	*holarctica*	[62]
Sweden	1981, 1995, 1999, 2000, 2002, 2003, and 2004	349	UG mostly	*holarctica*	[50]
	2000–2004	278	UG	*holarctica*	[46,51]
	2000	105	UG mostly	*NS*	[52]
	2003	475	NS	*NS*	[53]
	2006	90	NS	*NS*	[54]

(NS) Not specified; Clinical forms: ulceroglandular (UG).

Table 3. Tularemia outbreaks and sporadic cases related to other aquatic sources.

Country	Year of occurrence	Number of cases	Clinical forms (n)	Source of infection	*F. tularensis* subsp. involved	Reference
Canada	NS	1	UG	Suspicion of finger injury while cleaning freshwater fishes	*NS*	[70]
Finland	NS	1	PN	Near-drowning accident	NS	[65]
France	NS	1	PN with bacteremia	Near-drowning accident while fishing in a river	*holarctica*	[64]
	2008–2014	3	Otomastoiditis	Canyoneering in the same river	*holarctica*	[68]
Spain	1998	19	UG and GL	Crayfish fishing in a river	*holarctica*	[69]
Turkey	2007	3	OP	Swimming in the same lake	*NS*	[67]
	2010	1	GL	Swimming in freshwater	NS	[66]
USA	2000–2006 (Martha's Vineyard island)	59	PN (38), UG or GL (9), TY (2), OP (1)	Water environmental source likely	*tularensis*	[73,74]
	2016	1	UG	Finger injury while fishing in a freshwater lake.	*holarctica*	[71]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), pneumonic (PN), and typhoidal (TY).

developed ulceroglandular tularemia after a finger injury while cleaning pike and pickerel, which are freshwater fishes [70]. Similarly, in the USA, a patient contracted an ulceroglandular tularemia due to *F. tularensis* subsp. *holarctica* after a finger injury while fishing on a freshwater lake [71].

The particular ecology of Martha's Vineyard (Massachusetts), an island in the east coast of the USA should be highlighted. Two outbreaks of pneumonic tularemia occurred on this island, the first in 1978 [72] and the second from 2000 to 2006 [73,74]. *F. tularensis* subsp. *tularensis* was isolated from one dead patient and rabbits [73,75]. According to a case–control study, landscaping activities, especially lawn mowing and brush-cutting, were the most likely sources of exposure to *F. tularensis* through aerosols from environmental material [73,76]. However, the environmental reservoir of *F. tularensis* could not be characterized. Skunks and raccoons were found to be seropositive for *F. tularensis*, but other animal species tested had not or very rarely been infected with this pathogen [75]. A low prevalence of infection and a high degree of *Francisella* genetic diversity in ticks argued against the role of these arthropods as a source of human contamination [77]. In contrast, the role of the aquatic environment as a long-term reservoir of *F. tularensis* was strongly suspected. PCR detection of *Francisella* sp. DNA was frequently reported from brackish-water samples collected on the island [78].

The role of brackish water as a potential reservoir of *F. tularensis* is supported by a more recent study in Nunavik territory (Canada) demonstrating that tularemia seroprevalence in the human population was positively correlated with residence near the coast [79].

Other *Francisella* species and aquatic reservoir

The genus *Francisella* includes many other species with a proven or probable aquatic habitat. Genetic analyses have suggested that the ancestral *Francisella* species originated in a marine habitat [80]. Here, we review water-borne human infections caused by *Francisella* species other than *F. tularensis* (Table 4). Some of these species are opportunistic pathogens mainly

Table 4. Human infections caused by *Francisella* species other than *F. tularensis* related to aquatic sources.

