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EPIDEMIOLOGY OF TULAREMIA IN FINLAND

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Epidemiology of tularemia in Finland

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ACADEMIC DISSERTATION

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To Sofia and Peppi

Contents

Abstract

Tularemia is a zoonotic disease caused by the facultative intracellular bacterium *Francisella tularensis*. Recurrent outbreaks with hundreds of cases are reported in Finland and Sweden every few years. In other European countries the disease is quite rare, but sporadic outbreaks have been reported from various countries.

Specific risk factors associated with ulceroglandular and pneumonic tularemia were investigated in a population-based case-control study presented in this thesis (study I). In addition, the public health impact of tularemia in Finland was analyzed and information on clinical features of the disease and patient characteristics was collected. Reported mosquito bites and farming activities were independently associated with ulceroglandular tularemia, whereas exposure to hay dust was associated with pneumonic tularemia.

Spatial and temporal epidemiology of tularemia in Finland was investigated based on notifications to the National Infectious Disease Register (study IV). The prevalence of *F. tularensis* –antibodies in the adult general population was studied using serum samples from a nationwide population-based health survey (study IV). A serologic response to *F. tularensis* was found in 2% of the population.

Occurrence of *F. tularensis* in wild rodents was studied by screening a total of 547 wild small mammals from 14 locations around Finland for the presence of *F. tularensis* DNA by PCR analysis (study II). High copy numbers of *F. tularensis*-specific DNA were detected in tissue samples of only 5 field voles originating from one location.

The pathogenesis of *F. tularensis* infection in wild voles was studied in an experimental infection (study III). A rapid lethal clinical course, bacteremia and tissue necrosis were observed in the infected field voles and bank voles. The correlation between vole

population cycles and human tularemia outbreaks was assessed using surveillance data from 1995-2013 (study IV). Vole population peaks clearly preceded subsequent tularemia outbreaks one year later.

In conclusion, this thesis describes risk factors for tularemia as well as the clinical characteristics and epidemiology of the disease in Finland. Moreover, the kinetics of *F. tularensis* infection in voles and the occurrence of *F. tularensis* in wildlife in Finland are represented. Since 1995, more than 5000 cases of tularemia have been reported. About 2% of the adult population has antibodies against *F. tularensis*. Incidence and seroprevalence are highest in Northern Ostrobothnia. Ulceroglandular and pneumonic tularemia have different risk factors. In rodents, *F. tularensis* infection is rare and mostly fatal. Rodents act as amplification hosts of *F. tularensis*, and high rodent densities predict tularemia outbreaks in humans in the following year.

List of original publications

This thesis is based on the following publications:

I **Rossow H**, Ollgren J, Klemets P, Pietarinen I, Saikku J, Pekkanen E, Nikkari S, Syrjälä H, Kuusi M, Nuorti JP. Risk factors for pneumonic and ulceroglandular tularaemia in Finland: A population-based case-control study. Epidemiology and Infection 2014;142:2207-16.

II **Rossow H**, Sissonen S, Koskela KA, Kinnunen PM, Hemmilä H, Niemimaa J, Huitu O, Kuusi M, Vapalahti O, Henttonen H, Nikkari S. Detection of *Francisella tularensis* in voles in Finland. Vector Borne and Zoonotic Diseases 2014;14:193-8.

III **Rossow H**, Forbes K, Tarkka E, Kinnunen PM, Hemmilä H, Huitu O, Nikkari S, Henttonen H, Kipar A, Vapalahti O. Experimental infection of voles with *Francisella tularensis* indicates their amplification role in tularemia outbreaks**.** PLOS ONE 2014;9:e108864.

IV **Rossow H**, Ollgren J, Hytönen J, Rissanen H, Huitu O, Henttonen H, Kuusi M, Vapalahti O. Incidence and seroprevalence of tularaemia in Finland, 1995 to 2013: regional epidemics with cyclic pattern. Eurosurveillance 2015;20:21209.

The publications are referred to in the text by their Roman numerals.

Abbreviations

BSL	Biological safety level
CDC	Centers for Disease Control and Prevention, USA
CI	confidence interval
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
F. tularensis	*Francisella tularensis*
Ig	Immunoglobulin
kDa	Kilodalton
LPS	Lipopolysaccharide
LVS	Live vaccine strain
mOR	matched odds ratio
NIDR	National Infectious Disease Register, Finland
PAR	population attributable risk
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
subsp.	Subspecies
THL	National Institute for Health and Welfare, Finland
TLR	Toll-like receptor
WHO	World Health Organization
µl	Microliter

1 Introduction

The Centers for Disease Control and Prevention (CDC) consider the bacterium *Francisella tularensis* to be a Category A select agent with potential for use as a biological weapon (http://www.cdc.gov/Tularemia/). This classification is due to the very high infectivity combined with relative stability of *F tularensis* in aerosols and the high morbidity from respiratory tularemia. In addition to *F. tularensis*, only two bacteria belong to this category, namely *Yersinia pestis* and *Bacillus anthracis*, the causative agents on plague and anthrax. Both the former Soviet Union and the USA have had programs to develop weapons that would disseminate *F. tularensis* aerosols.

Tularemia, the zoonotic disease caused by *F. tularensis*, occurs naturally in many regions of the northern hemisphere. *F. tularensis* can be transmitted to humans directly from infected wildlife or more frequently through the bite of an arthropod vector. The disease is caused by two different subspecies: *F. tularensis* subsp. *tularensis* (type A), which is almost completely restricted to North America, and *F. tularensis* subsp. *holarctica* (type B), which is spread throughout the entire Holarctic region, including Finland. Type B is less virulent and usually nonlethal in humans, but it may still cause severe long-lasting disease. Tularemia often presents with non-specific symptoms, which may delay its diagnosis, especially in non-endemic areas. Finland repeatedly reports among the highest number of tularemia cases worldwide. The ecology of tularemia is complex and knowledge of its epidemiology is incomplete. The factors prompting tularemia outbreaks are still for the most part unclear. The main purpose of this thesis study was to describe the epidemiology of tularemia in Finland and to elucidate the risk factors for tularemia. The role of rodents in the ecology of tularemia and their susceptibility and response to *F. tularensis* were other targets of the study. Before this study, the occurrence of *F. tularensis* in Finnish wildlife as well as the prevalence of *F. tularensis* -antibodies in the population were unknown.

2 Review of the literature

2.1. *Francisella tularensis*

Francisella tularensis is a small (0.2–0.7μm), aerobic, Gram-negative (faintly staining), nonmotile, non-spore-forming coccobacillus belonging to the γ-subclass of *Proteobacteria* (Dennis et al. 2001, Keim et al. 2007, Sjösted 2007, Oyston 2008). Its outer membrane has an unusually high level of fatty acids with a unique profile and a structurally and antigenically unique lipopolysaccharide (LPS) that does not have general toxic or immunogenic properties (Okan et al. 2013, Jones et al. 2014). Virulent strains of *F. tularensis* have a thin capsule, which makes them resistant to the killing activity of serum complement (Clay et al. 2008, Jones et al. 2014). The size of the *F. tularensis* genome is about 1.9 Mb (Larsson et al. 2005) and it contains a pathogenicity island (FPI, required for phagosomal escape) that includes 19 genes essential for virulence (Nano et al. 2004). The taxonomic position of *F. tularensis* has repeatedly been changed. Originally, it was classified in the genus *Bacterium*, later in the genus *Pasteurella*, and it was subsequently placed in the genus *Brucella* (Ellis et al. 2002, Oyston et al. 2004). Finally, in 1947, it was assigned to the new genus *Francisella*. Based on modern 16SrRNA sequence comparisons, the most closely related but still distant human pathogens are *Coxiella burnetii* and *Legionella pneumophila* (Larsson et al. 2005) (Figure 1). *Francisella tularensis* was first discovered in Tulare County, California, USA, in 1911 from ground squirrels found dying of a plague-like illness (Oyston 2008).

Four subspecies of *Francisella tularensis* with different geographical distributions and virulence have been identified. The disease tularemia is caused by two of the subspecies: *F. tularensis* subsp. *tularensis* (type A), which occurs mainly in North America, and subsp. *holarctica* (type B), which is endemic in many countries of the Northern

Hemisphere, including Finland (Ellis et al. 2002, Johansson et al. 2004, Sjöstedt 2007). *Francisella tularensis* subsp. *mediasiatica* is rare and has been found only in Central Asia; *F. tularensis* subsp. *novicida* occurs in North America and Australia (Oyston et al. 2004, Matayas et al. 2007). Other species in the genus *Francisella* (Figure 2) are mainly water-related and have not been associated with tularemia-like illness. In addition to water-related species, *Francisella* –like bacteria have also been found to reside in ticks. These *Francisella*-like-endosymbionts are nonpathogenic and probably not even transmitted to mammals by tick bite (Keim et al 2007). The subspecies *holarctica* (former *palaearctica*) is further separated into three biovars: biovar I (erythromycin sensitive), biovar II (erythromycin resistant), and Japonica (Kudelina et al. 1980, Ellis et al. 2002). The subspecies *tularensis* has been divided into three distinct genotypes: A1a, A1b, and A2 (Kiersten et al. 2009). The subtype A1b is considered the most virulent.

Francisella tularensis is a facultative intracellular pathogen that infects multiple cell types, including phagocytes as well as epithelial and endothelial cells, but its primary target cell is the macrophage (Sjöstedt 2007, Oyston 2008). It enters macrophages with a unique system of pseudopod loops (Clemens et al. 2005), replicates in the cytoplasm, and is released by the induction of apoptosis (Oyston 2008). Unlike other Gram-negative bacteria, the LPS of *F. tularensis* does not act as a typical endotoxin, nor does *F. tularensis* produce any other toxins (Ellis et al. 2002, Oyston et al. 2004). The LPS of *F. tularensis* does not bind to host molecules such as LPS-binding protein and TLR4 to trigger a proinflammatory response (Barker et al. 2006). Instead, *F. tularensis* is even capable of downregulating proinflammatory cytokines (Telepnev et al. 2003). The virulence of *F. tularensis* is mostly based on its ability to evade host immune responses and to enter, persist, and replicate within macrophages (Steiner et al. 2014). When grown in cysteine-supplemented medium, *F. tularensis* produces hydrogen sulfide. It is oxidase negative and weakly catalase positive (Sjöstedt 2005). The subspecies *tularensis* ferments glycerol and maltose, but not sucrose, whereas the subspecies *holarctica* ferments maltose but not sucrose or glycerol (Foley et al. 2010). Repeated transfers on media may reduce virulence.

