CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Detection of *Francisella tularensis* Antibodies in Sera of European Hares (*Lepus europaeus*)

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled "***Detection of Francisella tularensis Antibodies in Sera of European Hares (Lepus europaeus)"*** independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague date

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Oluwo Babafemi Hafeez

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Abbreviations

• NADPH	Nicotinamide Adenine Dinucleotide Phosphate
• LPS	Lipopolysaccharide
• FPI	*Francisella* Pathogenic Island
• WW II	World War II
• US	United States
• CDC	Centers for Disease Control and Prevention
• PCR	Polymerase Chain Reaction
• EE.	Early Endocytic
• LE	Late Endocytic
• MR	Mannose Receptor
• MBM	Murine Bone Marrow
• FCP	*Francisella* Containing Phagosomes
• MIP-2	Microphages Inflammatory Protein 2
• ACPA	Anti-citrullinated Protein Antibodies
• MDM	Monocyte Denied Macrophage.
• ATPASE	P-type Adenosine Triphosphatase
• CYBA	Cytochrome B-245 Alpha Chain
• PMNs	Polymorphonuclear Leukocytes
• BMMs	Bone Marrow Derived Microphage
• LVS	Live Vaccine Strain
• O. D	Optical Density

Abstract

Francisella tularensis is a zoonotic organism of public health importance. In Czech Republic, hares which is a commonly hunted animal serves as the primary reservoir for *F. tularensis*. Thus, the likelihood of hunters been infected by tularemia is very high especially in areas with high prevalence. Humans often get the disease through close contact with an animal reservoir or through tick bites. The clinical manifestation varies depending on the geographical area considered. This extremely contagious microbe is regarded as a biological danger due to its ability to spread fast in aerosol.

The isolation of the *Francisella tularensis* in the laboratory is difficult as it is a highly pathogenic organism. Thus, the work with *F. tularensis* requires a high level of biosafety. In laboratories, PCR tests are used to confirm ongoing infection while the serology e.g., ELISA amongst other laboratory techniques is used to confirm if the individual was infected by the bacteria in the past and has developed antibodies against it. Thus, the ELISA is effective for seroprevalence studies. Infection with tularemia in specific parts of Czech Republic (Zlin Region, Central Bohemia Region and South Moravian Region) is the primary focus of this thesis and the three regions chosen are areas of major interest based on their geographical structure and relatively medium to high hunting activities done in these areas.

In total, 57 samples were collected for laboratory analysis. Eighteen (18) positives samples were gotten from South Moravian Region (Březí u Mikulova). Negative samples were gotten from the other two regions (Zlin Region and Central Bohemia Region). For the samples analysis, samples with OD at 0.9 (twice the value of negative control) and above are tagged as positive why the samples below 0.9 is the negative value. These results shows that hunters are at risk of contracting this disease because 18 positives out of 46 samples collected in just one region is a significant number for a bacteria caused disease of huge public health relevance. The high value of positive samples in the Southern Moravian Region could be due to the geographical structure or climatic condition which supports the co-habitation of hare and vector (ticks). In the Zlin Region, Central Bohemia no positive samples were gotten but the number of tested samples was too low to make any strong conclusions about these results.

Keywords: *Francisella* spp; Serological techniques; Pathogenicity; Biosecurity; ELISA; Host; Hares; Tularemia; Terrestrial form; Vectors; Detection.

1.1 Introduction

1.1.1 History of *Francisella tularensis* research

Francisella tularensis is the pathogen causing tularemia, often known as rabbit fever. It is the most fatal illness connected to rabbits and human. In 1910, the species was reported by George Walter McCoy who worked in the US Plague Laboratory in San Francisco, (McCoy 1910). *Francisella tularensis* was identified and given its primordial name *Bacterium tularense* when McCoy and Charles Chapin found and cultivated coccobacilli organisms from a rodent epidemic in Tulare County, California, in 1912 (McCoy et al. 1912). Edward Francis in 1920 after studying myriads of symptoms finalized that the *Bacterium tularense* (now *Francisella tularensis*) is the causative agent of tularemia (i.e., *Bacteria tularensis* in the blood). At the initial, it was categorized in the Proteobacteria subfamily, however further examination of 16S rDNA sequences revealed that it is not closely linked to other characterized species (Forsman et al. 1994). In 1959, it was moved to the genus *Francisella* (Olsufiev et al. 1959) from the initial genus *Pasteurella* (Larson et al. 1955). After further research on identification of the organism, it became known as *Francisella tularensis* in honor of the discovery by Francis (McCoy et al. 1912; Tärnvik et al. 2003).

As of 2002, three subspecies (biovars) of *Francisella tularensis* have been identified namely**:** (**a**)**.** *Francisella tularensis* subspecies *tularensis* **(b).** *Francisella tularensis* subspecies *holarctica.* **(c).** *Francisella tularensis* subspecies *mediasiatica* and a lone species **(d).** *Francisella novicida*.

1.1.2. Basic biology of *Francisella tularensis*

Francisella is still the sole recognized genus in the *Francisellaceae* as of 2007 till date. The bacterial pathogen *francisella* remains of keen interest in both medicine and veterinary science (Broekhuijsen et al. 2007). *Francisella* may infect rodents, burrowing birds, and lagomorphs (such as rabbits and hares) (Sam et al. 2020). Given that it may spread from animals to people, tularemia is classified as a zoonosis or zoonotic disease.

Francisella bacteria are aerobic (i.e., it need oxygen to grow), intracellular, non-spore forming, non-motile, highly virulent, and pleomorphic organism (Ryan et al. 2004). They are gram-negative coccobacillus that needs cysteine to grow (Michigan Department of Community Health 2015). At a low infectious dosage, the organism displays high virulence (i.e., a high tendency to cause disease). The illness can be spread via ticks, flies, mosquitoes, and contaminated aerosol particles. There are additional potentially hazardous pathways, such as the one detailed in a study on the transmission of tularemia through the fur of an infected dog (Siret et al. 2006). Direct contact with an infected animal is necessary for this.

The *Francisella tularensis* infects and proliferates in the macrophages, hepatocytes (liver cells), endothelium, epithelial cells, fibroblasts, chicken embryos, and amoebae (Sjostedt 2007). In the macrophages, the lifecycle occurs by phagocytosis and the bacterium is sequestered from the interior of the infected cell by a phagosome. Further, to complete its cycle, the *Francisella tularensis* breaks out of phagosome into cytosol and quickly proliferates. Finally, the infected cell undergoes apoptosis (cell death), and the progeny bacteria is released in a single burst event (Carruthers et al. 2020) to initiate new rounds of infection. Successful proliferation in the host cells is conditioned by expression of the regulatory proteins MglA, SspA, and PmrA that control FPI (*Francisella* pathogenicity island) genes expression (Brotcke et al. 2006, Sebastian et al. 2007, Wang et al. 2007, Raynaud et al. 2007, Thomas et al. 2007).

The molecular processes by which species of *Francisella* adjusts to live in host cells are yet unknown (Barker et al. 2007). Certain school of thought has pointed at the Francisella pathogenicity island (FPI) genes has been the mechanism at which they adapt to the environment (Brotcke et al. 2006, Golovliov et al. 2003, Gray et al. 2002, Lauriano et al. 2004, Santic et al. 2007).

1.1.3 Subspecies of *Francisella tularensis*

The organism *Francisella tularensis* have been classified and reclassified over the years. They were separated into two types (A and B) by one stream. *Francisella tularensis* subspecies *tularensis* (previously known as type A) causes serious disease condition in humans and animals. Thus, it is termed as the most virulent of the *Francisella tularensis* subspecies and it is mostly found in North America (Fig. 1) (Gurycova 1998).

The *Francisella tularensis* subspecies *holarctica*, formerly known as Jellison's subtype B, found in Europe (Fig. 1) is ten times less virulent than the *Francisella tularensis* subspecies *tularensis* (Olsufyev et al. 1983) and it has also been isolated in North America. Just like the *Francisella tularensis* subspecies *tularensis*, the *Francisella tularensis* subspecies *holarctica* can causes tularemia disease in humans.

According to their biochemistry, sensitivity to erythromycin, and geographic isolation, the three biovars of *F. tularensis* subsp. *holarctica* are distinguished from one another. These are biovars I, II, and japonica (Ellis et al. 2002). In Europe, Russia, the Far East, and North America, Biovar I is widely spread. Eastern Europe, Western Siberia, and Scandinavia are the only regions from which Biovar II, an erythromycin-resistant strain, has so far been identified. The biovar japonica is found basically in Japan (Olsufjev et al.1983).