Country	Year of occurrence	Number of cases	Clinical forms	Source of infection	*F. tularensis* subsp. involved	Reference
Australia	NS	1	UG	Cut in the toe in brackish water	*F. novicida-like*	[99]
	NS	1	NS, bacteremia	Cut with a fishhook while fishing	*F. hispaniensis*	[105]
Canada	NS	1	OP and PN	Skin abrasion from a saltwater crab	*F. philomiragia*	[84]
Spain	NS	1	OP	Holiday activities in the Mediterranean sea	*F. philomiragia*	[83]
Turkey	NS	1	TY	Swimming in the sea and taking mud baths	*F. philomiragia*	[81]
USA	1977–1985	5	PN, bacteremia	Near-drowning accident in saltwater or brackish water	*F. philomiragia*	[86]
	1995	1	NS, bacteremia	Use of water from a private well for food preparation and bathing	*F. novicida*-like	[100]
	NS	1	NS, bacteremia	Working in the brackish water of Assawoman Bay in Maryland	*F. philomiragia*	[85]
	NS	1	TY	Practice of jet-ski in a bay in the Atlantic Ocean	*F. philomiragia*	[82]
	NS	1	NS, bacteremia	Near-drowning accident while surfing in the Atlantic Ocean	*F. novicida*	[97]
	2001	1	NS	Exposure to hot spring water near a salt-lake	*F. novicida*	[89]
	NS	1	UG	Suspicion of infection through contact of an open wound in brackish water	Species close to *F. halioticida*	[113]
	2011	3	NS, bacteremia	Consumption of ice from ice machines	*F. novicida*	[98]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), oculoglandular (OG), pneumonic (PN), and typhoidal (TY).

causing diseases in immunocompromised patients exposed to an aquatic environment. Among them, infections caused by *F. philomiragia* or *F. novicida* have been most frequently reported.

Less than 20 human infections with *F. philomiragia* have been published in the English literature [81–88]. These infections occurred in healthy individuals that have survived a near-drowning accident [86] or in immunocompromised patients (especially people suffering from chronic granulomatous disease) after exposure to an aquatic environment [81–86]. Interestingly, human contamination usually occurred after exposure to salt- or brackish-water [81–86]. Infections with *F. philomiragia* most often occurred during recreational activities in sea, ocean or bay connected to the ocean [81–83,85,86]. A young patient was contaminated after a skin lesion caused by a saltwater crab [84]. In a review of 14 cases, Wenger *et al.* showed that most *F. philomiragia* infections occurred in patients living within 50 miles of a salt-water coastline [86], again suggesting a clear association between salt-water exposure and *F. philomiragia* infections.

The geographical distribution of *F. philomiragia* is probably wide as human infections with this species have been described in the USA [82,85,86], Canada [84], Europe [83] and Turkey [81]. Frequent detection of *F. philomiragia* in water samples by culture or PCR indicates that the aquatic environment is likely the primary reservoir of this bacterium [78,89–93]. In two studies, in Norway [92] and the USA [78], *F. philomiragia* was only found in salt- or brackish-water but not in fresh-water, suggesting that water salinity is a major element in the natural life cycle of *F. philomiragia*. However, this species was also isolated from spring water near a salt lake in the USA [89] and cooling towers in China [91], reflecting its distribution in different aquatic reservoirs. *F. philomiragia* DNA was also detected in ballast water from cargo traveling all around the world [90], which might be a mode of diffusion of this species at the global scale. Although the aquatic reservoir appears to be predominant, *F. philomiragia* was isolated in a sick muskrat and in brackish water surrounding it [94] suggesting that a mammal reservoir may exist for this bacterium. *F. philomiragia* was also detected in *Dermacentor* ticks [95].

F. novicida is also a rare human pathogen. A dozen cases have been published in the English literature so far [96]. For all cases with an identified mode of contamination, an aquatic source was involved [89,97–100]. In the USA, a case of *F. novicida* bacteremia occurred after a near-drowning accident in the Atlantic Ocean [97], another case after exposure to hot spring water near a salt lake [89], and the last one after exposure to water from a private water well [100]. A striking outbreak of *F. novicida* bacteremia occurred among inmates in the USA after consumption of ice from ice machines from which *F. novicida* DNA was detected by PCR [98]. Finally, in Australia, a patient developed an ulceroglandular form of infection with *F. novicida* after cutting himself in brackish water [99]. Most of the patients suffering from *F. novicida* infection were immunocompromised or had underlying health conditions [98,100]. The identification of *F. novicida* has never been reported in animals or arthropods [96]. The only known reservoir of this bacterium is the aquatic environment as attested by its repeated isolation from water samples [78,89,101,102]. As for *F. philomiragia*, salinity seems to impact *F. novicida* survival in water, as this bacterium was detected by culture or PCR only in sea-water [101,102], brackish-water [78] and spring water near a salt lake [89].