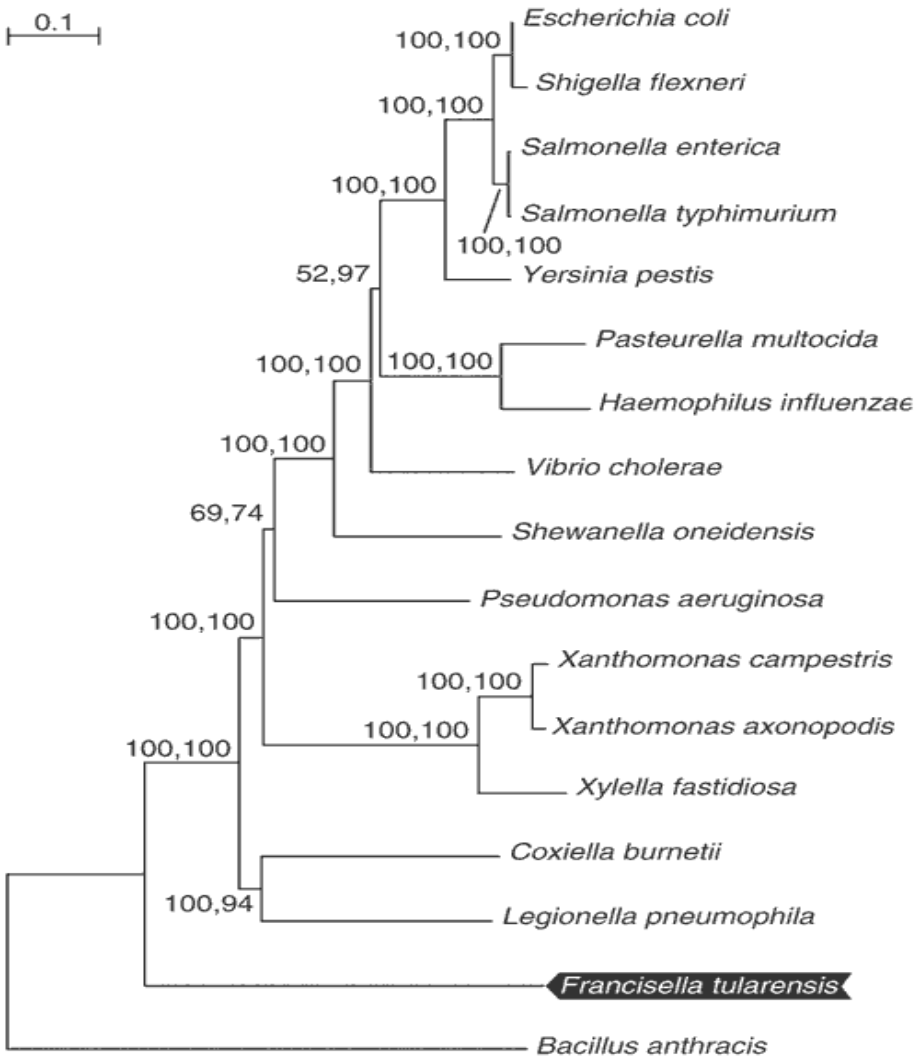

Figure 1. *Phylogenetic relationship of 16* ϒ*-proteobacterial species inferred from a concatenated alignment of the proteins encoded by dnaA, ftsA, mfd, mraY, murB, murC, parC, recA, recG, and rpoC.* Bacillus anthracis *was used as the outgroup. The topology, branch lengths, and bootstrap support are according to the reconstruction with the neighbor-joining method. Values at nodes are bootstrap support values for the neighbor-joining and maximum parsimony methods (in that order). Reprinted from Larsson et al. 2005 with permission.*

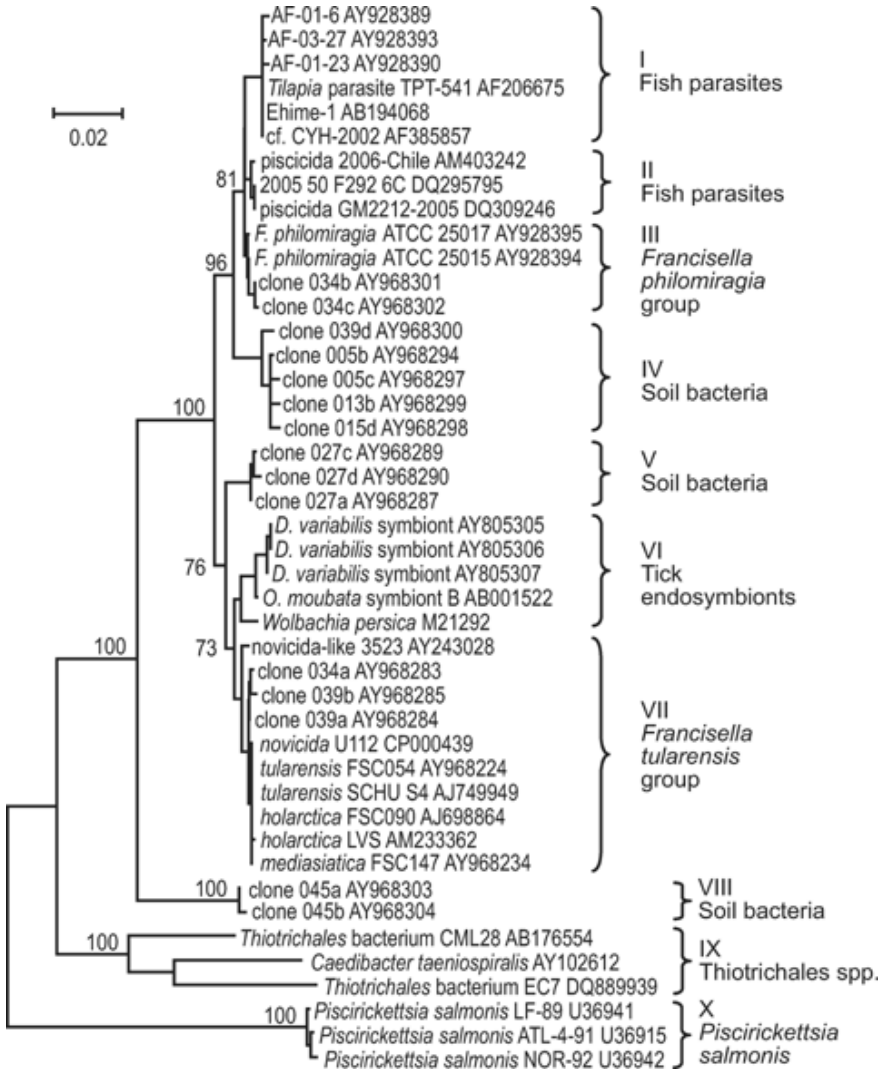

Figure 2. *Phylogeny of* Francisella *and representative relatives based upon 1070 bp of the 16S RNA gene sequences. The scale bar indicates the genetic distance of 0.02 nucleotide changes per site. Reprinted from Keim et al. 2007 with permission.*

2.2. Ecology and epidemiology of tularemia

2.2.1. Life cycle of F. tularensis *in nature*

Tularemia is a zoonotic infection that naturally occurs in the northern hemisphere. *Francisella tularensis* is widespread and has an extensive host range; it can infect several wildlife species, domestic animals, and humans (Dennis et al. 2001). Outbreaks, however, are rare in most areas. *Francisella tularensis* does not produce spores, but it can survive in water, soil, straw, and animal carcasses for several months (Pomanskala 1956, Gilbert et al. 2012). Survival is longest under winter conditions (Pomanskala 1956). It has been shown that *F. tularensis* multiplies in amoebae at the same level as in macrophages (Abd et al. 2003). Since amoebae are commonly found in water and soil as a part of their normal biofilms, and the infection process in amoebae resembles that in macrophages, it can be assumed that amoebae serve as environmental hosts for *F. tularensis* (Abd et al. 2003, Broman et al. 2011). Furthermore, mosquito larvae feed on aquatic protozoa and hence can become infected with *F. tularensis* in their natural aquatic environment (Lundström et al. 2011). As adults, mosquitos then transmit the bacterium to susceptible hosts (Thelaus et al. 2013, Bäckman et al. 2015).

The time-dependent evolution of *F. tularensis* is low. This bacterium persists in the environment in highly localized subpopulations in an inactive stage over years (Svensson et al. 2009, Johansson et al. 2014). The genetic diversity within the subspecies *holarctica* however, is broad, and multiple genetic clades are found in isolates obtained during natural tularemia outbreaks indicating environmental point sources of tularemia (Svensson et al. 2009, Karlsson et al. 2013, Johansson et al. 2014, Sissonen et al. 2015). The distribution of tularaemia caused *by F. tularensis* subsp. *holarctica* has been associated with closeness to water (and hence mosquito breeding sites), and recently, a positive correlation between the incidence of tularemia and the proportion of a municipality area covered by inland water has been found in Sweden (Desvars et al. 2015). It is suggested that aquatic protozoa enable *F. tularensis* to persist in natural waters at the specific locations where outbreaks occur and outbreaks are prompted by ecological or other

environmental factors like weather conditions favorable for mosquitos (Ryden et al. 2012, Bäckman et al. 2015, Sissonen et al. 2015).

2.2.2. Francisella tularensis *in wildlife*

Among wildlife species, lagomorphs and rodents are generally the most susceptible to this disease (Mörner 1992). Clinical signs of tularemia in wildlife are not well documented; investigations have only been conducted on dead animals (Hestvik et al. 2015). *Francisella tularensis* infection in lagomorphs and rodents is probably usually acute and fatal (Mörner 1992, Mörner et al 1988). In post-mortem examination, multifocal necrosis in the liver, spleen, and bone marrow are typically found (Mörner et al. 1988, Wobeser et al. 2007, Nelson et al. 2014), but chronic lesions such as granulomas have also been found in infected hares (Gyuranecz et al. 2010). Since 2000, the Finnish Food Safety Authority (Evira) has screened 100–250 hares and brown hares, and a few other animal species yearly for tularemia. The proportion of tularemia-positive samples has been 0–25%, depending on the year. All the animals tested by Evira have been found dead in nature.