1.1.4 *Francisella tularensis* subspecies *mediasiatica*

The subspecies was exclusive to some regions of Turkmenistan and Kazakhstan in Central Asia. The name of the subspecies is derived from this geographic region. The virulence of this subspecies was previously poorly understood, and no instances of this subspecies have been documented previously in humans although some evidence shows its virulence in hares is like virulence of subspecies *holarctica* (Keim P.S et al. 2007). Due of its low abundance, this subspecies has generally been ignored by most studies but in 2013, *Francisella tularensis* subspecies *mediasiatica* was discovered in the Middle Asian subspecies in Russia's Altai area (Mokrievich A.N et al. 2013).

1.1.5 *Francisella tularensis subspecies novidica*

The *Francisella novicida* has generated lots of debates over the years. Before 1989, scientist have been debating on whether to categorize it as a specie on its own or a subspecies of *Francisella tularensis*. The idea of calling it a subspecies was initiated because of its genetic similarity and biochemical traits (Hollis et al. 1989) with the *Francisella tularensis*, but from 1989 it became generally accepted as a species on its own.

The reason for the exemption as a subspecies of *Francisella tularensis* is because the metabolic requirements of isolates of species *Francisella novicida* are less pathogenic compared to those of isolates of the other three subspecies. *Francisella novicida* produces relatively little sickness in humans, and the few instances that do occur are usually linked to people who have impaired immune systems or other medical conditions.

Francisella novicida infections are uncommon in human making precise diagnosis challenging (Brett et al. 2012; Birdsell et al. 2009). Only 12 cases have been documented (Hollis et al. 1989; Clarridge et al. 1998; Leelaporn et al. 2008; Birdsell et al. 2009; Brett et al. 2012. Respicio-Kingry et al. 2012; Sjödin et al. 2012; Whitehouse et al. 2012). A patient from Australia has been reported to have an infection that resembles *Francisella novicida*, however a genome comparison reveals that this strain that caused the disease is the *Francisella tularensis* (Whipp et al. 2003; Sjödin et al. 2012). Diseases caused by *Francisella novicida* do not present as common tularemia symptoms (Hollis et al. 1989; Birdsell et al. 2009). It is likely that *Francisella novicida* resides in an environmental niche and its spread in nature via methods that do not need a mammalian.

The pathogenicity of *Francisella novicida* and *Francisella tularensis* subspecies in laboratory animals varies as well. Although having different pathogenicity, *Francisella novicida* is frequently employed in laboratories as a stand-in for *Francisella tularensis* since the two species share 97% of the same nucleotides. The *Francisella novicida* has only been found to cause disease in immunosuppressed humans and it has been isolated in Australia and very sparsely in North America (Ellis et al. 2007; Oyston et al. 2004; Petersen et al. 2005). Also, it is not a naturally occurring zoonotic bacteria and it has never been identified in arthropod vectors (Sjoedstn 2004) or arthropod host. Indeed, the only source of *Francisella novicida* isolated so far is salt water (Larson et al. 1995; Petersen et al. 2009a; Whitehouse et al. 2012).

Additionally, because several *Francisella*-like endosymbionts have been found in ticks by PCR and sequencing, it does not appear that insufficient testing procedures are to blame for the absence of *Francisella novicida* discovery in arthropods.

Arthropod vectors typically acquire infection via bacteremia animal hosts; the absence of *Francisella novicida* in arthropods is consistent with *Francisella novicida* predicted inability to generate bacteremia in wild animals. (Scoles et al. 2004; Goethert et al. 2005; Kugeler et al. 2005; Machado-Ferreira et al. 2009; De Carvalho et al. 2011; Ivanov et al. 2011; Kreizinger et al. 2013). Unlike *Francisella tularensis* subspecies *tularensis* and *Francisella tularensis* subspecies *holarctica*, *Francisella novicida* needs several genes to develop in the host and cause illness in humans. Symptoms seen in *Francisella novicida* are subclinical in non-immunocompromised patients. It is common to see fever whereas in non-immunocompromised patients' fever isn't common (Birdsell 2009; Clarridge 1996; Hollis 1989; Leelaporn 2008).

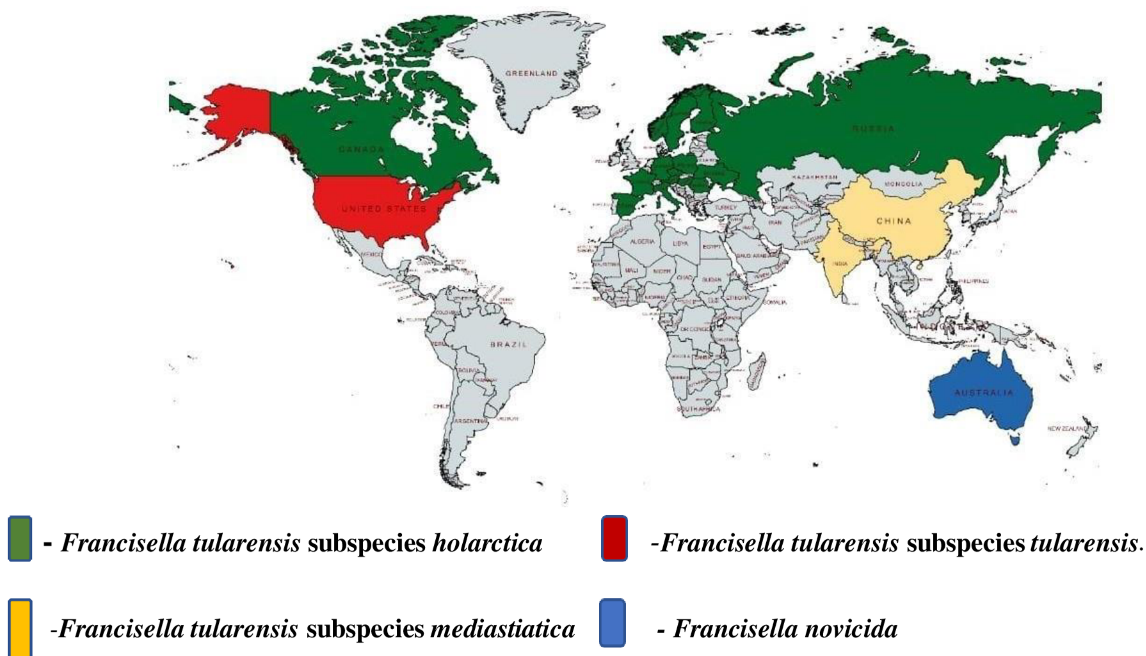


Figure 1. Geographical Distribution of *Francisella* subspecies and species: The colored map shows *Francisella tularensis* subspecies *holarctica* endemic in Europe and some part of North America while *Francisella tularensis* subspecies *tularensis* are endemic in North America region. *Francisella tularensis* subspecies mediastiatica endemic in Asia and *Francisella novicida* found in Australia.

1.2 Epidemiological distribution of *Francisella tularensis*

Since the fall of 1936, the Czech Republic has seen some cases of tularemia (Černý 2001), a zoonotic disease of the northern hemisphere (Quinn et al. 1994). More than 400 instances were found to be present between 1971 and 2000. Tularemia persisted in a few areas with spontaneous outbreaks, but it was not stable (Pikula et al. 2003). All those afflicted had interacted with hares (Pikula et al. 2003). Tularemia has now been identified as an endemic disease in some parts of the Czech Republic (Pikula et al. 2003). There are about 100 documented cases per year, with a few outliers (Fig. 3). (Kubelová et al. 2012).

The peak season, which is the autumn, is often when tularemia infection hikes. After examining two 15-year periods, it was shown that there was a strong correlation between the geographic distribution and number of tularemia natural foci in the Czech Republic from 1971 to 1985 and from 1986 to 2000. A link between the population density of European brown hares (*Lepus europaeus*) and the number of tularemia natural foci was also discovered in the Czech Republic. (Pikula et al. 1996). The incidence of natural foci of tularemia increases with population density of European brown hares.

Under ideal circumstances, *Francisella tularensis* subspecies are frequently found in endemic regions of the northern hemisphere (Pikula et al. 2003; 2004). *Francisella tularensis* subspecies *tularensis* is spread by ticks, which are vectors or the disease in rodents and lagomorphs (Pikula et al. 2002, 2004b; Zhang et al. 2006; Treml et al. 2007; Hubalek et al. 1998). *Francisella tularensis* subspecies *mediasiatica* rarely cause disease in people, but subspecies *tularensis* does so (Euler et al. 2012; Georgi et al. 2012). Thus, *Francisella tularensis* subspecies *mediasiatica*, is said to have minimal pathogenicity (Broekhuijsen et al. 2003).