Other *Francisella* species have been rarely associated with human infections originating from aquatic sources. *F. hispaniensis* was first isolated in 2003 from a Spanish patient suffering from bacteremia [103,104]. The source of contamination was not identified [103]. However, a few years later, *F. hispaniensis* was isolated in the blood of an Australian immunocompromised patient after he cut himself with a fishhook while fishing [105].

The genus *Francisella* also includes species that are pathogenic for marine animals. *F. noatunensis* subsp. *orientalis* and *Francisella noatunensis* subsp. *noatunensis* are widely described as warm- and cold-water fish pathogens, responsible for 'piscine francisellosis.' This disease causes high morbidity and mortality in many fish species worldwide and is responsible for economic losses in aquaculture [106–109]. *F. noatunensis* does not seem to be pathogenic in humans [106]. *F. halioticida* can infect *Haliotis* mollusks (abalones) [110,111] and *F. marina sp. nov.* was recently identified as causing disease in Spotted Rose Snapper fishes [112]. Interestingly, a novel *Francisella* species very close to *F. halioticida* was isolated in the USA, in a diabetic patient, from an infected skin wound developed after contact with brackish water [113]. Finally, other *Francisella* species are endosymbionts of marine ciliates such as *F. endociliophora* [114].

Over the past ten years, new *Francisella* species have been isolated from the aquatic environment. *F. salina* and *F. uliginis* were isolated from sea-water in the USA [101,102]. Water from cooling towers also seems to be a reservoir of *Francisella* species as attested by recent isolation from these air conditioning systems of *F. frigiditurris* in the USA [102] and *F. guangzhouensis* in China and Germany [91,115–117]. These two latter species have been transferred to the new genus *Allofrancisella* [115].

Table 5 summaries studies dealing with the detection of *Francisella* species in water samples, either using culture, PCR or both methods. It shows the broad spectrum of *Francisella* species found in aquatic reservoirs.

Regarding PCR methods, it is important to notice that *Francisella* species that are not yet characterized may not be PCR-amplified from water samples due to the use of inadequate primers [78]. Even if there is PCR amplification with a new species, it may not be accurately identified as a novel species due to a lack of resolution within the utilized amplicon. Full identification of a novel species requires whole genome sequencing.

Mechanisms of *F. tularensis* survival in water environments

The high frequency of water-borne tularemia cases implies the persistence of *F. tularensis* in the aquatic environment. However, the mechanisms of *F. tularensis* survival in this environment have not been elucidated so far.

Long-term survival of F. tularensis in water

Experimental studies suggest long-term survival of *F. tularensis* in various water environments. Several authors described the survival of *F. tularensis* in water microcosm from 1 to 70 days [118–120]. *F. tularensis* survival seems to be influenced by both water temperature [118] and salinity [119]. Gilbert and Rose observed that *F. tularensis* subsp. *holarctica* remains cultivable after a stay in water for one day at 5°C or 25°C, but up to 28 days at 8°C [118]. Berrada and Telford showed that both type A and type B strains of *F. tularensis* remain cultivable after a stay of 8–10 days in fresh-water, but 30–42 days in brackish-water, both at 21°C [119]. In another study, *F. tularensis* subsp. *holarctica* remained cultivable after a stay in fresh water at 8°C up to 70 days [120].

Interestingly, when *F. tularensis* became uncultivable on agar plates, bacteria could still be detected and were metabolically active in water [118,120]. This phenomenon was observed by Gilbert and Rose [118] and Forsman *et al.* [120] for *F. tularensis* subsp. *holarctica.* The latter authors described the persistence of metabolic activity for *F. tularensis* in water up to 140 days [120]. This state is defined as 'viable but non-culturable' (VBNC) and could be responsible for long-term survival of bacteria in the water environment. The VBNC state has been defined as a state from which bacterial cells cannot be cultured but maintain a metabolic activity and cellular integrity [118]. In addition, the VBNC state may be reversible, as bacteria may become cultivable under certain conditions. This reversion in the ability to grow on acellular media is called 'resuscitation' of VBNC bacteria. The VBNC state has been described for a wide range of bacteria such as *Vibrio* sp. [121,122], *Campylobacter* sp. [123], *Escherichia coli* [122] and *Legionella pneumophila* [124]. Depending on the bacterial species, the infectious nature and pathogenic potential of VBNC cells are variable, as well as the ways of their resuscitation. Forsman *et al.* reported that *F. tularensis* VBNC cells were no longer virulent in mice, and could not be resuscitated [120]. Finally, a recent study demonstrated that *F. tularensis* subsp. *holarctica* possesses a mechanosensitive channel that protects this bacterium from hypo-osmotic shock when it is released from an infected animal to water [125].