Serological studies on wildlife populations in Europe have demonstrated that exposure to *F. tularensis* is relatively common. Antibodies against *F. tularensis* have especially been detected in wild carnivores (Kuehn et al. 2012) and beavers (Mörner et al. 1983), but not in other rodents or lagomorphs (Mörner et al. 1988, Kaysser et al. 2008, Gyuranecz et al. 2010). This supports the hypothesis that these animal species are highly susceptible to tularemia. Foxes and raccoon dogs have been suggested to be proper biological indicator species for the presence of *F. tularensis* in the wild (Kuehn et al. 2012).

2.2.3. Tularemia in domestic animals

Only a few reports on tularemia in domestic animals can be found in the literature. Infection with *F. tularensis* is probably relatively common in endemic areas, but disease outbreaks in domestic animals are rare (Nordstoga et al. 2014). Most domestic animals are

considered relatively resistant to infection. Of the domestic species, cats, rabbits, and sheep are most susceptible to tularemia. Clinical manifestations of the disease in animals resemble those in humans (Foley et al. 2010, Nordstoga et al. 2014). Feline tularemia is most commonly reported. Apparently, cats acquire the disease by hunting infected rodents (Foley et al. 2010). The most common signs of tularemia in cats are anorexia, lethargy, fever, and mandibular lymphadenopathy (Woods et al. 1998, Foley et al. 2010, Carvalho et al. 2014). Tularemia should be considered a differential diagnosis of unexplained febrile illnesses in outdoor cats living in areas where the disease is endemic (Magnarelli et al. 2007). Infection with subsp. *tularensis* can be lethal if untreated (Baldwin et al. 1991, Berman-Booty et al. 2010).

In post-mortem examination, multifocal necrosis in the spleen, liver, and lymph nodes are the most typical findings (Baldwin et al. 1991, Berman-Booty et al. 2010). Dogs are considered less susceptible to tularemia, and signs of the disease in them are rarely reported (Foley et al. 2010, Carvalho et al. 2014). Only one report of canine tularemia has been published from Europe (Nordstoga et al 2014), and only sporadic cases from North America (Gustafson et al. 1996, Meinkoth et al. 2004). Tularemia should be considered a differential diagnosis in hunting dogs with signs of fever, lethargy, anorexia, and pain. Symptoms in dogs are unspecific and self-limiting, and infection is probably often overlooked (Nordstoga et al. 2014).

2.2.4. Epidemiology of human tularemia

The epidemiology of tularemia varies worldwide and the incidence from many countries is not known. There are differences in the epidemiology of tularemia between the US and Europe, but also between different European countries. Tularemia has been reported from many countries of the Northern Hemisphere (Figure 3a & 3b), most frequently Finland and Sweden where it occurs endemically (Tärnvik et al. 2004, Splettstoesser et al. 2009). Recent outbreaks have also been reported from Kosovo (1999–2000), Massachusetts, USA (2000), France (2004), Turkey (2004–2005), Germany (2005 and 2012), Spain (2007), and Norway (2011) (Table 1). Both German outbreaks occurred among

participants in a hare hunt (Hauri et al. 2010, Otto et al. 2015), whereas the outbreaks in Kosovo, Turkey, and Norway were linked to rodents and contaminated drinking water (Reintjes et al. 2002, Grunow et al. 2012, Wilke et al. 2009, Larssen et al. 2011). In Massachusetts and Spain, landscaping was identified as a risk factor for exposure (Feldman et al. 2001, Feldman et al. 2003, Allue et al. 2008). In Italy, most cases of tularemia occur in winter and spring, and contaminated water is thought to be the main source of infection (D'Alessandro et al. 2014). Contaminated drinking water was also found to be the source of infection in the Republic of Georgia in 2006 (Chitadze et al. 2009), and a waterborne outbreak associated with crayfish fishing was reported from Spain in 1998 (Anda et al. 2001).

Although *F. tularensis* has a broad geographical distribution (Figure 3) and a wide range of animal hosts, such as rabbits, hares, voles, and other rodents, tularemia outbreaks are rare and occur for unknown reasons locally in geographically restricted areas (Sjöstedt 2007, Svensson et al. 2009). The life cycle of *F. tularensis* in nature is not entirely known. In addition to mammals, subspecies *holarctica* has also been isolated from surface water, mud, and mosquito larvae collected in endemic areas (Broman et al. 2011, Lundström et al 2011), which supports the hypothesis that *F. tularensis* subspecies *holarctica* could persist in natural waters, possibly in aquatic amoebae. However, there is also evidence that rodents play an important role in the ecology of tularemia, since there appears to be a correlation between tularemia outbreaks and preceding peaks of vole cycles, at least in Finland, Sweden (Tärnvik et al. 1996), and Hungary (Gyuranecz et al. 2012). Furthermore, outbreak investigations suggest that high rodent population densities may initiate tularemia outbreaks in humans (Reintjes et al. 2002, Allue et al. 2007, Grunow et al. 2012). Exposure to rodents or their droppings was suspected to be the source of infection in a large tularemia outbreak in Kosovo in 1999–2000 (Reintjes et al. 2002, Grunow et al. 2012). Relatively high prevalences of *F. tularensis* infections in wild rodents have been detected at human tularemia outbreak sites in Germany (Kaysser et al. 2008), China (Zhang et al. 2006), Slovakia, and Austria (Gurycova et al. 2001). Between the epidemics, however, the pathogen is only rarely found in rodents (Vest et al. 1965, Arata et al. 1973, Broman et al. 2011, Gyuranecz et al. 2011).

The highly virulent subspecies *tularensis* is principally found in North America, where mice, rats, rabbits, hares, and squirrels may carry these bacteria and ticks are considered the most important vector transmitting tularemia among wild animal populations and from animals to humans (Matayas et al 2007, Sjöstedt 2007, Foley et al. 2010).

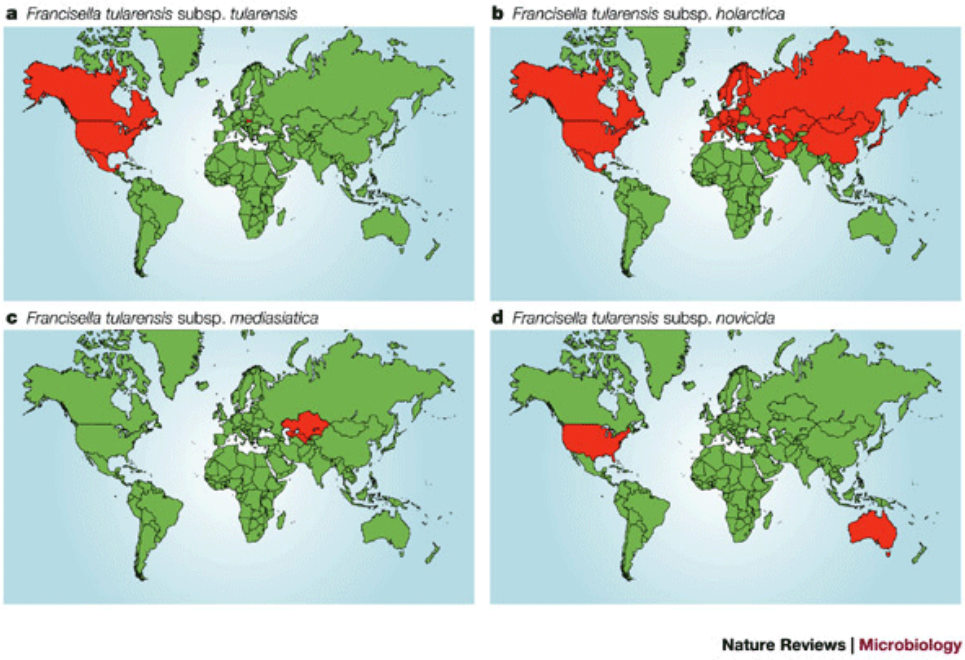

Figure 3. *Worldwide distribution map of the four main* Francisella *subspecies.* *Countries where tularemia has been reported are shown in red. Reprinted from Oyston et al. 2004 with permission.*

Table 1. *Characteristics of tularemia outbreaks in European countries 2000–2014.*

Country and outbreak year	Number of cases	Source of infection	Most common clinical forms	References
Kosovo 1999–2000	327[1]	Food and water contaminated by *F. tularensis*-infected rodents and their feces	Oropharyngeal	Reintjes et al. 2002
Sweden 2000	464[2]	Mosquito bites	Ulceroglandular	Eliasson et al. 2002
Finland 2000	926[2]	Mosquito bites and hay dust	Ulceroglandular	Rossow et al. 2014 (study I)
Turkey 2004–5	135[1]	Contaminated drinking water	Oropharyngeal	Wilke et al. 2009
France 2004	15[1]	Aerosols from a dog's fur disseminated by the dog shaking itself	Pneumonic	Siret et al. 2006
Germany 2005	11[1]	Infected hares shot at a hare hunt	Typhoidal	Hauri et al. 2010
Finland 2007	403[2]	Mosquito bites	Ulceroglandular	Jounio et al. 2010
Spain 2007	507[1]	Rodents	Typhoidal	Allue et al. 2008
Sweden 2010	484[2]	Not reported	Not reported	Johansson et al. 2014

Norway 2011	180[2]	Contaminated drinking water	Oropharyngeal	Larssen et al. 2011
Germany 2012	7[1]	Infected hares shot at a hare hunt	Typhoidal	Otto et al. 2015

[1]Number presented in the cited publication

[2]Number of cases reported from the whole country in the epidemic year

2.3. Clinical features of tularemia

2.3.1. Clinical manifestations

Tularemia is a challenging disease, not only for the patients, but also for clinicians, clinical microbiologists, and public health professionals. *F. tularensis* is widespread and highly infectious for humans, but the disease is beyond outbreaks in endemic areas uncommon, sources of exposure are various, and the clinical spectrum is broad (Weber et al. 2012). Often, the clinical symptoms are nonspecific. The clinical manifestations of *F. tularensis* infection in humans mainly depend on the route of infection and can be divided into two main groups: ulceroglandular and typhoidal (Oyston 2008) (Table 2). Humans can become infected through arthropod bites, direct contact with carcasses of infected animals, by inhalation of infective aerosols generated though agricultural, landscaping, or laboratory activities, or by ingestion of contaminated food or water (Dennis et al. 2001, Weber et al. 2012). One documented case of pneumonic tularemia with bacteremia occurred after the inhalation of contaminated brackish water in a near-drowning accident in France (Ughetto et al. 2015).