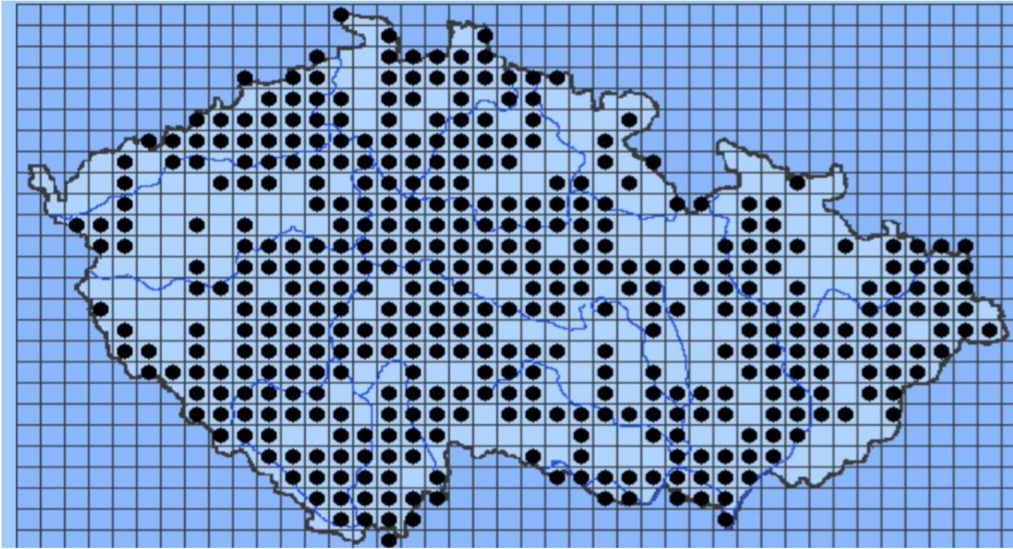


Figure 2. Map Showing distribution of hares in selected areas of Czech Republic (Anděra 2023).

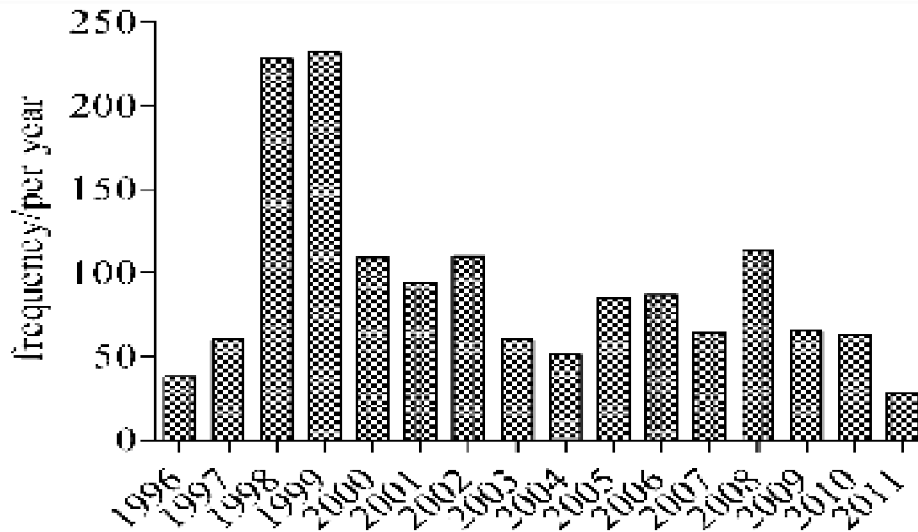


Figure 3. A graph showing the number of reported cases of tularemia in Czech Republic from 1996 - 2011. (Kubelková et al. 2012).

1.3 Tularemia in Game, Food, Companion, and Wild Animals

In the Czech Republic, brown hares (*Lepus europaeus*) are common game animals hunted by people. It is one of the biggest species of hares and is suited to temperate and open terrain. Hares are herbivores that mostly eat grasses and herbs. Its larger legs and bigger noses set them apart from other leporids (rabbits) (Chapman et al. 1990). Much of continental Europe and a small portion of Asia are home to the European hare. Its distribution reaches southern Scandinavia, eastern Europe, northern, western, and central Asia, and northern Spain. According to studies the averages European hare population density in study regions in the Czech Republic varied between 6.8–17.0 individuals/100 hectare. So, possibility of contracting one is very high. (Petr 2018).

Tularemia is spread largely by the bites of ticks and insects. It is impossible to overstate the importance of vectors (Ellis 2002). *Francisella tularensis* commonly infects rodents, squirrels, hares, rabbits, ticks, and mosquitoes (Nigrovic et al. 2008), birds, fish, amphibians, arthropods, and protozoa (Mörner 1992). Also, the disease tularemia has been documented in dogs, cats, and food animal like sheep, buffalo, and cattle. In dogs and cats the probability of contracting the disease is moderate to intermediate (Woods et al. 1998). Tularemia is seldomly reported in cats as the clinical signs mimics other infectious diseases (Woods et al. 1998; Cooper et al. 1973; Gliatto et al. 1994). While cats are more susceptible with cases seen in Europe, Asia (Zidon 1964), and North America (Gliatto et al. 1994; Rhyan et al. 1990), dogs are more resistant to tularemia (DeBey et al. 2002).

The possibility of dogs contracting this disease can be due to their activities e.g., hunting with humans or sheep ranching (Johnson 1944; Zidon 1964). The transmission to humans from dogs is mechanical as it is normally from contaminated mouth parts following killing of infected hares, consumption of infected rabbits or through blood transmission from infected ticks. Cat illness frequency is regarded as a significant risk factor for human tularemia infection in North America (Larson et al. 2014). Animals that are afflicted are sluggish, inert, and passive; diseased wildlife occasionally exhibits no outward symptoms of illness (Gustafson et al. 1996; Baldwin et al. 1991). Tularemia may readily infect both humans and dogs (it should be noted that young rabbits quite often fall prey of these domestic animals). Hunters of rabbits may become more susceptible to tularemia if they have frequent close contact with hunted animals.

Asides from the companion animals, food animals like sheep whose farming is gaining popularity in the Czech Republic (Bucek et al. 2016) can also be affected. This specie has greater chances of contracting the disease than the cattle and buffalo. Individual epizootics of tularemia have killed more than 1000 sheep (Frank et al. 1961) and huge economic lose due to the residual effect have also resulted from animals that survived infection (Hopla et al. 1994; McNabb 1930).

Also, wild animals basically carnivores and wild animal keepers (rangers) are at risk but very minimal because carnivores do not commonly exhibit bacteremia (Olsen 1975). Mink and fox rangers have used rabbits and hares carcasses as feeds which has caused tularemia outbreaks. (Jellison 1970; Gorham 1950). The first documented outbreak of tularemia in wild animals involves foxes in Minnesota (Schlotthaer et al. 1935). Sick, slow hares are easy preys in the wild for carnivores, so there are possibilities of contracting the tularemia disease. In a previous study, a high percentage of carnivores having antibody titers from serological surveys indicates past exposure to *Francisella tularensis* (McKeever et al. 1958; Lesser et al. 1936).

There have only been a few sporadic occurrences of the tularemia causing agent in European carnivores among wild animals. Cases in red fox in Germany in 2018, the raccoon dog in 2012 (Schulze et al. 2016), and the stone marten in Switzerland in 2012 (Origgi et al. 2013) have all been reported. Tularemia, a disease that affects domestic carnivores like cats, is sporadic in North America and is virtually always brought on by the *Francisella tularensis* subspecies *tularensis* (Larson et al. 2014).

Also, it seems probable that certain aquatic mammals sometimes contract the disease from water that is contaminated with *Francisella tularensis*. For instance, tularemia outbreak in beavers in Montana in 1939 and 1940 may have been caused by water-borne francisella bacteria (Jellison et al. 1942). According to research, *Francisella* bacteria are widely distributed in streams and muds in the northwest United States, and fatal tularemia infections of muskrats and beavers are likely to have happened there (Parker et al. 1951).

The main arthropod responsible for transmitting tularemia in the wild is the rabbit tick (*Haemaphysalis leporispalustris*). In addition to the rabbit tick, other blood-sucking arthropods including lice, and fleas can also transmit the illness from one rabbit to another (Francis 1937).

The rabbit tick, rabbit louse, and rabbit flea do not bite people; hence they cannot cause transmission of *Francisella tularensis* to people. The rabbit tick mostly cause tularemia outbreak among wild animals and birds. (Green et al. 1942). Tularemia is also reported to be transmitted by the tick species *Dermacentor variabili*s and *Dermacentor andersoni* (Avashia et al. 2004). Both serve as the disease's origins in both humans and numerous animal species.

Tularemia post-mortem symptoms are discernible but not immediately apparent, such as tiny white patches on the liver or spleen of infected animals (Figure 3; Pictures A & D). Hunters may, however, spot white lesions on the viscera of dead animals during field inspections. It should be mentioned that until the viscera have been analyzed in authorized laboratories, it is not advisable according to for hunters to remove or dissect the viscera of dead animals that are clearly diseased. (CDC 2007).