Long-term survival in water has also been reported for other *Francisella* species. Berrada and Telford showed that *F. novicida* (like *F. tularensis*) remains cultivable after a stay of up to 30–42 days in brackish-water, at 21°C [119]. The fish-pathogen *Francisella* species are able to survive in water in the absence of a suitable fish host. Indeed, *F. noatunensis* subsp. *orientalis* remains cultivable after a stay up to 2 days in freshwater and up to 3 days in seawater [126]. *F. noatunensis* subsp. *noatunensis* remains cultivable after up to 12 days in freshwater and up to 50 days in seawater [127]. Interestingly, *F. noatunensis* was also able to enter in a VBNC state after a period of stay in the water, and these VBNC were not pathogenic to cods [127].

Long-term survival of *F. tularensis* in natural aquatic environments is suggested by a number of studies detecting this bacterium by culture or PCR in environmental water samples [12,16,17,19,20,32,40,41,43,44,47,78,128–132]. Not surprisingly, long-term detection of *F. tularensis* in water environments was reported in countries were water-borne tularemia cases are frequent and predominant. In Turkey, two environmental studies identified *F. tularensis* subsp. *holarctica* in water samples collected from the aquatic environment or from village water supply systems highlighting the role of different water sources as common and persistent reservoirs of *F. tularensis* in this country [32,130]. In Sweden, *F. tularensis* subsp. *holarctica* was PCR-detected in water samples collected during outbreak and non-outbreak periods, in tularemia endemic areas [131]. However, *F. tularensis* was also detected from water samples in countries where tularemia is not or rarely a water-borne disease [128,129,132]. Hightower *et al.* isolated *F. tularensis* strains from water in Ukraine and considered that the aquatic environment was the third main *F. tularensis* reservoir after arthropods and mammals in this country [129]. In Germany, following the reemergence of tularemia in 2004, an ecological study in outbreak areas found a river water sample PCR-positive for *F. tularensis* suggesting a natural aquatic reservoir for this bacterium [132]. In the Netherland, tularemia also re-emerged in 2011 leading to the surveillance of *F. tularensis* prevalence in the environment [128]. Surface water samples were PCR-positive for *F. tularensis* subsp. *holarctica* in 10% of the randomly collected samples and 88% of the samples collected in areas where tularemia cases among hares or humans had been reported [128]. These studies show the almost

Table 5. Detection of *Francisella* species in water samples.