The incubation period of tularemia is about 3–5 (range 1–21) days and the disease onset is acute with non-specific influenza-like general symptoms, especially fever, chills, and headache (Tärnvik et al. 2007). Two *F. tularensis* subsp. cause clinical infections in humans: *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type

B), which is more widespread (Johansson et al. 2004). Although disease forms are similar with both, subsp. *tularensis* generally causes a more fulminate and severe disease than subsp. *holarctica* (Tärnvik et al. 2003, Johansson et al. 2014). Infection with the most virulent strains can have a case fatality rate of 30% if untreated (Dennis et al. 2001). However, tularemia responds well to antibiotic treatment.

Ulceroglandular and glandular tularemia

Ulceroglandular tularemia, the most common form, is caused by vector-borne transmission or other percutaneous inoculation of *F. tularensis* (Tärnvik et al. 2003). At the onset, patients experience systemic flu-like symptoms, including fever, chills, headache, and myalgia (Dennis et al. 2001, Nigrovic et al. 2008). At the same time, a painful maculopapular lesion, which later ulcerates, develops at the inoculation site (Ellis et al. 2002, Tärnvik et al. 2003, Nigrovic et al. 2008). This is accompanied by substantial regional lymphadenopathy (Tärnvik et al. 2003, Hanke et al. 2008, Nigrovic et al. 2008). The lymphadenopathy is actually the usual cause for medical attention, whereas the primary ulcer can remain even unnoticed (Tärnvik et al. 2003). Cases without skin or mucosal ulceration are diagnosed as glandular tularemia (Tärnvik et al. 2007). In the oropharyngeal form, the primary ulcer is in the pharynx, and in the oculoglandular form in the eye (Tärnvik et al. 2007). In all of these syndromes, regional lymph nodes are extensively enlarged. Without treatment, lymph node suppuration frequently occurs (Tärnvik et al. 2003, Tärnvik et al. 2007).

Pneumonic and typhoidal tularemia

Pneumonic tularemia may occur directly after inhalational exposure to aerosolized *F. tularensis* (primary pneumonia) or secondarily as a complication of any other disease form through the hematogenous spread of *F. tularensis* (Tärnvik et al. 2003, Tärnvik et al. 2007, Nigrovic et al. 2008). Primary pneumonia of type A tularemia presents the most serious form of *F. tularensis* infection, with a mortality rate of up to 60% if untreated (Dennis et al. 2001, Tärnvik et al. 2003). Pneumonic type A tularemia is a fulminant illness characterized by high fever, chills, dyspnea, chest pain, cough, and general weakness (Tärnvik et al. 2007, Nigrovic et al. 2008). Infection with *F. tularensis* subsp. *holarctica* through the respiratory route usually presents as an atypical pneumonia or the

typhoidal form, which is very difficult to diagnose due to nonspecific symptoms and numerous other potential causes with similar symptoms (Dahstarnd et al. 1971, Syrjälä et al. 1985, Tärnvik et al. 2003, Cunha 2006, Thomas et al. 2010, Weber et al. 2012).

Table 2. *Clinical forms of tularemia, the routes of infection and main symptoms.*

Disease form	**Route of infection**	**Main symptoms**
Ulceroglandular/ glandular	Arthropod bites, direct contact	Cutaneous ulcer, lymph node enlargement, fever, myalgia
Oculoglandular	Direct inoculation on conjunctiva, dust	Unilateral conjunctivitis, swelling of the eyelids and local lymph nodes
Oropharyngeal	Ingestion	Pharyngitis, fever, swollen cervical lymph nodes
Respiratory	Inhalation	Atypical pneumonia (fever, headache, myalgia, weight loss)
Typhoidal	Unknown, inhalation	Severe systemic symptoms without specific organ manifestations

2.3.2. Pathogenesis and pathology

Francisella tularensis, especially subsp. *tularensis*, is highly virulent and only 10–50 CFU are sufficient to cause disease in humans and other mammals. Infection can be transmitted through arthropod bites, direct contact with the carcasses of infected animals, by inhalation of infective aerosols, or by ingestion of contaminated food or water. After invasion, *F. tularensis* multiplies predominantly within macrophages. It is taken up by macrophages by inducing them to produce asymmetric pseudopod loops and then degrade the phagosomal membrane to escape to the cytoplasm (Clemens et al. 2005, Oyston 2008). In the cell, *F. tularensis* inhibits phagosome fusion and survives by suppression of acidification (Clemens et al. 2007, Sjöstedt 2007). After replication in the cytoplasm, it induces apoptosis to release large numbers of bacteria from the dying cell (Oyston 2008). Intracellularly, it disseminates first to the local lymph nodes and then via lymphatic vessels or hematogenously to multiple organs, particularly the liver, spleen, lung, and kidney (Thomas et al. 2010). The LPS of *F. tularensis* does not act as a typical endotoxin; it induces very little proinflammatory response compared to other Gram-negative bacteria (Sandström et al. 1992, Forestal et al. 2003, Barker et al. 2007, Steiner et al. 2014). *Francisella tularensis* is even capable of downregulating proinflammatory cytokines (Telepnev et al. 2003). Lesions caused by *F. tularensis* may resemble those seen in tuberculosis (Syrjälä et al. 1986), but due to faster bacterial replication, tularemia develops much more rapidly. In histopathological examination, tularemia typically presents with necrotizing granulomas found in the patient's lymph nodes and other target organs (Navarro et al. 2011, Yildirim et al. 2014, Strehl et al. 2014).

2.3.3. Laboratory diagnostics

Laboratory diagnosis of *F. tularensis* infection is mainly based on serology. Serology can be used for all clinical forms of tularemia. Antibodies against *F. tularensis* reach a detectable level 1–2 weeks after the onset of symptoms (Koskela et al. 1985, Tärnvik et al. 2007). Agglutination assays are the standard serological tests for tularemia diagnostics, but ELISA tests have also been developed for this purpose (Koskela et al. 1985, Schmitt et al. 2005, WHO 2007). The specificity of tularemia serology is high in both agglutination

assays and ELISA, and cross-reactions are rare (Tärnvik et al. 2007). The antibody levels reach their peak at 4–7 weeks after infection and stay detectable with declining titers for several years (Koskela et al. 1985). If suitable tissue specimens are available, culture or PCR can also be used for diagnostics. Culture and PCR-based methods are most useful in suspected ulceroglandular tularemia, when a wound specimen can be taken from the primary lesion (Johansson et al. 2000, Eliasson et al. 2005). PCR is more sensitive than culture for demonstration of *F. tularensis* in wound specimen (Johansson et al. 2000). Successful culture is also more dependent on selection of the right transport medium.

Culture however is rarely used in routine clinical diagnostic of tularemia because *F. tularensis* is difficult and hazardous to grow in culture (Tärnvik et al. 2007, Nigrovic et al. 2008). It is a relatively slow growing bacterium, requires a cysteine-enriched agar, and culture work must be done under BSL-3 conditions (Dennis et al. 2001, WHO 2007, Nigrovic et al. 2008). *F. tularensis* grows best at a temperature of 37 ^{o}C and a pH of 6.9 (Foley et al. 2010). Colonies appearing after 24–48 hours of incubation are small, flat, pale gray, shiny, easily emulsified, and surrounded by alpha-hemolysis (Foley et al. 2010). They reach the maximum size in 3–4 days (Sjöstedt 2005). The identity of colonies can easily be tested by slide agglutination or by direct fluorescent antibody staining (Tärnvik et al. 2007). Isolation of *F. tularensis* from blood culture is very rare, and most cases have been reported from the USA (Haristoy et al. 2003, Karagöz et al. 2012). *F. tularensis* is one of the most infectious pathogens by the airborne route and therefore relatively commonly associated with laboratory-acquired infections.

2.3.4. Treatment and prevention

Antibiotic therapy should be started as soon as tularemia is suspected. A delay in proper treatment increases the risk of a severe disease course and complications, such as lymph node suppuration (Tärnvik et al. 2007, Weber et al. 2012). For the treatment of tularemia, streptomycin has been the golden standard for decades (Tärnvik et al. 2003). Other aminoglycosides such as gentamycin are also effective against tularemia, but these are all associated with toxicity and a parenteral administration route. Doxycycline is a safer alternative, but the risk of relapse is considerable if the treatment period is less than 14

days (Tärnvik et al. 2003, Tärnvik et al. 2007). At present, ciprofloxacin and other quinolones are recommended as the first choice in treating uncomplicated tularemia (Ikäheimo et al. 2000, Tärnvik et al. 2007). The development of antibiotic resistance is not a problem with *F. tularensis*, since *Francisella* is not a member of the commensal human flora and no person-to-person transmission occurs. However, there is widespread natural resistance to erythromycin among strains of subsp. *holarctica* (Ikäheimo et al. 2000).

In case of accidental exposure (e.g. laboratory personnel), antibiotic treatment should be initiated within 24 hours (WHO 2007). It is also important to keep in mind that tularemia patients are not a source of infection for other people; direct human-to-human transmission of tularemia has never been reported. An attenuated live vaccine (LVS) was used for laboratory workers in the former USSR, US, and Japan, but it did not protect well against respiratory tularemia. Currently, no licensed vaccines against tularemia are available, but new vaccines are under development (Conlan 2011). Preventive measures against tularemia infection include protecting against mosquito bites, minimizing exposure to hay dust during landscaping activities and avoiding the handling of dead rodents and hares.

3 Aims of the thesis study

1) To determine and evaluate the risk factors of different clinical forms of human tularemia during an outbreak and to assess the public health impact of tularemia in Finland

2) To obtain information about the prevalence of *F. tularensis* -carriage in wild rodents in Finland

3) To study the pathogenesis of *F. tularensis* subsp. *holarctica* –infection in an experimental trial, and thereby evaluate the potential of field voles (*Microtus agrestis*) and bank voles (*Myodes glareolus*) to spread the bacterium

4) To describe the spatial and temporal epidemiology of human tularemia in Finland, to determine the prevalence of *F. tularensis* antibodies in the adult population, and to assess the role of vole population cycles in the temporal and spatial pattern of human tularaemia outbreaks

4 Materials and methods

Detailed descriptions of materials and methods are given in the original publications I–IV. Some methods not described in the original articles are explained here more thoroughly. For others, the reference to the original publication is given in Roman numerals.