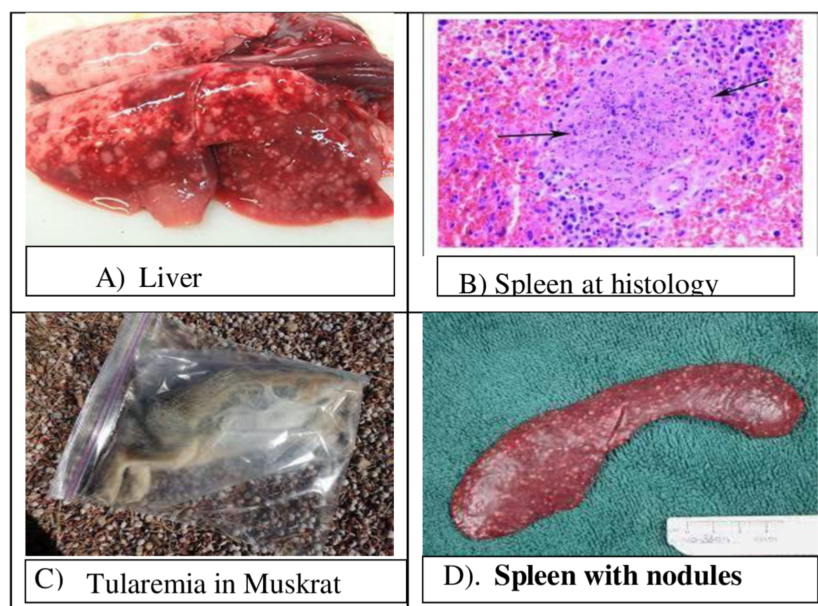


Figure 4. Picture showing how to handle an animal suspected to have died of *Francisella tularensis* infection and visceral of a patient infected by tularemia (Histology and Pathology).

A) Picture showing a white multiple nodes on liver (Université de Montréal). B) Spleen of a tularemia positive Hare showing multiple foci of necrosis (Michigan DNR Wildlife Disease Laboratory). C) Picture showing a typical case of Tularemia in Muskrat (Dr. Rochelle Jensen). D) Spleen showing areas of white necrosis and neutrophils indicating a bacterial infection (Kansas State Veterinary Diagnosis Laboratory).

1.4 Transmission cycle of *Francisella tularensis*

The *Francisella tularensis* cycle is divided into terrestrial and aquatic forms (Maurin et al. 2016). *Francisella tularensis* subspecies t*ularensis* is connected to the terrestrial cycle. The aquatic cycle is connected to *Francisella tularensis* subspecies *holarctica*, which is spread by mosquitoes (infected when breed in contaminated water or swampy places) and passed from small mammals living near water to semi-aquatic animals. As was already noted, contact with contaminated water or inhalation of infected aerosols are important ways that *Francisella tularensis* subspecies *holarctica* is spread. Another method of transmission involves direct contact with the tissues and bodily fluids of affected animals (Sting et al. 2013). The danger of infection is higher for hunters and forest workers since skinning involves close touch (Becker et al. 2016).

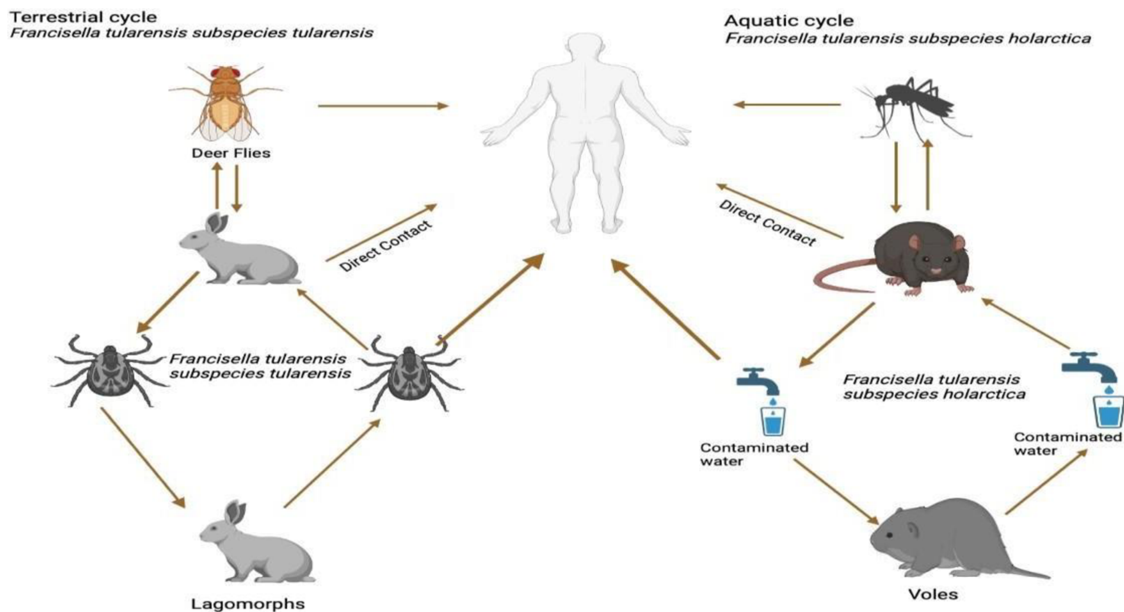


Figure 5. Showing Terrestrial and Aquatic cycles of Tularemia (Gökeen et al. 2017) **(Redrawn).**

The terrestrial and aquatic cycle is vividly explained by the diagram (Fig 5) which shows the role that arthropods (Ticks, Flies), lagomorphs, water bodies play in the transmission of *F. tularensis* subspecies *tularensis* and *F. tularensis* subspecies *holarctica.*

1.4.1 Terrestrial Cycle of Tularemia:

The primary reservoirs of *Francisella tularensis* subspecies *tularensis* are tabanid flies, mites, hares, and rabbits (Christine et al. 2011). Arthropods which include Ixodid ticks (hard ticks) and hares in central Europe, mosquitoes in northern Europe (e.g., Sweden and Finland) (Tab. 1), have been the focus of most investigations as vectors of the organism *Francisella tularensis* subspecies *tularensis* (Petersen 2009). Regional distribution of these vectors, with mosquitoes being more prevalent in northern Europe and ticks being relatively uncommon, has a significant impact on the spatial variances in research activities.

Arthropods spread the *Francisella tularensis* infection between small mammals like rabbits and beavers to maintain the reservoir of the disease. In the terrestrial cycle, spread of tularemia is by tick or animal contact, middle-aged males predominate because they are more likely to be exposed through work or leisure activities (Maurin et al. 2016). Less commonly, contact with polluted water causes the development of glandular (Balci et al. 2014; Ulu-Kilic et al. 2013), oculoglandular (Helvaci et al. 2000, Ulu-Kilic et al. 2013, Ozdemir et al. 2000, 2005; Yeşilyurt et al. 2013; Bilgul et al. 2011) and pneumonic (Karagöz et al. 2013) forms of tularemia.

Table 1. Human cases of Tularemia in Europe with mosquitoes and hares as vectors.

Country	Year of Occurrence	No.of cases	Clinical forms	Subsp. Involved	Vector	References
Czech (Brno)	1959 – 1999 2000	577	Ulceroglandular & Glandular	*ssp. holarctica*	Hare,Rodents	Černÿ 2001. Pazdiora et al. 2002, Tomake 1937. Pikula 2003, Treml et al. 2001
France	2011	51	Ulceroglandular	*ssp. holarctica*	Aerosols,Tick, Contact with infected tissues	Decors et. al. 2011. Maurin et al. 2011; Li. et al. 2011.

Germany	2004 -2005	39	Ulceroglandular	*ssp. holarctica*	Hare	Hauri et al. 2010, Kaysser et.al. 2008, Müller 2007
Sweden	1981,1995, 1999,	349	Ulceroglandular commonly	*ssp. holarctica*	Mosquito	Rydén et al. 2012
Sweden	2000 - 2004	278	Ulceroglandular	*ssp. holarctica*	Mosquito	Svensson et al. 2009, Eliasson et al. 2002
Sweden	2000	105	Ulceroglandular commonly	Not Specified	Mosquito	Eliasson et al. 2002
Sweden	2003	475	Not Specified	Not Specified	Mosquito	Payne et al. 2005
Sweden	2006	90	Not Specified	Not Specified	Mosquito	Wik 2006
Finland	2000	1	Ulceroglandular	Not Specified	Mosquito	Byström et al. 2005, Jounio et. al.2010
Finland	2003	1	Ulceroglandular	Not Specified	Mosquito	Tärnvik 2003, Eliasson 2002.
Finland	2007	50	Ulceroglandular	ssp. *holarctica*	Mosquito, Aerosols	Seppänen 2011, Rydén 2012.
Kosovo	2001 -2010	25-327	Ulceroglandular	ssp. *holarctica*	Contaminated Water, Food	Grunow et al. 2012
Bulgaria	2003 -2005	24	Ulceroglandualar	ssp. *holarctica*	Contaminated Water, Food, Tick	Kantardjiev et al. 2006.