Country	Year of sampling	Type of water samples (n)	Testing methods	Findings (n samples)	Reference
China	2008	Cooling towers (NS)	Culture and strain identification by fatty acid analysis, and 16S rRNA, 23S rRNAs, *recA, rpoA*, *rpoB*, *rpoD*, *rpoH*, *groEL*, *dnaK*, *gyrB*, *sdhA*, and *fopA* genes sequencing	*F. guangzhouensis* (4)	[117]
	2009–2011	Cooling towers (312)	Culture and strain identification by 16S rRNA, *rpoB* and *sdhA* genes sequencing	*Francisella* strains phylogenetically close to *F.* philomiragia (1) or *F. guangzhouensis (8)*	[91]
	2008 and after	Cooling towers (NS)	Culture and strain identification by mass spectrometry, fatty acid analysis, and 16S rRNA, *rpoB, mdhA,* and *sdhA* genes sequencing	*Francisella* strains phylogenetically close to *F.* guangzhouensis (5). Description of *Allofrancisella inopinata* gen. nov., sp. nov. and *A. frigidaquae* sp. nov.; transfer of *F. guangzhouensis* to A. *guangzhouensis* comb. nov.	[115]
Germany	2005–2006	NS (28)	PCR targeting 16S rRNA and *fopA* genes	*F. tularensis* (1)	[132]
	2012	Cooling tower (NS)	Culture and strain identification by 16S rRNA, *fopA, gyrA, rpoA, groEL, sdhA,* and *dnaK* genes sequencing	*Francisella* strain phylogenetically close to *F. guangzhouensis* (*1*)	[116]
Netherlands	2013–2017	Surface water collected from areas with reported human or hare tularemia cases (127) or unrelated to recent tularemia cases (339)	PCR targeting *ISFtu2* and *fopA* genes	*F. tularensis* in 88% of the case-related samples and in 10% of the randomly collected samples	[128]
Norway	2010	Seawater (149) or freshwater (64)	PCR sequencing of 16S rRNA; and for positive samples *sdhA* and *purCD* PCR	*Francisella* sp. in seawater (38) but not in freshwater samples. *F. philomiragia*-related species mostly.	[92]
Sweden	2003– 2005	Surface water (341)	PCR sequencing of *lpnA,* 16S rDNA, *lpnA,* and FtM19InDel	*F. tularensis* (108), mainly subsp. *holarctica*, rarely subsp. *mediasiatica*	[131]
Turkey	2008 - 2009	Rivers, spring waters or village fountains in tularemia-endemic areas (154)	Culture and strains identification by 16S rRNA gene sequencing; and PCR targeting IS*Ftu2*	*F. tularensis* subsp. *holarctica* isolation (4); or *F. tularensis* positive PCR IS*Ftu2* (17)	[130]
Ukraine	1941– 2008	NS (NS)	Culture	*F. tularensis* (393)	[129]
USA	2003	NS (23)	PCR sequencing of 16S rDNA; and for positive samples *ISFtu2*, *23 kDa, tul4, fopA* and *sdhA* PCR	*F. philomiragia* (1)	[93]
	NS	Seawater (NS)	Culture (CHAB-PACCV medium) and strains identification by PCR sequencing of 16S rRNA and *sdhA* genes	*F. philomiragia*-like (2) and *F. novicida*-like (1). The two *F. philomiragia*-like strains were latter characterized as new *Francisella* species by Challacombe *et al.*: *F. salina* and *F. uliginis.*	[101,102]
	2005– 2007 (Martha's Vineyard island)	Fresh-water (35) or brackish-water (42)	Culture and PCR targeting 16S rRNA gene; for positive samples *sdhA, tul4*, IS*Ftu2*, and *fopA* PCR.	No positive fresh-water samples. *Francisella* DNA detected by PCR in brackish-water samples (19). Mainly *F. philomiragia*; few *F. novicida*- or *F. tularensis*-like strains. *F. philomiragia* grown from one brackish-water sample.	[78]
	NS	Hot or cold spring waters near a salt lake (NS)	Culture and strain identification by fatty acid analyses, ribotyping and 16S rRNA gene sequencing	*F. philomiragia* and *F. novicida* (NS)	[89]
	NS	Cooling tower (NS)	NS	*F. frigiditurris* (NS)	[102]
Cargo ships*	2007–2008	Ballast water from 5 general cargo ships (NS)	PCR sequencing of 16S rRNA gene	*F. philomiragia* and *F. noatunensis* in ballast water from 4 cargo ships	[90]

(NS) Not specified; * Cargo ships from Columbia, Republic of the Congo, USA, Canada, and Iran.

constant presence of *F. tularensis* in randomly collected water samples at different time periods. This observation can only be explained by repeated contaminations of the aquatic environment, particularly from the animal reservoir during epizootics, but also by the persistence of these bacteria in the water environment during non-epizootic periods.

Table 5 summarizes studies dealing with *F. tularensis* detection in water environments. PCR was more effective than culture for detection of *F. tularensis* in water samples, possibly because of a low bacterial inoculum, the encroachment of *F. tularensis* by other bacterial species, or a VBNC state of bacteria. However, PCR results should be interpreted with caution since it has been shown that *F. tularensis* specific primers (such as those targeting *fopA* or *tul4* genes) may also amplify DNA from *Francisella* species other than the tularemia agents [78].