4.1. Ethics (III & IV)

Experimental procedures and facilities were approved by the Finnish Animal Experiment Board (Permit ESAVI/6162/04.10.03/2012), which followed the Finnish legislation for animal experiments. The Health 2000 Survey was approved by the Ethical Committee for Research in Epidemiology and Public Health at the Hospital District of Helsinki and Uusimaa.

4.2. Cases and controls in the case-control study (I)

Case patients for the case-control study were directly identified through active surveillance of diagnostic laboratories, and control subjects matched for birth, sex and postal code were listed from the National Population Information System. A case patient was defined as a person with laboratory-confirmed tularemia within the study period and who lived in one of the three endemic health districts (Fig. 1 in I). Patients with laboratory-confirmed tularemia and physician-diagnosed pneumonia were classified as pneumonia cases. Patients with laboratory-confirmed tularemia who reported enlarged lymph nodes and/or skin ulcers were classified as ulceroglandular cases (I).

4.3. Animals

4.3.1. Collection of wild animals (II & III)

Small mammals were trapped from 14 locations around Finland during six years by the Natural Resources Institute, Finland. The trapped small mammals were predominantly rodents, mainly bank voles and field voles (II).

4.3.2. Animals for experimental infections (III)

Laboratory-borne adult visibly healthy field and bank voles were obtained from the Natural Resources Institute, Finland and kept in individually ventilated and HEPA-filtered isolation cages during the experimental infections (III).

4.4. Bacterial strain used in experimental infections (III)

The *F. tularensis* strain used to experimentally induce tularemia in voles had originally been isolated from a cutaneous ulcer of a 49-year-old woman and identified as subsp. *holarctica* by 16S rRNA gene sequencing. The strain was cultured and grown on chocolate agar plates at +35 °C in 5% CO_2 for five days. MacFarland 1.0 suspension was prepared in sterile isotonic saline and diluted in a ten-fold series to an estimated concentration of 1000 cfu/ml. The definite concentration in each experiment was determined by plate counting. The diluted suspension was kept on ice and used for inoculations within 1–2 h of preparation. The viable count of *F. tularensis* in the remaining dilution was comparable to that of the fresh dilution.

4.5. Human serum samples (IV)

Serum samples from a multidisciplinary epidemiologic health survey were obtained from the National Institute for Health and Welfare (THL) and stored at -70 °C. Sera from seven patients who had laboratory-confirmed tularemia between 1 and 16 years ago were used as positive control sera. Control sera were obtained from the Department of Medical Microbiology and Immunology, University of Turku (IV).

4.6. Database material (I & IV)

In Finland, laboratory-confirmed tularemia is a notifiable disease by the diagnosing laboratory. Clinical microbiology laboratories report cases to the National Infectious Disease Register (NIDR), which is maintained by THL. The notifications include information on the date and type of specimen, date of birth, gender, place of treatment, and place of residence. Diagnostic criteria for reporting include 1) the isolation of *F. tularensis* in a clinical specimen, 2) a >4-fold rise in the serum antibody titer, or a single antibody titer of >160 when using an agglutination assay, or 3) the presence of specific IgM and IgG antibodies in the serum when an ELISA-based assay is used.

4.7. DNA extraction and PCR analyses (II & III)

DNA was extracted from tissue samples of the wild-caught and experimentally infected animals and from the excreta of the experimentally infected animals with commercial kits. For tissue samples, the Wizard Genomic DNA Purification Kit (Promega, Madison, USA), following the protocol for animal tissue, was used. The QIAamp DNA Stool kit (Qiagen, Hilden, Germany) was chosen for fecal samples and the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), using the protocol for purification of cellular, bacterial or viral DNA from urine, for urine samples.

The DNA samples were analyzed with a semi-quantitative real-time PCR assay (qPCR) targeting the 23kDa gene of *F. tularensis*. All PCRs were run in duplicate with an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). DNA from tissue samples was analyzed using 1:100 dilutions, and for urine and fecal samples, three 10-fold (undiluted, 1:10, 1:100) dilutions were examined.

A part of the bacterial 23S ribosomal RNA gene from three initially *F. tularensis* PCR-positive samples was amplified and sequenced with an automated ABI 3130XL DNA sequencer (ABI) using the BigDye chemistry (ABI) and the Sequencer 4.0 program (Gene Codes Corporation, Ann Arbor, MI USA) (II).

4.8. Histology and immunohistochemical staining (III)

Tissue sections were collected immediately after euthanasia, fixed in 10% buffered formalin, routinely paraffin wax embedded, cut into 3–5 µm sections, and subjected to staining with hematoxylin-eosin (HE) or used for immunohistology (IH). IH was performed using a mouse monoclonal antibody against *F. tularensis* LPS (clone T14; Meridian Life Sciences, Memphis, USA) and the horseradish peroxidase method (Envision; Dako, Glostrup, Denmark) with diaminobenzidine as the chromogen, after antigen retrieval with citrate buffer (pH 6.0) microwave pretreatment (III).

4.9. Serologic testing (IV)

4.9.1. Enzyme-linked immunosorbent assay

All serum samples were screened for *F. tularensis* antibodies with an enzyme-linked immunosorbent assay (ELISA) as previously described by Koskela et al. (1985). Whole bacterium lysate prepared from *F. tularensis* live vaccine strain (LVS) was used as the antigen in the ELISA assay. Microtiter plates (Thermo Fisher Scientific, Vantaa, Finland)

were coated with *F. tularensis* lysate (10 µg/ml) in phosphate buffered saline (PBS) at 37 °C overnight, and washed twice with washing solution (H_2O, 0.05% Tween 20, Merck, Hohenbrunn, Germany). The wells were saturated with 1% normal sheep serum (Bio Karjalohja Oy, Karjalohja, Finland) in PBS at 37 °C for two hours, and washed twice with washing solution. Serum samples were diluted 1:100 in 1% normal sheep serum in PBS. The wells were incubated with the diluted samples at 37 °C for 90 minutes, and washed three times with the washing solution. The wells were incubated with rabbit anti-human IgG antibody (1:3000; Dako, Glostrup, Denmark) at 37 °C for two hours, and washed three times with the washing solution. After washings, p-nitrophenyl phosphate (1 mg/ml; Reagena, Toivala, Finland) was added for 30 min before the reaction was stopped with 1 M NaOH, and absorbances (OD405) were measured with the BEP III system (Siemens Healthcare Diagnostics Products GmbH, Erlangen, Germany). Results are expressed as arbitrary enzyme-immunosorbent units (EIU) on the scale from 0 to 100 units.

4.9.2. Western blotting

Western blotting was conducted as earlier described by Schmitt et al. (2005). A formalin-inactivated LVS suspension in LSB (125mM TRIS pH 6.8, 4% SDS, 20% Glycerol, 10% 2-ME, 0.02% Bromophenol Blue) was boiled for 15 minutes, centrifuged with an Eppendorf tabletop centrifuge 20 minutes at 10 000 rpm, and electrophoresed at 130 V for about 1.5 hours using a 4–20% separating gel (Mini-Protean TGX 4–20%, BIO-RAD, Berkeley, California, USA). Bacterial antigens were transferred onto a nitrocellulose membrane at 200 mA for approximately one hour. Unspecific binding sites on the membrane were blocked with 4% skimmed milk in PBS overnight at 4 °C. The dried and cut membrane strips were incubated with sera diluted 1:100 in 4% skimmed milk in PBS for one hour. After washing the membranes three times with washing solution (TEN +0.05% Tween20), the strips were incubated with a monovalent goat anti-human IgG peroxidase conjugated immunoglobulin (Li-cor, Odyssey) diluted 1:10 000 in blocking buffer at room temperature for one hour. Following another three rinses with washing solution, the membranes were scanned with an Odyssey imaging system. Positive sera showed a typical LPS band pattern at a dilution of 1:100. Samples were screened from the highest EIU (83.7) in ELISA down to EIU 26.8 (including 55 serosurvey samples + 7

positive control samples). The last positive sample in WB had an ELISA EIU value of 28.5. The following 10 samples were WB negative and screening was stopped due to the low EIU values of the remaining samples.

4.10. Statistical analyses and study questionnaires (I & IV)

In study I, conditional logistic regression was used to calculate matched odds ratios (mORs) and their 95% confidence intervals (CIs) for three main outcomes: 1) all tularemia cases, 2) ulceroglandular cases and 3) pneumonia cases. A P-value of ≤ 0.2 was used as the screening criterion for the selection of variables for multivariate analyses. Due to the survey design, the variables had varying proportions of missing responses. In Model 1, missing data were assumed to be missing at random and only subjects with complete information on variables in the final model are included. Model 2 was a Bayesian full likelihood analysis in which missing data were taken into account and became a multidimensional additional parameter. To include a variable in the final model, the required posterior probability was >50% in Gibbs' variable selection. The Kolmogorov-Smirnov test was used to compare distributions and the chi-squared test to assess the associations between variables among case patients (independent observations). Adjusted population-attributable risks (PARs) and their 95% confidence intervals for each independent risk factor were calculated on the basis of mORs from Model 2. Analyses were performed using Stata version 9.2 (Stata Corporation, USA) and Winbugs 1.4.3 (MRC-bsu.Cambridge and Imperial College) (I).

In study IV, statistical analyses were performed using IBM SPSS Statistics version 22. The original study questionnaire from the Health 2000 survey is available at: http://www.terveys2000.fi/data.html. Univariate chi-squared tests were used to test the independence of the categorical variables. Logistic regression was used to model the relationship between seroprevalence rates and explanatory variables. The notified/total tularemia incidence ratio was calculated by assuming a stationary population at risk and prevalence pool. Then, the prevalence odds of tularemia, $p/(1-p) = I*D$, where I is the

"total" incidence (notified + unnotified) and the D is mean residual life time of the mean age of acquiring tularemia.

Poisson regression was used to analyze the association between tularemia outbreaks and the phase of the vole cycle. Calculations were performed according to the hospital district. The effect of vole cyclic phase factor was assumed to be year independent. Possible overdispersion was corrected by the Pearson chi-squared scale parameter method and possible autocorrelations of Pearson residuals from the model were checked by autocorrelation plots (IV).