1.4.2 Aquatic Cycle of Tularemia

Waterborne tularemia (Aquatic tularemia) is the most typical kind for both people and animals, drinking water continues to be the major source of illness although there are other routes. Blood-sucking mosquitoes, and tabanid flies spread the *Francisella tularensis* subspecies *holarctica*. Also, activities like fishing, swimming, and drinking of contaminated water (Fig. 5) are route through which humans can contract this infection tularemia.

Basically, in North America the disease is mostly spread by water, mosquitoes, and beavers. In countries like Turkey, Kosovo, Bulgaria, Georgia, Macedonia, Norway, Sweden, Italy, and Germany, tularemia has also been linked to water supplies (Akalin et al. 2009) (Tab. 1). Turkey had a resurgence of tularemia in 1988 in the form of oropharyngeal tularemia. (Tab. 1). The source is basically from contaminated water (Akalin et al. 2009). Between 1988 and 2018, 28 cases of tularemia in this country were reported. Both outbreaks and cases are related to drinking polluted water (Willke et al. 2009; Balci et al. 2014; Meric et al. 2008; Helvaci et al. 2000; Ulu- Kilic et al. 2013).

Due to the oral route of tularemia infection, nearly all patients in this epidemic presented with oropharyngeal tularemia. In both infants and adults, it was discovered that the sex ratio of men to females with oropharyngeal tularemia was one or even less. The causes of this are still unknown, but some evidence points to occupational hazards as the primary reason for increased exposure in men (Willke et al. 2009; Balci et al. 2014; Meric et al. 2008; Akalin et al. 2000; Ulu-Kilic et al. 2013; Meric et al. 2010; Leblebicioglu et al. 2008; Sencan et al. 2009; Gürcan et al. 2004).

Francisella tularensis mostly contaminate water in wells and springs and since these water sources are not adequately disinfected and the well and springs are hideouts for hares and rabbits. Thus, they are sources of contracting the disease. Animal carcasses, human waste, or animal feaces might also be at blame for this pollution (Akalin et al. 2009). Only *Francisella tularensis* subspecies *holarctica* has been linked to people in Europe, even though several of these outbreaks contained subspecies of *Francisella tularensis* subspecies *tularensis*.

In Kosovo, 2001 to 2010, around 357 cases of ulceroglandular form of tularemia were reported (Grunow et al. 2012) (Tab. 1). Food and water pollution are seen as the primary causes of this outbreak and the propagation of *Francisella tularensis*. It is believed that a rat and mice invasion that occurred during the Kosovo War is what started the outbreak. This host must have introduced *Francisella tularensis* to basements and wells. The bacteria were isolated in a rat discovered in a well in a community that was affected.

In Bulgaria, there were 24 documented water-related fatalities between 2003 and 2005 and 285 documented water-related fatalities in 1997 (Kantardjiev et al. 2006; Christova et al. 2004; Velinov et al. 2001) (Tab. 1). It was possible to isolate *Francisella tularensis* using water samples from several wells. This thus emphasis that drinking water may expose people to contaminants. Most of the patients developed oropharyngeal form of tularemia. Scandinavia has also had reports of waterborne tularemia. Although glandular, pulmonary, and ulcerative tularemia mimicking typhoid fever were also noted, oropharyngeal tularemia affected most cases.

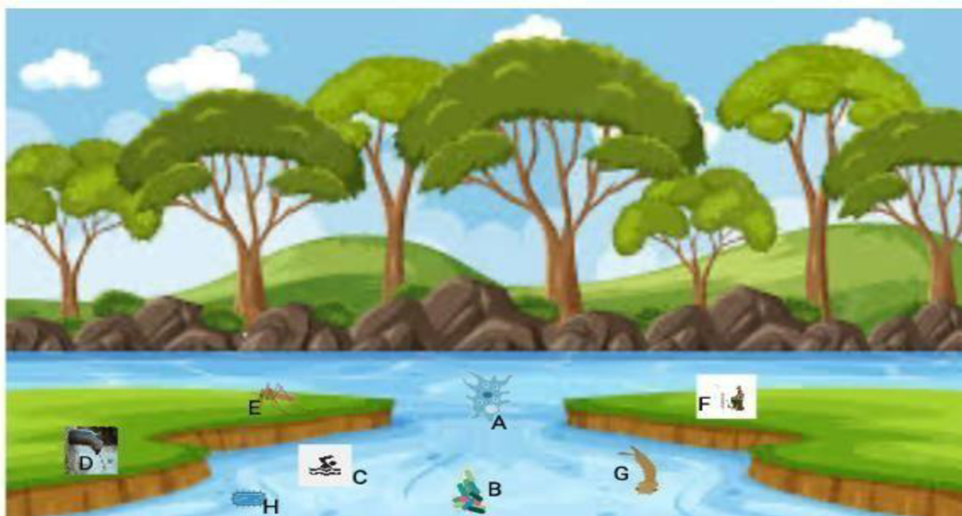


Figure 6. Pictorial representation of mode of transmission of *F. tularensis* (i.e.,how humans or animals can contract the bacteria) (Maurin et al. 2016). **(Redrawn)**

(A). Bacterium in Amoeba **(B)**.Bacterium in biofilms **(C).**Infection during swimming **(D).**Aquatic reservoir by drink in contaminated water **(E).**Infection after a mosquito bite **(F).**Infection during fishing activities. **(G).**Bacterium in mosquito larvae **(H).**The bacterium surviving in water

1.5 Forms of Tularemia Infection in Humans

Tularemia can be categorized based on their symptoms. Typhoid, oropharyngeal, ulcerative, glandular, pneumonia, adenitis, and ophthalmopathy. This illness typically takes 3–5 days to incubate following exposure (Nigrovic et al. 2008). Acute or subacute pneumonia is a common presentation of the pneumonic and typhoid types. Patients frequently arrive after the incubation period with severe fever (pyrexia) and ulcers that have a necrotic basis and well-defined boundaries. Subtle local lymph node swelling may happen a few days after exposure.

1.5.1 Glandular and Ulcero-glandular form

These two forms of tularemia are most prevalent. More than 95% of the cases in European nations can be counted. This kind of illness can spread by vectors, direct contact with diseased animals, or indirect contact with items like veterinary supplies. Open wounds remain a major entry point for this bacteria organism even though there are other routes. On occasions, the illness can be contacted even with apparent clean skin (Anda et al. 2001). The phrase "glandular tularemia" is exclusively used to describe situations in which the main ulcer cannot be seen (Fig 6: picture A). In ulceroglandular tularemia, the bacteria penetrate via the skin after being bitten by or scratched by an arthropod. Ticks are often the vector; however, deer flies and mosquitoes can also spread *Francisella tularensis*. A skin ulcer forms where the infection first started (Fig 6: picture B) (Kavanaugh 1935).

1.5.2. Oculoglandular form

The eyes remain a major point of entrance of *Francisella tularensis*. Rubbing the eye with *Francisella tularensis* contaminated or infected fingers can expose to the infection or simply the splash (Fig 6: picture C) of infected blood, this kind of tularemia can be treated with the use of antibiotics such as gentamycin, tetracyclines, doxycycline, and most recently quinolones (Maurin et al. 2000; Sawyer et al. 1966; Welling et al. 1977; Syrjälä 1991; Kavanaugh 1935; Foshay 1940; Guerrant et al. 1976; Steinemann et al. 1999).

Although in the latest outbreak in Kosovo, high rate of this form (4.2%) was recorded (Pérez- Castrillin et al. 2001), fewer than 1% of all human cases of tularemia fall into this group. Fever, conjunctivitis which manifests as an eyebrow response, extensive destruction, photophobia, and mucopurulent removal except for red conjunctiva and granulomatous lesions in conjunctival wings are all symptoms of oculo-glandular tularemia (Chappel et al. 1981).

1.5.3 Oropharyngeal form

This form of tularemia is less common depending on how much contaminated food or drink is consumed. Pharyngitis and uveitis are among the disease's clinical signs. (Hughes et al. 1957; Foshay 1940; Luotonen et al. 1986). The mouth, throat pustules and mucous membrane becomes erythematous during physical examination (Fig 6: picture D). Also, excessive regional cervical lymphadenopathy, fever, sore throat, myalgia, inflamed pharynx, tonsils are a symptom of the illness and lymph node edema is typically unilateral (Foshay 1940; Hughes 195; Luotonen et al. 1986).

The typhoidal type is characterized by the absence of any cutaneous or mucous membrane lesions, significant lymph node enlargement is observed (Francis et al. 1927). It is the second most prevalent kind of tularemia, which accounts for 10-15% of cases and is also known as septicemic tularemia (Fig 6: picture E). Pneumonia is a typical symptom. Although the exact method of transmission is not entirely established, it is most likely obtained by ingestion. The usage of the word "typhoidal" appears to be misleading. (Avery et al. 1967; Syrjälä et al. 1985). Only when no route of infection can be established may the term still be acceptable.