Survival in biofilms

An essential mechanism for survival and persistence of bacteria in the water environment is biofilm formation. Biofilms are defined as naturally formed adherent communities of bacteria within an extracellular polymeric matrix [133]. A number of bacterial species, such as *Vibrio cholerae* [134], *Legionella pneumophila* [135], *Helicobacter pylori* [136] *and Pseudomonas aeruginosa* [137] form biofilms to promote their survival under environmental water conditions. *In vitro* studies have demonstrated that both *F. tularensis* subsp. *holarctica* [138,139] and *F. tularensis* subsp. *tularensis* [139] can form biofilms. The aquatic *Francisella* species, *F. novicida* [139–141] and *F. philomiragia* [142] have also been capable of biofilm formation experimentally. *F. novicida* was also demonstrated to be able to form biofilm in chitin surface, the second most abundant biopolymer in nature, providing the structure of arthropods, insects, and fungi [139]. *F. philomiragia* was shown to form more biofilm at 25°C than at 37° C, which is compatible with its natural aquatic reservoir [142]. The fish pathogen *F. noatunensis* was also demonstrated to form biofilm *in vivo*. [143]. Until now, biofilm formation has not been associated with virulence in *Francisella* species [133]. Thus, biofilm formation is most likely a way of environmental survival and persistence in these species [133]. To our knowledge, *Francisella* biofilm has never been described in natural environmental water microcosm.

Survival in amoebae

Free-living amoebae are ubiquitous organisms in soil and water environments. Several human pathogens, such as *L. pneumophila* and some *Mycobacterium* species resist phagocytosis and digestion by the free-living amoebae and may survive in water environment inside amoebae [144]. Moreover, these bacteria may survive for long periods in amoeba cysts [144]. This mode of survival may apply to *Francisella* species, especially *F. tularensis*. Multiple experimental studies have focused on the interaction between *Francisella* species and several amoeba species [145–154]. Berdal *et al.* demonstrated that *F. tularensis* could penetrate in the amoeba *Acanthamoeba castellanii* and be released from it [152]. Then, other authors described the multiplication of *F. tularensis* subsp. *tularensis* in *A. castellannii* 24 h post-infection [150]. *F. novicida* and *F. philomiragia* were also able to multiply within *Hartmanella vermiformis* and *A. castellanii* [142,146,148,150,151]. *F. noatunensis* was also able to infect and replicate within the amoeba *Dictostelium discoideum* [149]. *Francisella* cells were localized within vacuoles in amoeba trophozoites [146] but were also able to survive in amoebal cysts for several weeks [150]. This latter finding suggested that amoeba could be a long-term reservoir of *Francisella* spp. in water environments. Other authors described an enhanced survival of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and *F. novicida* in co-culture with amoebae such as *A. castellanii*, *A. polyphaga*, *Vermamoeba vermiformis* or *Ochromonas danica* [145,147,153]. These authors described the presence of *Francisella* sp. inside and outside the amoeba in the co-culture model, suggesting that bacterial survival could be related to intra-amoebal replication, to favorable interaction between extracellular bacteria and amoeba, or both [145,147,153]. Interestingly, Gustafsson *et al.* demonstrated that growth supernatant of *A. palestinensis* (without the presence of amoebae) increased multiplication of *F. tularensis* [154]. After five days in co-culture with amoeba, *F. tularensis* subsp. *holarctica* was also shown to enter in a VBNC state [147]. In contrast to *L. pneumophila,* passage through amoebae did not increase *Francisella novicida* virulence [148]. Despite some disagreements about interaction mechanisms, all these reports argue that *Francisella* spp. are resistant to free-living amoeba and that protists may contribute to the survival of *Francisella* sp. in the water environment. However, to our knowledge, *Francisella* species have never been detected within amoebae in environmental water samples, in contrast to *L. pneumophila* and *Mycobacterium* spp. [155].

Survival in mosquito larvae

Mosquito larvae may also represent a long-term *F. tularensis* reservoir in the aquatic environment. It has been shown that these larvae can ingest *F. tularensis* subsp. *holarctica* that are present in water and ingested bacteria survive throughout the different maturation stages of these arthropods up to adult mosquitoes [57,63,138,156,157]. The fact that

mosquito-borne tularemia cases have occurred over years in Sweden and Finland should be considered a further evidence of the existence of a long-term aquatic reservoir of *F. tularensis*. However, it should be notified that mosquitoes could also be infected at the adult stage after a blood meal on an infected host [57]; in this case tularaemia is not water-borne.