4.11. Study design in experimental infections (III)

The experimental infections were conducted at the biosafety level 3 laboratory of the Faculty of Veterinary Medicine, University of Helsinki, Finland. In the pilot study, field voles were infected with four dose/route combinations: either 120 or 1200 cfu of *F. tularensis* subsp. *holarctica*, and either an intranasal or a subcutaneous delivery route. The animals were checked twice a day and euthanized immediately if they exhibited signs of illness. A full post-mortem examination was performed and tissue specimens collected for histological and PCR analyses. In the main experiment, 12 field voles and 12 bank voles were injected subcutaneously with a suspension containing 70 cfu of *F. tularensis* subsp. *holarctica*. Three animals of each species served as non-infected controls. Three infected voles of each species were euthanized on days one and three post-infection. The remaining voles were euthanized if symptomatic. At necropsies, samples of urine, feces, spleen, and kidney were aseptically collected and frozen at -80 °C for PCR analysis. Tissue specimens from lungs, liver, spleen, bone marrow, kidneys, stomach, duodenum, jejunum, colon, and the inoculation site were fixed in 10% buffered formalin for histological and immunohistological assessment (III).

5 Results and discussion

5.1. Epidemiology of tularemia in Finland

5.1.1. Descriptive epidemiology

Up to 1995, over 5000 notifications of laboratory-confirmed tularemia cases have been reported to the NIDR in Finland. The annual number of notified cases has ranged from 14 to 926; epidemics have typically occurred in different districts every 2–4 years (Table 3). The average annual incidence on the basis of these notified cases was 5.1/100 000 population. During the largest epidemic on record in 2000, the tularemia incidence in Finland reached 18/100 000 population. Incidence rates and case numbers were typically highest in the healthcare districts of Northern and Southern Ostrobothnia and Central Finland (Figures 4 & 5). The mean age of patients was 45 years (range 0–93 years) (Figure 6), and 55% of the cases were males. Notifications were strongly seasonal, with the majority of cases diagnosed during the summer and early autumn. The cumulative number of cases per month for 1995–2014 showed a peak in August-September (Figure 7). Almost 80% of the cases were notified during August or September. In epidemic years, 3–5 localized outbreaks occurred concomitantly at different sites. The municipalities with the highest cumulative case numbers were Oulu, Haukipudas, Pori, Seinäjoki, and Kiiminki. The diagnosis of tularemia was confirmed in about 96% of the cases by serology, in more than 3% of the cases by culture, and in the remaining cases by PCR.

Most European countries report only sporadic cases of tularemia, and larger epidemics rarely occur. Major outbreaks with several hundreds of cases regularly occur in Finland and Sweden, and less frequently in Russia. In France, Germany, Italy, and Poland, for example, the average tularemia incidence is about 100 times lower than in Finland or Sweden (Splettstoesser et al. 2009). Approximately 60–80% of all tularemia cases in

Europe are reported from Finland and Sweden. Other countries with notable case numbers are Norway, Russia, the Czech Republic, Hungary, and Slovakia (Tärnvik et al. 2004, Splettstoesser et al. 2009). In all countries, tularemia outbreaks are typically localized and most of the cases occur within small areas. This type of spatial pattern indicates environmental point sources of infection. The local ecological factors crucial for the persistence of *F. tularensis* in the environment, however, are still not very well known. The age and sex distribution and seasonality of tularemia in Finland, as well as the disease outcomes, are compatible with epidemiological data from Sweden (Desvars et al. 2015). Little long-term epidemiological information is available from other countries, but based on outbreak investigations, tularemia in Germany and France is mainly associated with hare hunting (Siret et al. 2006, Hauri et al. 2010, Maurin et al. 2011, Otto et al. 2015). Outbreaks in Norway, Turkey, and Kosovo have been linked to drinking water contaminated by rodents (Reintjes et al. 2002, Wilke et al. 2009, Larssen et al. 2011). Mosquito-borne transmission dominates in both Finland and Sweden (I, Eliasson et al. 2002).

Table 3. *Number of confirmed tularemia cases by healthcare district reported to the National Infectious Disease Registry 1995–2014.*

Health Care District	***1995***	***1996***	***1997***	***1998***	***1999***	***2000***	***2001***	***2002***	***2003***	***2004***	***2005***	***2006***	***2007***	***2008***	***2009***	***2010***	***2011***	***2012***	***2013***	***2014***	***Total***
Åland	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
Southern Karelia	0	0	1	0	0	0	0	1	3	0	1	5	1	0	4	1	1	0	0	0	18
Southern Ostrobothnia	32	23	4	1	2	167	6	0	75	4	15	45	5	26	136	15	11	65	4	2	638
Southern Savo	6	22	2	0	0	2	1	3	1	1	1	5	1	0	1	1	0	0	0	0	47
Helsinki and Uusimaa	51	14	4	3	4	47	1	13	63	25	5	59	17	4	41	10	5	8	2	1	377
Eastern Savo	0	3	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	1	0	0	7
Kainuu	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	5
Kanta-Häme	10	1	0	1	0	8	0	1	5	0	1	2	1	1	3	2	2	0	0	0	38
Central Ostrobothnia	9	4	4	6	3	38	0	1	16	0	0	2	4	24	17	1	4	33	2	0	168
Central Finland	181	57	6	22	14	134	2	43	222	35	13	70	20	15	1	14	1	40	4	1	895
Kymenlaakso	62	19	2	25	0	11	9	16	8	23	4	8	1	1	13	1	0	1	0	2	206
Lapland	0	3	1	0	0	2	0	0	3	0	0	1	2	0	1	0	2	2	0	0	17
Western Ostrobothnia	0	2	1	0	1	3	0	0	3	0	0	0	7	1	1	0	1	0	0	0	20
Pirkanmaa	54	70	5	40	1	17	1	4	71	13	4	83	13	2	20	2	2	6	0	0	408
Northern Karelia	4	1	0	1	0	1	0	0	1	3	3	0	0	0	1	1	0	0	0	0	16
Northern Ostrobothnia	30	141	68	7	57	411	4	16	161	29	10	31	313	21	83	26	35	37	1	0	1481
Northern Savo	1	23	1	1	0	9	1	2	15	1	2	6	5	1	7	3	3	2	0	0	83
Päijänne Tavastia	2	2	0	1	0	1	1	1	5	11	1	9	1	0	3	0	0	1	0	0	39
Satakunta	12	11	9	8	2	45	3	5	133	2	1	124	7	5	48	7	2	1	0	2	427
Vaasa	5	1	0	0	0	22	0	0	21	0	0	11	4	13	18	5	6	33	1	0	140
Southwest Finland	8	0	1	1	2	7	0	0	12	4	0	12	0	2	6	2	0	2	1	1	61
Total	467	397	109	117	87	926	29	106	820	151	62	475	403	116	405	91	75	233	15	9	5093

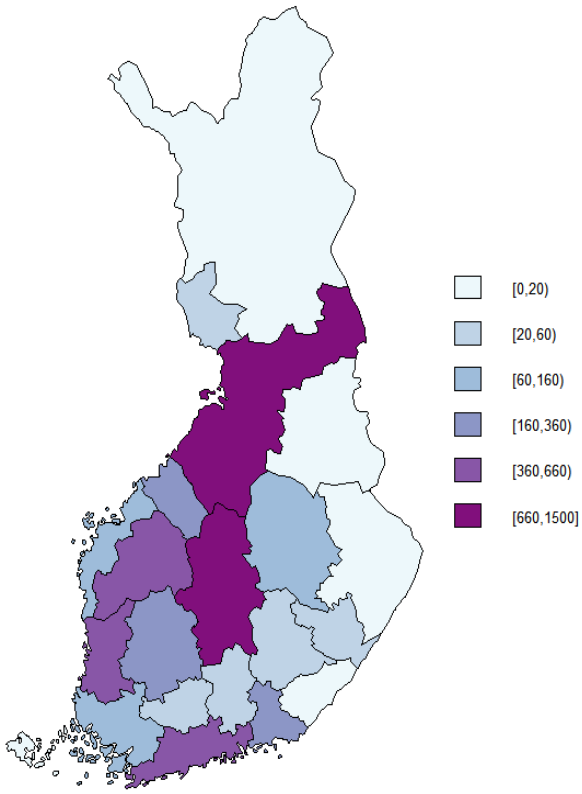

Figure 4. *Total number of laboratory-confirmed cases of* Francisella tularensis *infections according to the healthcare district reported to the National Infectious Disease Registry 1995–2013.*

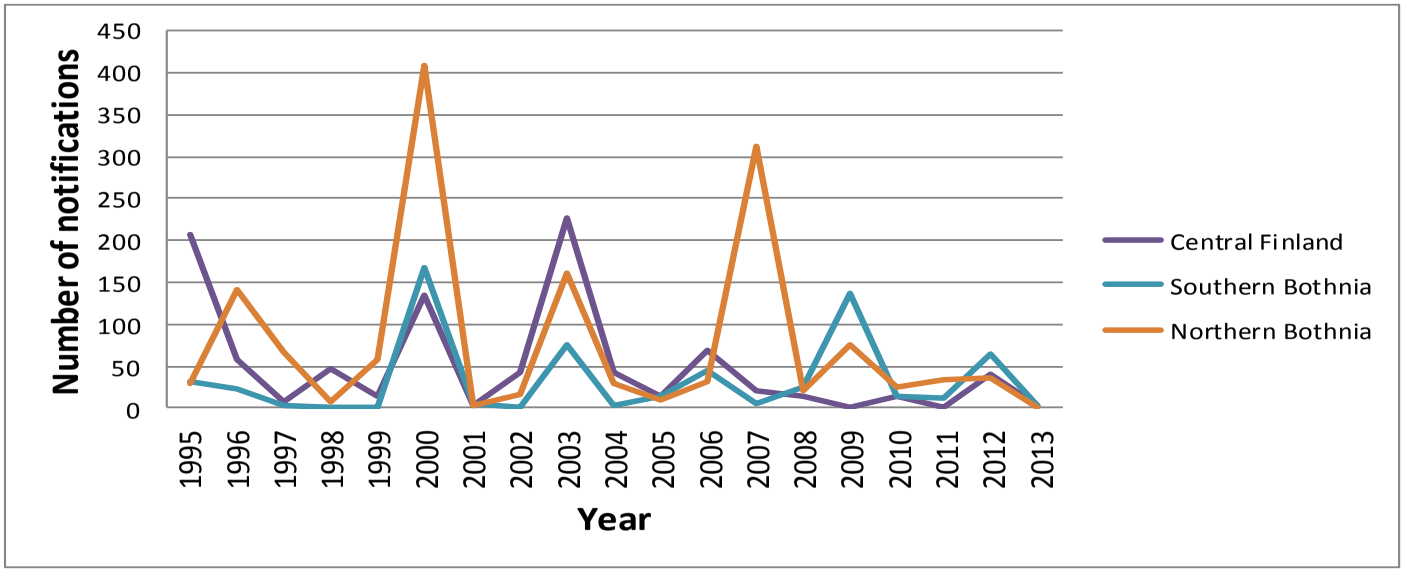

Figure 5. *Cumulative number of notified tularemia cases in three endemic healthcare districts, Finland, 1995–2013.*