1.5.4 Respiratory form

This results from breathing in aerosolized *Francisella tularensis* (Francis et al. 1927). This frequently occur when agriculture is practiced primarily in arid, dusty lands. Farmers tends to be more susceptible to contracting this through handling contaminated hay (Eliasson et al. 2002), although other occupations like mowing the lawn can also occasionally result in the disease (Feldman et al. 2003; McCarthy et al. 1990).

However, outbreaks may still include a sizable patient population, but less often than in cases of ulceroglandular tularemia. It is thought that rat or hare carcasses that were abandoned in the fields after passing away from tularemia are a source of bacterial aerosols. Respiratory tularemia can cause pneumonia symptoms as coughing, chest pain, dyspnea, and hyperpnea. First signs may include hyperthermia and nonspecific symptoms like nausea and vomiting (Tärnvik et al. 2003).

It is occasionally possible to have no respiratory symptoms. Pneumonia can appear as a subsequent respiratory symptom or therefore in any type of tularemia due to bacterial spread. The *Francisella tularensis* subspecies *tularensis* and *Francisella tularensis* subspecies *holarctica*, has considerable differences in the disease's progression in respiratory tularemia (Tärnvik et al. 2003).

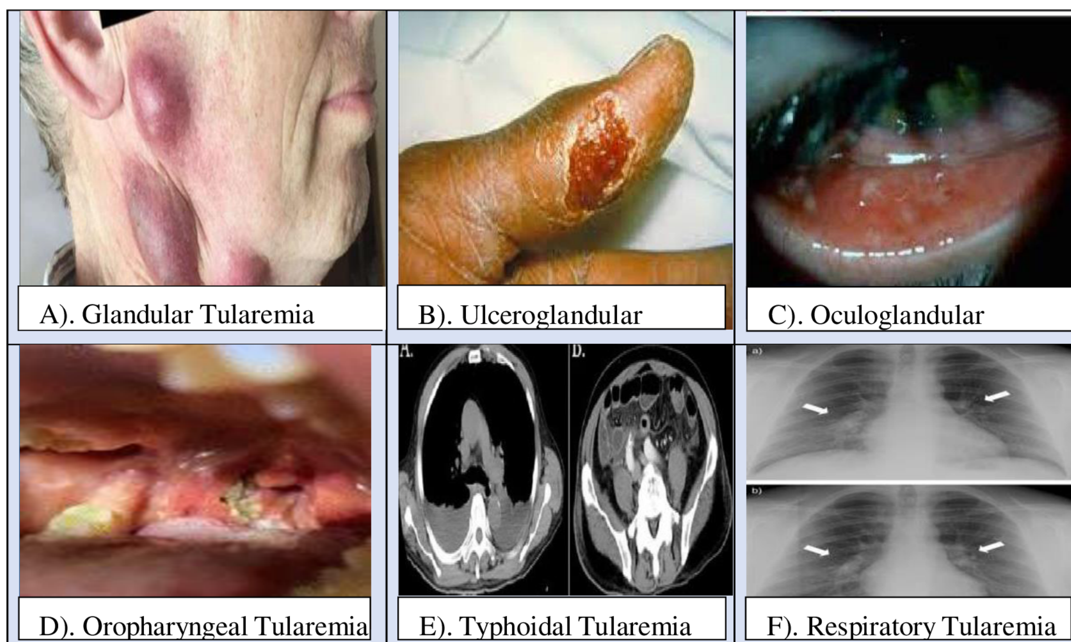


Figure 7. Pictures showing symptoms in tularemia patients.

(A). Picture showing swollen in the neck region of a 43-year-old farmer with glandular formof tularemia (Kerry O et al. 2023). **(B).** Picture showing area of ulcerations in finger of a patient with ulcero-glandular form of tularemia (Center for disease control and prevention). C). Picture showing caseous necrosis in eyes of a patient with oculo-glandular form of tularemia (Sebnem et al. 2014). D). Picture showing erythematous ulceration in the oral cavity (mouth) of a patient with oropharyngeal form of tularemia (American Journal of Medicine). E). Picture of chest CT scan showing bilateral pleural effusion and abdominal inflammation of the ascending colon in a patient with typhoidal form of

tularemia (Nakamura et al. 2018). F). Radiograph showing hilar enlargement in a 24-year-old farmer 13 days and 10 weekspost onset of infection (Tärnvik 2008).

1.6 Tularemia infection in humans

Tularemia is primarily a hunter's disease and hunting are an activity commonly practiced in Czechia. Apart from hunting, humans contract the disease by consuming inadequately prepared or contaminated hare meat (Green 1942). Infection from handling a sick hare often manifests at the site of a cut or puncture in the skin, however it can occasionally manifest in the eye due to contact with hands or splashing of cleaning solutions when cleaning. There is a lot of evidence to suggest that the disease-causing bacterium can also penetrate intact human skin (Yeatter et al. 1943).

Francis (1937) provided the following description of a typical tularemia case: About 3 days after infection contact, disease begins with headache, chilliness, vomiting, agonizing aches all over the body, and fever. The patient goes to bed believing that he has the flu, an ulcer develops from the hand sore, the glands in the armpit or near the elbow swell, become sensitive, and hurt. They may later turn into an abscess. Debility, weight loss, and perspiration are present. The illness lasts for approximately 3 weeks, and then there is a sluggish recovery that lasts for about 2 or 3 months. Many patients recover without any negative side effects, but 5% of them pass away, particularly when pneumonia complicates the situation. Those who have recovered from the sickness are permanently immune, despite the lack of evidence for a natural immunity in man. Tularemia can be suspected when signs like fever, skin ulcer, and enlarging lymph nodes in the neck region, history of tick, fly, or wild rabbit bites or contact with other animals are noticed (Francis 1937).

Further, tularemia in humans can be categorized based on their symptoms to following forms: typhoid, oropharyngeal, ulcerative, glandular, pneumonia, adenitis, and ophthalmopathy. This illness typically takes 3–5 days to incubate following exposure (Nigrovic et al. 2008). Acute or subacute pneumonia is a common presentation of the pneumonic and typhoid types. Patients frequently arrive after the incubation period with severe fever (pyrexia) and ulcers that have a necrotic basis and well-defined boundaries. Subtle local lymph node swelling may happen a few days after exposure.

1.7 Public health importance of *Francisella tularensis*

Francisella tularensis, is one of the most infectious pathogenic bacteria known, requiring inoculation with or inhalation of as few as 10 organisms to cause disease. *Francisella tularensis* is so infective that examining an open culture plate can cause infection. The bacteria *Francisella tularensis* is of huge public health importance. Apart from the fact that it can spread quickly, it has drawn attention as a biological weapon. People who breathe in contaminated aerosol typically get severe respiratory illnesses, including deadly pneumonia and systemic infections (Fig 6; Pic F).

The tularemia causing bacteria are widely distributed in nature and can be isolated and cultured in great quantities in a laboratory under strict biosecurity controls. Tularemia may induce potentially serious and deadly infections in people due to its high zoonotic importance.

1.8 Prevention and biosecurity measures against *Francisella tularensis*

Tularemia naturally exists in many parts of the world and for this reason it is necessary to put measures in place to curtail it whenever there is an outbreak. Basically, people use insect repellent containing DEET or repellent containing Permethrin to prevent vector bites (CDC 2018). Also, proper hygiene to prevent bacteria protects against this disease. Soaps, and hot water for regular hand washing, especially after handling animal bodies. Proper cooking of the food and ensuring that water comes from a safe source are measures commonly adopted.

On the field, its essential to use hand gloves when collecting sample from any animals. Further, it should be noted that the activated *Francisella tularensis* should only be cultured in a biosafety level 4 laboratory. If there are changes in the behavior of domestic animals (e.g., rabbits and hares or livestock), it's advisable to call a veterinarian to do a proper check.

Further, the creation of a vaccine that might be used to protect individuals against illness brought on by the intentional release of Francisella tularensis is one of the key goals of current research being conducted internationally (Europe and the USA). It could be advantageous if this vaccination can shield scientists dealing with especially severe strains of Francisella tularensis. Research and development on vaccines started in the 1930s in the former Soviet Union. The live attenuated vaccine strain (LVS), which was created from a virulent strain of *Francisella tularensis* subspecies *holarctica*, has recently received the greatest attention from researchers and has been the most extensively utilized vaccine strain.

Although the precise origin of the LVS is unknown, strain 15, which was initially created in Gamaleye, is known to be its progenitor (Eigelsbach 1961; Sandström 1994 Wayne 2004). Primates and rabbits are the best at replicating human illnesses among the animal species that have been examined as disease models.