Discussion

Although water-borne tularemia was first described in the 1930s [9], this route of human contamination has been largely underestimated. Tularemia cases linked to the aquatic reservoir are common and can occur as large epidemics. Thus, tularemia is a major public health problem in countries where water-borne tularemia cases predominate. These cases may occur through consumption of *F. tularensis*-contaminated drinking water, such as in Turkey [11–36] and its neighboring countries [38–42], and in Norway [43–45] where large-scale tularemia outbreaks caused by *F. tularensis* subsp. *holarctica* are regularly reported. Drinking water as a source of human infections with type B strains of *F. tularensis* have also been occasionally encountered in Sweden [46,47] and central Europe [48,49]. Human infections have occurred after consumption of contaminated water from the community water supplies, especially in countries with bad sanitary conditions, old water networks and inappropriate water treatment. Consumption of unsanitized surface water or well water was also a source of contamination. Consequently, it is of primary interest for practitioners to keep in mind that tularemia may correspond to sub-acute or chronic pharyngitis associated with cervical lymphadenopathy, especially in patients living or traveling in countries where water-borne tularemia cases are frequent. In addition, medical questioning regarding tularemia exposure should include a statement about unsanitized water consumption. Until now, this mode of human contamination has been observed in restricted areas and only linked to *F. tularensis* subsp. *holarctica*. However, similar cases could occur throughout the Northern hemisphere where type B strains are encountered. *F. tularensis* subsp. *tularensis* has also been associated with the water environment [72–74], and could also cause tularemia cases related to drinking water.

Mosquito-borne tularemia is also related to contamination of the aquatic environment by *F. tularensis*. Tularemia outbreaks caused by *F. tularensis* subsp. *holarctica* in Sweden [46,50–54] and Finland [58–60] are primarily related to mosquito bites. Because of skin inoculation of bacteria, the ulceroglandular form of tularemia predominates. Infections usually occur during the warm season at the time of maximum activity of mosquitoes. Scandinavian practitioners are now particularly aware of this situation and usually diagnose tularemia early in the course of the disease. In contrast, this mode of transmission is most often unknown by physicians in other parts of the word, and mosquito-borne tularemia cases could be missed in patients returning from Scandinavian countries. Also, potential transmission of tularemia through mosquito bites outside Scandinavia has not been thoroughly evaluated. A probable autochtonous mosquito-borne tularemia case was reported in Germany [62]. In Alaska, mosquitoes tested positive for *F. tularensis* DNA [63] arguing that these arthropods could also transmit tularemia to humans in this US state. In this time of global warming, mosquito vectors could spread to new geographic areas, leading to a rise in mosquito-borne tularemia cases [158].

Finally, tularemia cases may occur through other types of aquatic exposure such as near drowning accident [64,65], swimming [66,67], canyoning [68] and fishing activities [69–71], due to penetration of *F. tularensis* through the skin, conjunctiva, or digestive and respiratory tracts. These cases have been reported in Europe [64,65,68,69], Turkey [66,67] and North America [70,71], suggesting a wide aquatic distribution of *F. tularensis*. Type B strains of *F. tularensis* were likely involved in all these cases, except one case occurring in Canada for which type A and type B strains could be involved [70]. These data suggest that *F. tularensis* subsp. *holarctica* could be more frequently associated with aquatic reservoirs than *F. tularensis* subsp. *tularensis* There is currently no explanation regarding the preferential association of type B strains with water. Williamson *et al.* demonstrated that type B strains can resist to hypoosmotic shock when released into water [125]. It would be interesting to compare the osmotic shock resistance of type A versus type B strains. Noticeably, almost all human contaminations occurred after contact with fresh-water, suggesting that *F. tularensis* may better survive in such aquatic environment. *F. tularensis* subsp. *tularensis* may also infect humans through water exposure, as attested by the particular ecology of the Martha's Vineyard island in the USA [72–74]. In this case, brackish-water was considered as a potential reservoir of *F. tularensis* [78]. Altogether, available data indicate that human contamination with *F. tularensis* may occur from a wide diversity of aquatic sources and activities.