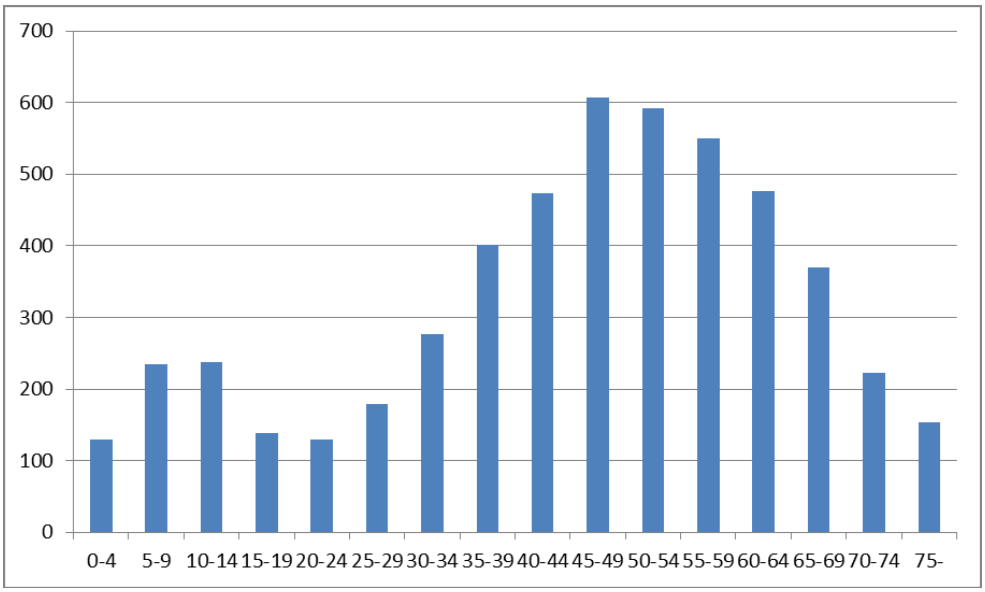

***Figure 6**. Cumulative number of notified tularemia cases according to age group, Finland 1995–2014.*

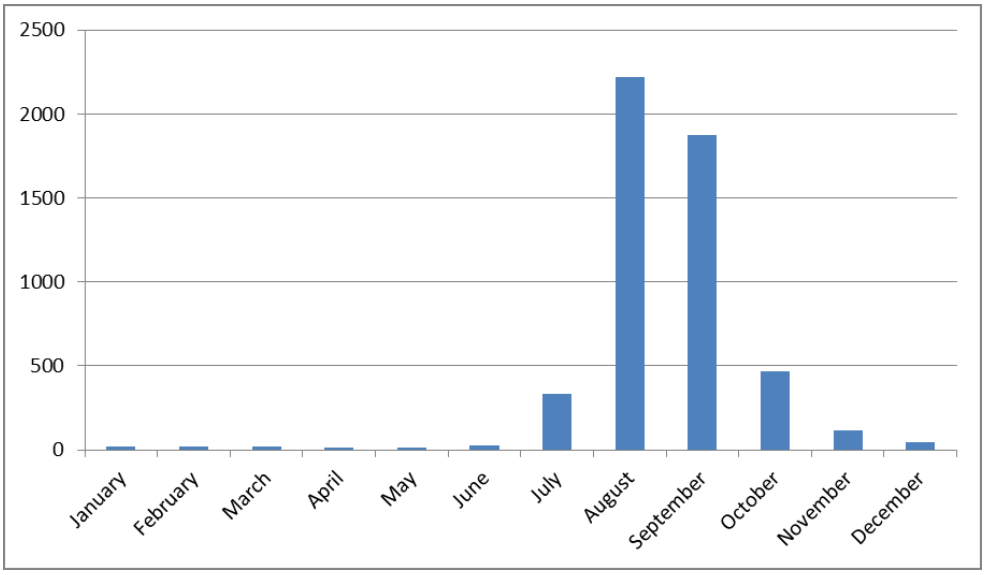

***Figure 7**. Cumulative number of tularemia cases according to month of notification, Finland, 1995–2014.*

5.1.2. Risk factors for tularemia and F. tularensis *seropositivity*

A population-based case-control study was conducted during the largest epidemic on record in 2000. Factors associated with the different clinical forms of tularemia had not been previously evaluated in controlled studies. The typical symptoms (Table 1 in I) of case patients with ulceroglandular tularemia included fever (92%), lymphadenopathy (90%), cutaneous ulcer (82%), and myalgia (78%). Case patients with pneumonic tularemia most frequently reported fever (100%), weight loss (67%), myalgia (61%), and cough (60%). Pneumonia patents also frequently reported other symptoms such as headache and exhaustion.

In general, mosquito bites, farming activities, and handling dead animals were associated with an increased tularemia risk. Risk factors for the different disease forms and patient characteristics, however, were various. In case patients with ulceroglandular disease, both genders were represented equally and age ranged from 4 months to 84 years (mean 42 years), whereas all pneumonia case patients were adults (their age ranged from 22 to 71 years, mean 52 years), and 70% of them were males (Table 1 & Figure 1 in I).

Of the case patients with ulceroglandular tularemia, 98% reported arthropod bites within the two weeks before illness compared with 70% of controls. Of the specific arthropods, however, only mosquito bites and horse fly bites were significantly associated with disease. Of the case patients, 93% reported mosquito bites compared with 69% of the controls; horse fly bites were reported by 14% of cases and 5% of controls. In univariate analysis, other factors associated with ulceroglandular tularemia were outdoor activities, forestry work, farming activities, and handling dead animals (Table 2 in I).

Mosquito-borne transmission has been considered the major transmission route of *F. tularensis* subsp. *holarctica* in Finland and Sweden (I, Lundström et al. 2011, Rydén et al. 2012, Thelaus et al. 2013, Bäckman et al. 2015), and some of the largest epidemics have reported a link to mosquito bites (I, Eliasson et al. 2002). Mosquitos may already acquire the bacterium as larvae from their aquatic habitat (Lundström et al. 2011, Rydén et al. 2012, Thelaus et al. 2013, Bäckman et al. 2015), and the persistence of the bacterium in natural waters of endemic areas, possibly in

association with amoebae, could explain the uneven geographical distribution of tularemia (Abd et al. 2003, Broman et al. 2011, Thelaus et al. 2013, Bäckman et al. 2015). The study participants were asked about being bitten by the different arthropods present in Finland. Mosquito and horse fly bites were associated with tularemia infection in univariate analysis, but after adjustment for other variables in multivariate analysis, only mosquito bites remained statistically significant. On the other hand, few subjects were exposed to arthropods other than mosquitoes, reducing the statistical power to calculate these associations. Study participants may also have had difficulties in identifying the various arthropod species or even noticing being bitten by an arthropod, possibly resulting in misclassification of exposure.

Exposure to hay dust was the only exposure significantly associated with pneumonic tularemia (Table 3 in I). In the 2 weeks prior to illness onset, 55% of the pneumonia patients were exposed to hay, compared with 15% of controls. Some farming activities, such as harvesting hay, have the potential of aerosolizing environmental pathogens, and several airborne tularemia outbreaks have been linked to farm work (Dahlstarnd et al. 1971, Syrjälä et al. 1985, Allue et al. 2008). However, this was the first study quantifying the association in a controlled design. Most patients with pneumonic tularemia were men, supporting the link with a farming occupation. Some small outbreaks of airborne tularemia have been associated with other outdoor activities such as lawn moving (McCarthy et al. 1990, Feldman et al. 2001) or hunting (Siret et al. 2006, Hauri et al. 2010). In conclusion, respiratory tularemia has to be considered an occupational hazard for farmers in endemic areas. Due to the nonspecific nature of symptoms and clinical findings, the diagnosis of respiratory tularemia remains challenging (Rossow et al. 2011).

In the serosurvey study, 1045 sera from randomly selected persons, representative for the Finnish population in each region, were screened with an enzyme-linked immunosorbent assay (ELISA) for the presence of IgG antibodies against *F. tularensis,* and positive results were further confirmed by immunoblotting. A serologic response to *F. tularensis* was found in 2% (95% confidence interval: 1.1–3.5%) of the population (IV). No significant difference in the age or sex ratio was found between seropositive and seronegative groups. The mean age of seropositive persons was 55 years and 50% were males. No association was found between tularemia antibodies and long-term health problems. No single background factor was significantly associated with seropositivity. Geographically, the seroprevalence was highest in Northern Ostrobothnia (Figure 5 in IV). Based

on the number of notifications to the NIDR and the seroprevalence we found in our study, approximately 9.5% of all *F. tularensis* infections are notified.

5.2. *Francisella tularensis* infection in rodents

5.2.1. Pathogenesis and pathologic features

Host responses and the pathologic features of experimentally induced *F. tularensis* infection in rodents have previously been described in a small number of studies (Conlan et al. 2003, Conlan et al. 2008, Bandouchova et al. 2009). In all studies, the course of disease has been fatal regardless of the dose or route. Previous studies, however, have mainly been conducted on laboratory mice and may not apply to wild rodents. Tularemia outbreaks in humans have been linked to high rodent densities (Allue et al. 2008, Reintjes et al. 2002, Grunow et al. 2012, Wilke et al. 2009, Larssen et al. 2011), and exposure to rodents or their droppings was suspected as the infection source in a large outbreak in Kosovo. Even chronic shedding of *F. tularensis* has been suggested when rodents are infected orally (Bell et al. 1975). Nevertheless, the exact role of rodents in bacterial maintenance, and the nature of their association with human disease have remained unclear.