It appears that mice are affected equally by illnesses brought on by strains of all subspecies, including the *F. novicida* species. (Wayne 2004). The live vaccine strain (LVS) has been widely utilized in recent studies on the virulence and defense mechanisms of *F. tularensis* in mice. Despite the lack of clinical trials for the LVS vaccine, there is some

indication from research involving a small number of volunteers that intradermal immunization may either protect against a future challenge with fully virulent *F. tularensis* or lower the severity of the illness (Saslaw et al. 1961a; Saslaw et al. 1961b). There is some evidence that the immunization is efficacious when given orally and by airborne technique (Saslaw et al. 1961; Eigelsbach 1966; Hornick et al. 1966). In experiments carried out in 1950s and 1960s, volunteers were given the LVS vaccination before being tested with 200–20,000 people using the airborne approach around a year later. All the control subjects received streptomycin or tetracycline as treatment when any of the control subjects developed tularemia for the first time. They all completely recovered from the sickness.

Despite being exposed to up to 2000 germs, most vaccination recipients avoided serious clinical illness. Immunized people showed different disease symptoms after exposure to 20 000 organisms compared to non-immunized participants after receiving the same dose of SCHU S4 (Eigelsbach 1967). In addition to these studies, there is evidence that the LVS vaccine has changed the incidence of laboratory-acquired tularemia.

Burke's 1977 study evaluated the incidence of laboratory-acquired tularemia between the years 1950–1959 and 1960–1969 following the introduction of the routine worker immunization program. The number of instances of typhoidal tularemia per 1000 at-risk workers dropped from 5.7 to 0.27. Although, ulceroglandular tularemia prevalence remained steady, immunization decreased the illness's clinical signs and symptoms. Immunizing lab staff may reduce the risk of infections contracted in laboratories (Rusnak et al. 2004).

In a study done in the Ohio State, Penitentiary with the killed Foshay vaccine and LVS, inmates were exposed to aerosolized SchuS4 (Saslaw et al. 1961). It was discovered that 57% of individuals immunized with Foshay vaccine developed disease and only 17% of LVS immunized men experienced tularemia symptoms (Saslaw et al. 1961). We can easily say through these statistics that the LVS has maintained its potency in protecting against *Francisella tularensis* as against the Foshay vaccine.

On the other hand, the animal model of the LVS hasn't been so successful. When given intradermally, the LVS establishes a sublethal infection in mice, while given intraperitoneal or respiratory route, it has a lethal effect (Cowley et al. 2011).

The oral administration of the LVS has a lethal dose effect less than 50 (i.e., safe for use with this route) this in a way can be said to be the most effect route of administration and it has demonstrated to protect against *Franciscella tularensis.* (KuoLee et al. 2007).

1.9 Detection of *Francisella tularensis*

There are different methods of detecting *Francisella tularensis* in the laboratory. It is expedient to determine which method best suites the purpose of detection (Tab. 2). The commonly used method of detection includes:

- Bacteria Isolation using culture and selective media.
- Serological test
- Molecular techniques

Francisella tularensis can be isolated from blood, sputum, pharyngeal or conjunctival exudates, ulcers, lymph nodes, and gastric washings to identify or diagnose tularemia. It won't grow on common medium, albeit a rare strain will occasionally thrive on blood agar after first isolation. Cultures media which includes Francis medium, McCoy and modified Thayer-Martin agar are used to isolate Francisella tularensis in a biosafety 4 laboratory (Nulens et al. 2002). The media are prepared at 37°C for 48hours in either 5% CO2 or ambient air. On Francis medium and modified Thayer-Martin agar confluent, milky, mucoid colonies are formed. Slow growth can take up to three weeks and identification is based on morphology.

The serological test includes tube agglutination test (TAT), microagglutination, slide agglutination test and enzyme linked immunosorbent assay (ELISA). The slide agglutination test can be used to detect Francisella tularensis. The slide agglutination test involves the production of flakes when 1 drop of blood is mixed with 1 drop of antigen. The TAT method is used to detect antibody in blood samples that contains a fixed quantity of antigen. The tubes that have a clear supernatant fluid are positive.

The enzyme link immunosorbent assay (ELISA) is a sensitive method for quantifying and detecting individual proteins in samples that may contain complex protein mixtures is the enzyme-linked immunosorbent assay (ELISA). Specific proteins adsorbed on the surface of microplate wells are found using antibodies. The method makes it possible to analyze high number of samples quickly and at small volume.

The process of ELISAs can be carried out in different ways depending on the sample specifics and the sensitivity required.

1.9.1 Direct ELISA Techniques:

The application of antigen to ELISA plates is the first step in both direct and indirect ELISA procedures (Lin 2015). According to our methodology, the antigen is first added to the plates, which are then incubated for an hour at 37°C or overnight at 4°C in a humid environment. Following the incubation phase, the plates must be washed of any potential unbound antibodies and any unbound ELISA plate sites must be blocked with Bowen serum albumin (Engvall et al. 2010). The second step is crucial because it lowers the number of false positives and stops non-specific antibodies from adhering to the plate. After adding the solution and the enzyme-conjugated primary detection antibody, the plate is once again washed.

The main detection antibody directly binds to the target protein in a direct ELISA. The plate is then thoroughly cleaned again to get rid of any unbound antibodies. The plate changes color when a substrate or chromophore, such as alkaline phosphatase (AP) or horseradish peroxidase (used for our procedure) is introduced. The oxidation of substrates by HRP or the hydrolysis of phosphate groups from the substrate by AP causes the sample to change color (Kohl et al. 2017). In addition to avoiding secondary antibody cross-reactivity, direct ELISA has the benefit of being quicker than indirect ELISA since it requires fewer stages. Its shortcomings include a low sensitivity compared to other ELISA variants and a high reaction cost (Shah et al. 2016).

1.9.2 Indirect ELISA Techniques:

The step in the indirect ELISA is identical to the direct, except for the additional wash steps and types of antibodies added after the buffer is removed (Lin 2015). Basically, the indirect makes use of the primary detection which sticks to the protein of interest and secondary enzyme linked antibody complementary to the primary antibody (Tabatabaei et al. 2022).

The primary antibody is added first followed by a wash step and the enzyme conjugated secondary antibody is added and incubated. This basically are the step different from the direct ELISA method (Kohl et al. 2017).

In indirect ELISA a 96-well microplate (Engvall 2010) is normally coated with 100 ml per well of bacterial cells (*F. tularensis*). In sequel, analyte is immediately applied to a microtiter plate. The introduction of the sample (biological fluid, such as serum, saliva, etc.) results in the formation of a complex where any antigen-specific antibodies that may be present bind to the analyte (Fig 7). Additional washing removes non-binding proteins and other components from the sample combination. Using a secondary antibody linked to a reporter molecule, the bound antigen specific antibody is identified. The signal may be assessed by comparing the output signal to a standard curve.)

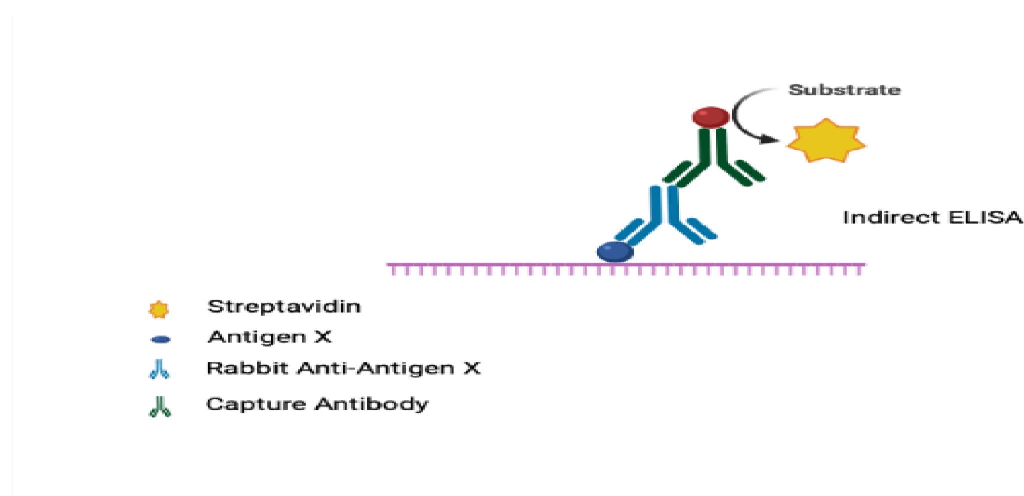


Figure 8. Diagram showing binding of antibodies with antigen in Indirect ELISA test**.**

1.9.3 Polymerase Reaction Techniques:

The PCR is categorized as a molecular technique. It is a straightforward enzyme technique which enables the amplification of a particular DNA fragment from a complicated pool of DNA. The PCR test was created by Dr. Kary Mullis, who claimed that it lets you pick the piece of DNA you're interested in and have as much of it as you want (Mullis 1990).

This technique is frequently employed to quickly produce millions or billions of whole or partial duplicates of a given DNA sample. Unlike the ELISA, the PCR has been reported as a highly sensitive and specific test in several studies (Khalifehgholi et al. 2013; Lu et al. 1999).