Francisella species other than *F. tularensis* are primarily considered aquatic bacteria. *F. philomiragia* and *F. novicida* have been occasionally involved in human infections, especially in immunocompromised patients, but also in specific situations such as near-drowning accident [81–86,89,97–100]. Infections caused by these species were frequently associated

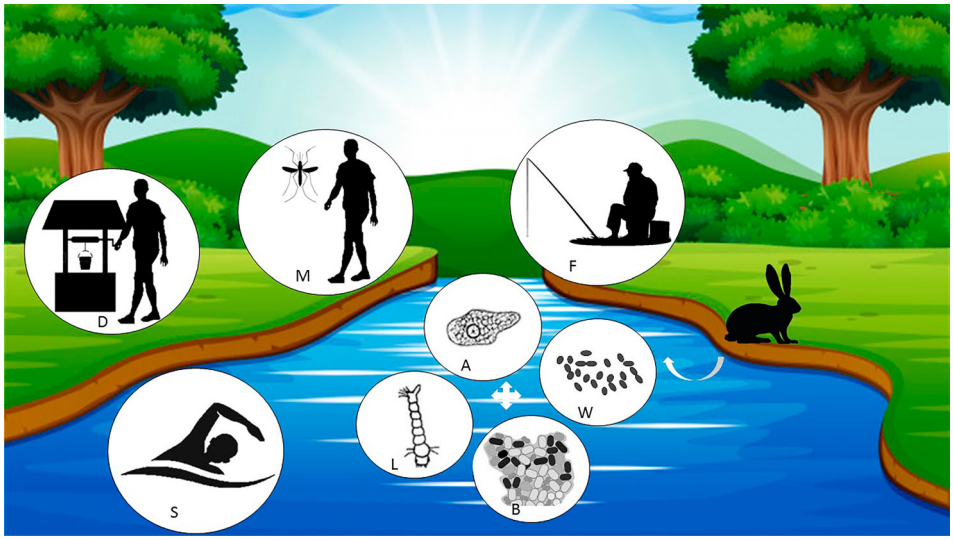


Figure 1. Potential aquatic sources of human infections with *Francisella tularensis*. *Francisella tularensis* is released into water from animals. The bacterium is able to survive in water (W), in mosquito larvae (L), in biofilms (B), or in cooperation with amoeba (A). Human can be contaminated from the aquatic reservoir by drinking contaminated water (D), after a mosquito bite (M), or during swimming (S) and fishing (F) activities.

with exposure to brackish-water or salt-water [81–86,89,97,99]. In line with this observation, *F. philomiragia* and *F. novicida* have been frequently isolated from brackish- and salt-water samples [78,89,90,92,101,102]. In recent years, novel *Francisella* species have been detected in environmental or clinical samples, the majority of them being associated with an aquatic reservoir. Among them, *F. hispaniensis* [105] and *F. halioticida* [113] are rare human pathogens. These findings strongly suggest that the *Francisella* species are mainly aquatic bacteria.

Several mechanisms could be involved in the survival of *F. tularensis* in aquatic environments. Experimental and epidemiological studies have demonstrated that both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* can survive for long periods in water microcosms [32,78,118–120,125,128–132]. Interestingly, after a long stay in water, *F. tularensis* has been shown experimentally to evolve to a VBNC state [118,120], which could account for long-term survival of this bacterium in water. Both type A and type B strains of *F. tularensis* can form biofilms *in vitro* [138,139], another potential survival mechanism of these bacteria in aquatic environments. Experimentally, both subspecies are able to multiply in amoebae or at least interact with these protozoa to enhance their survival [145,147,150,152,153]. Finally, *F. tularensis* subsp. *holarctica* is also able to infect mosquito larvae *in vitro* and survive during larvae maturation up to the adult stage [57,63,138,156,157]. It should be mentioned, however, that all these mechanisms have been evaluated experimentally, but not yet confirmed in natural water environments. It is very likely that all these mechanisms exist and are entangled. Infected animals and animal carcasses may contaminate water environments, in which protozoa, mosquito larvae and biofilm communities may become contaminated with *F. tularensis* and serve as reservoirs for this bacterium.

In conclusion, our goal was to demonstrate that *F. tularensis* is likely able to survive for prolonged periods in various aquatic environments, which likely constitute a significant reservoir for this bacterium. Figure 1 summarizes the probable tularemia aquatic cycle as it can be pictured from current literature data. In terms of public health, it is important to remember that providing people with access to safe drinking water via treatment of municipal and private sources remains a priority. Better characterization and control of the aquatic reservoir of *F. tularensis* would also be of tremendous importance following a bioterrorist attack. Following a bioterrorist attack, *F. tularensis* could survive for months in the environment, leading to a high number of secondary tularemia cases. Water and mosquito reservoirs should be monitored in the overall tularemia surveillance, in addition to the wildlife reservoir.

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