As a part of this thesis, the susceptibility and response of field voles (*Microtus agrestis*) and bank voles (*Myodes glareolus*) to *F. tularensis* subsp. *holarctica* was studied in an experimental trial (III). In a pilot study, voles were infected intranasally and subcutaneously with *F. tularensis* subsp. *holarctica*. Infected animals showed symptoms of severe acute illness on day 5 post-infection; approximately 65% of the animals were found dead and the remaining ones had to be euthanized due to endpoint symptoms (e.g. lethargy, anorexia, hunching). The pathologic, histopathologic, and immunohistochemical examination revealed findings characteristic for septicemia in all cases. Gross pathologic lesions were generally lacking, apart from splenomegalia in some individuals. Analyses of the pathogenesis showed that bacteria are taken up locally by macrophages and neutrophils and then disseminate to several organs by lymphohematogenous spread. Consequently, they can be found both within monocytes and cell free in vessels of almost all organs, leading to necrosis of infected cells (III). Thus, extensive necrosis, particularly in the lymphatic tissues (i.e. spleen and lymph nodes), was the most significant finding in *F. tularensis*-infected voles (Figure 2

in III). Interestingly, this is not associated with an evident inflammatory response. Bacterial aggregates were found in several organs, including within the glomerular tufts of the kidneys and in the intestinal mucosa (Figure 4 in III), which indicates that *F. tularensis* is excreted in urine and feces.

Study III presents an experimental model that mimics natural *F. tularensis* subsp. *holarctica* infection of wild voles, and demonstrates that both field voles and bank voles are highly susceptible to the bacterium. Infected animals died with bacteremia, following a rapid clinical course and generally with very high bacterial loads in organs. PCR analysis also proved that infected voles excrete *F. tularensis* in their urine and feces around the time of death (Figure 3 in III). However, it cannot be ruled out that some individuals might have survived infection if the voles were exposed to *F. tularensis* orally. In the surviving individuals, shedding of bacteria might have lasted longer, possibly leading to more widespread contamination of the environment.

5.2.2. Occurrence of Francisella tularensis *in wild-caught voles and other rodents*

The occurrence of *F. tularensis* in wildlife had not been previously studied in Finland, and data from only a few other European counties had been published (Gurycová et al. 2001, Kaysser et al. 2008, Broman et al. 2011, Gyuranecz et al. 2011, Grunow et al. 2012). Most of the previous investigations were carried out during or shortly after an outbreak at the outbreak site, whereas as a part of this thesis the occurrence of *F. tularensis* in rodents was studied in random locations with respect to tularemia occurrence in humans (Figure 1 in II). The presence of *F. tularensis* among wild-caught small mammals was examined with PCR screening of 547 individuals (II). The majority of the trapped animals were field voles (N = 237) and bank voles (N = 182), but lemmings, water voles, and a few other species were also included (Table 1 in II). Large amounts of *F. tularensis* DNA were detected in liver samples of 5 field voles (II). All PCR-positive animals originated from Konnevesi, Central Finland. Four out of five infected animals were males. One infected field vole was collected in fall 2008, whereas the rest were from spring 2009. Partial sequencing of the 23S rRNA gene from three positive samples confirmed the subsp. as *Francisella tularensis* subsp. *holarctica*. Comparison of the *F. tularensis* load in four organs of the five *F. tularensis*-positive animals showed that *F. tularensis* can be detected in samples of liver, lung, and spleen tissue, but the kidney is not always positive (Table 2 in II).

5.2.3. Linking human tularemia outbreaks to wildlife and arthropod vectors

Francisella tularensis persists in the local ecosystems in a dormant stage and causes epidemics only when the ecological and environmental conditions are suitable for bacterial replication and transmission (Sjöstedt 2007, Ryden et al. 2012, Johansson et al. 2014). In most countries of Northern Europe and Scandinavia, tularemia outbreaks occur in late summer and early autumn (I, IV, Eliasson et al. 2002, Johansson et al. 2014, Desvars et al. 2015). Only Norway reports high case numbers during the winter months (Larssen et al. 2011, Larssen et al. 2014). Tularemia is typically associated with outdoor activities, and farmers and hunters are at particular risk of infection (I, Dahlstarnd et al. 1971, Eliasson et al. 2002, Calanan et al. 2007, Martín et al. 2007, Allue et al. 2008, Hauri et al. 2010, Mailles 2014). In Finland and Sweden, the disease is classically mosquito-transmitted and most cases are reported during August and September, related to the occurrence of mosquitos (I, IV, Eliasson et al. 2002).

Airborne outbreaks, mainly associated with potentially aerosol-generating activities such as farming, gardening or hunting, rarely occur and remain very local (Dahlstarnd et al. 1971, Feldman et al. 2001, Siret et al. 2006, Hauri et al. 2010, Otto et al. 2015). In Norway, water-borne outbreaks are most common (Berdal et al. 2000, Larssen et al. 2011, Larssen et al. 2014), and in Kosovo, outbreaks have been linked to rodent contamination (Reintjes et al. 2002, Grunow et al. 2012). Rodents and lagomorphs can act as local amplifiers for *F. tularensis* and after death contaminate the environment, including the breeding sites of mosquitos (III, Thelaus et al. 2013, Bäckman et al. 2015). Aquatic protozoa most likely enable *F. tularensis* to persist in natural waters at the specific locations where outbreaks occur (Abd et al. 2003), but outside epidemics, *F. tularensis* replicates, based on the absence of time-dependent evolution, rarely (Johansson et al. 2014). Weather conditions favorable for mosquitos impact on the transmission to humans, and thus, the size of outbreaks (Ryden et al. 2012).

Tularaemia caused by subspecies *holarctica* has been associated with proximity to natural water, and a positive correlation between the tularemia incidence and the proportion of a municipality area covered by inland water has been found in Sweden (Desvars et al. 2015). In Finland however, such a correlation does not occur. Quite the contrary, the cumulative tularemia incidence in the Finnish

Lakeland is below the average (Figure 4, IV), but a correlation between vole population dynamics and human tularemia outbreaks has been indicated (IV). Rodent hosts enable bacterial replication bursts during outbreaks (III, Figure 8). A high vole density may thus be one, but not the only precondition for tularemia outbreaks. Not all local environmental and ecological factors critical for disease outbreaks are yet known very well. The variation in the magnitude and especially the locality of human tularemia outbreaks clearly deserves further analyses. Although tularemia is, because of its complex ecology, a difficult disease to control, infection surveillance in wildlife could possibly help to predict and prevent human tularemia outbreaks (Carvalho et al. 2014, Hestvik et al. 2015).

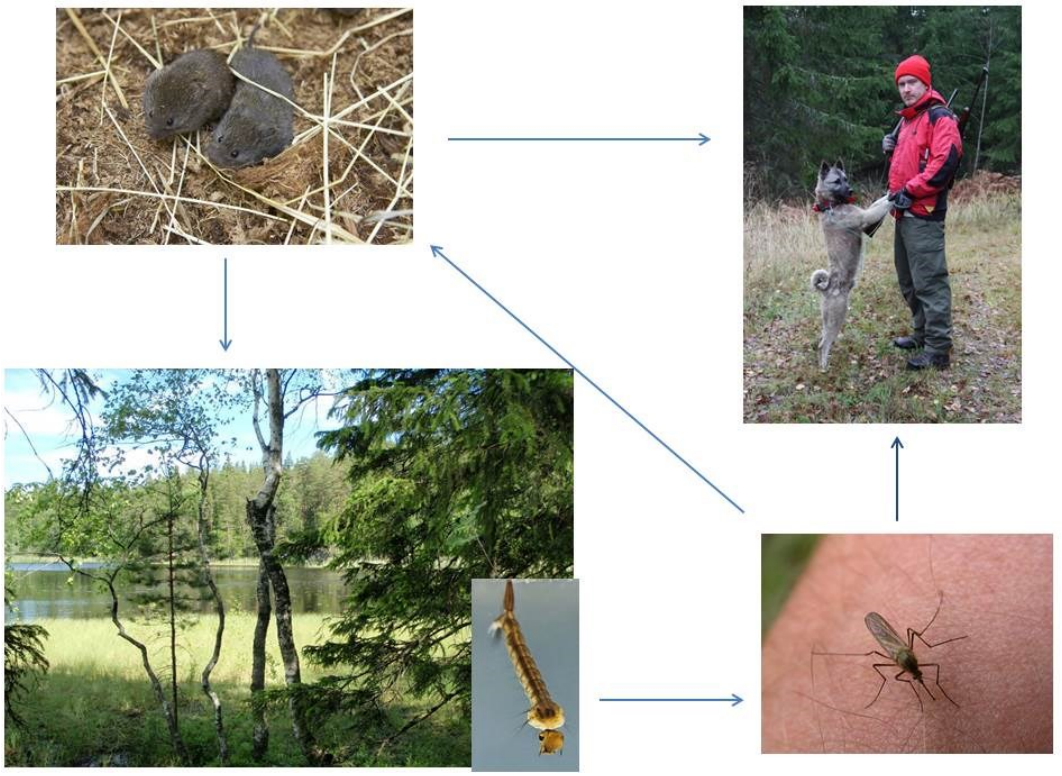

Figure 8. *Life cycle of* F. tularensis *in Finland.*

6 Concluding remarks

Tularemia is endemic in Northern and Southern Ostrobothnia and Central Finland. Recurrent regional and seasonal outbreaks involving hundreds of cases occur in the endemic areas every 3–4 years. In other regions, outbreaks of this size are quite rare. The *F. tularensis* -antibody prevalence is 2% on the population level. Incidence and seroprevalence are highest in Northern Ostrobothnia.

Tularemia causes severe illness and places a burden on the healthcare system during epidemics. Although the majority of the tularemia disease burden is attributable to mosquito bites, risk factors for ulceroglandular and pneumonic forms of tularemia are different, enabling targeted prevention measures. The risk of tularemia infection can be reduced by protecting against mosquito bites, using disposable gloves if handling dead rodents and lagomorphs, and by minimizing exposure to hay dust potentially contaminated with bacteria by wearing masks for respiratory protection while performing farming and landscaping activities. Physicians in endemic areas should consider tularemia in patients with acute febrile illness.

Francisella tularensis infection in voles is rather rare outside epidemics. Field and bank voles are highly susceptible to *F. tularensis*, with infection leading to bacteremia, tissue necrosis, and death. Although septicemic voles may pose a substantial risk to humans during tularemia outbreaks and serve as amplification hosts for *F. tularensis*, we did not find evidence supporting the role of voles as a true reservoir of *F. tularensis* between outbreaks.

A correlation can be shown between vole population dynamics and human tularemia outbreaks. Human tularemia outbreaks typically occur a year after the vole population peak. The dynamics of the vole population thus may be a factor of epidemiological importance, although not the only prerequisite for tularemia outbreaks.

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