This enables researchers to amplify DNA (or a portion of it) from avery tiny sample to a suitable level for in-depth analysis. PCR not only yields data more quickly but also has a greater throughput, enhancing the laboratory's ability to analyze samples. Thermocycling is the bases of operation of most PCR techniques. Thermal cycling involves repeatedly heating and cooling reagents to facilitate a variety of temperature- dependent processes, such as DNA synthesis and DNA replication by enzymes. Two major reagents are used in PCR (which are short fragments of single-stranded DNA called oligonucleotides). Clinical materials such as throat swabs, scrapings from ulcers, lymph node aspirates or biopsies, and respiratory specimens are used to isolate *F. tularensis* (e.g., pleural fluid). Blood cultures frequently come back negative.

Table 2. Test methods available for the diagnosis of tularemia and their limitations

Method	Population free from infection	Individual animal free from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection surveillance	Immune status in individual animals or populations postvaccination
	Detection of the agent					
Antigen detection	-	-	-	++	-	-
Bacterial isolation	-	-	-	+++	-	-
Real-time PCR	+	-	-	+++	+++	-
Conventional PCR	+	-	-	+++	+	-
	Detection of immune response					

TAT	++	++ +	++	+++	+++	-
SAT	+++	+++	+++	++	+++	-
MAT	++	+++	++	+++	+++	-
ELISA	++	+++	++	++	+++	-

+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction; SAT = slide agglutination test; TAT = tube agglutination test; MAT = microagglutination test; ELISA = enzyme-linked immunosorbent assay. (Versage et al. 2003; Barns et al. 2005)

2. 0 Aim of Thesis

This study aims at describing the seroprevalence of *Francisella tularensis* antibodies in European bown hare (*Lepus europaeus*) which are one of the most common game animals in Czechia and know reservoir of *Francisella tularensis*.

3.0 Materials and Method

3.1.1 Sample Collection

In preparation of this experiment, blood samples were collected by hunters during the winter season of 2021. Eight (8) European brown hare (*Lepus europaeus*) blood samples were collected at Central Bohemian Region in Kněžice u Městce Králové (ZIP 28902) on 18th December 2021, forty-six (46) samples were collected at South Moravian Region in Březí u Mikulova (ZIP 69181) on 26th December 2021 and three (3) samples were collected at Zlín Region in Kladeruby (ZIP 756430) on 28th December 2021. Antigens were given out of benevolence by the Military Research Institute of the Czech Republic. A total of fifty-seven (57) hare blood samples were analyzed. The blood samples to be analyzed were collected in a plain collecting tube and spined using centrifuge at 6500RPM. The blood spined was separated into three phases: blood cells, white blood cells and platelets, serum.

The serum was carefully removed and dispensed into a new tube. The serum was stored at -20°Celsius. The serum could be a source of infection; thus, serum was handled with the use of a hand gloves. The samples were kept in the freezer to prevent degradation.

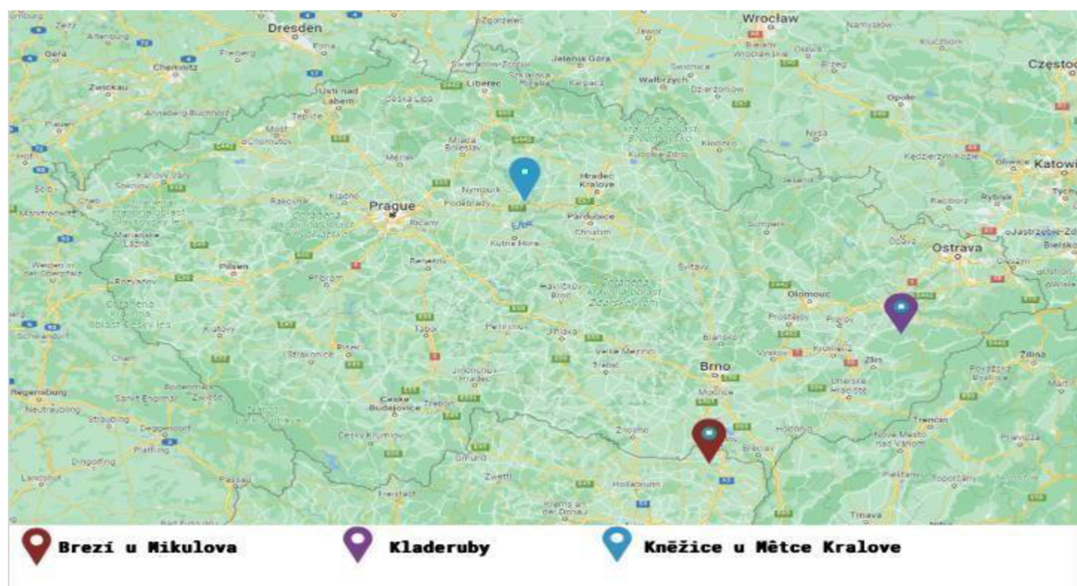


Figure 9: Map Showing Area of Sample Collection

- *1 x PBS dilution:*

The Phosphate buffer saline is an isotonic solution (pH of 7.4). To prepare a working solution of 1 x PBS (1000ml), we mixed 100mls of 10 x PBS to 900mls of distilled water.

- *Serum dilution:*

A serum is separated from coagulating blood. It is amber in color and rich in protein. The serum was diluted to achieve 1:800 i.e., 1µl of serum in 799µl of PBS. Using the dilution 1:800 gave the best result. Other dilution caused a low signal or too high background.

- *Antigen dilution:*

Antigens are substances e.g., bacteria, virus, fungi, allergens that cause the body to produce antibodies. Antigens were prepared by mixing 1µl antigen into 999 µl 1 x PBS. This mixture makes a total of 1000µl.

- *Preparation of Washing buffer:*

The washing buffer helps to eliminate excess materials found in the wells of the microtiter plate. This removal occurs without disruption of the antigen-antibody binding reaction. They are often used during the coating process and between reagent addition steps. The washing buffer was prepared by mixing 250ml of PBS (Ph of 7.4) with 125µl of Tween-20 (0.05%)

- *Preparation of Blocking buffer:*

This is done by making a homogenous solution of 2% Bovine Serum Albumin powder mixed with 1x PBS i.e., Prepared by adding 2g of Bovine Serum Albumin in 100ml of PBS.

• *Preparation of PROTEIN A solution:*

Protein A which was prepared (diluted) using 1 µl: 1999 Protein A in 2% 100 µl of Bovine Serum Albumin was added to each well. The protein A is used in our ELISA protocol to determine or detect the circulating IgG antibodies to a bacteria organism (*Francisella tularensis*) instead of the secondary antibody. The dilution 1:1999 gave the best result. Other dilution caused a low signal or too high background.

Protein A has a specific affinity to the Fc domain on IgG antibodies from many species and therefore it allows the use of our technique of sera from wide spectrum of different animals.

3.1.3 Laboratory Protocol Used (Indirect Elisa)

The indirect ELISA was standardized and performed as described in literatures (Mateo-de-Antonio et al. 1993; Carpenter 1997) with modifications. The loading concentration of the *Francisella* antigen was prepared by diluting the storage concentration of *Francisella* antigen (1µl antigen into 999µl PBS). Incubation was performed in a humid chamber (Fig.10). 1000 µl of antigen was prepared. From this solution, 100 µl was pipetted into the ELISA plates and kept in a humid chamber (Fig. 10) and kept in the fridge at 4°C overnight. On the following day the ELISA plate is brought out and washed 3 times using a washing buffer (Washing buffer prepared as explained above).

Each well of the antigen-coated plates was blocked with 100µl of 2% blocking buffer on the second day. The plates are then kept in a humid chamber (Fig. 10) and refrigerated over-night. On the third day after three washes with washing buffer, 100 µl of test 1:800 diluted sera samples were pipetted in each well. Samples were analyzed in triplicates. The plates were sealed in a humid chamber (Fig. 10) and kept overnight.

On the fourth day, the plates were emptied again and washed three times using the usual washing buffer. Protein A which was prepared (diluted) by taking 1µl: 1999 Protein A in 2% 100 µl of Bovine Serum Albumin was added to each well. On the 5th day, the content of the plate is thrown away and washed using the washing buffer.

Afterwards a 50µl substrate (TMB Microwell Peroxidase, KPL SureBlue TM USA) was added in each well. The plates were kept for 10-minutes to allow development of color change reaction. (Fig. 9). The reaction was stopped after 10-minutes. Color development was halted by the addition of 50 µl/well of 2M H_2SO_4 into each well. This stops the reaction and changes the color of the wells from blue to yellow. The absorbance measurements were made at 450 nm, using an automatic ELISA plate reader (550, Bio-Rad, Life Sciences, Hercules, USA). Samples with an absorbance value equal to or higher than 0.9 (O.D value) were considered as positives (Fig. 10). Blank sample (with no added serum) was used as a negative control.

Figure 10. Picture of Antigen *F. tularensis* in the ELISA plate.

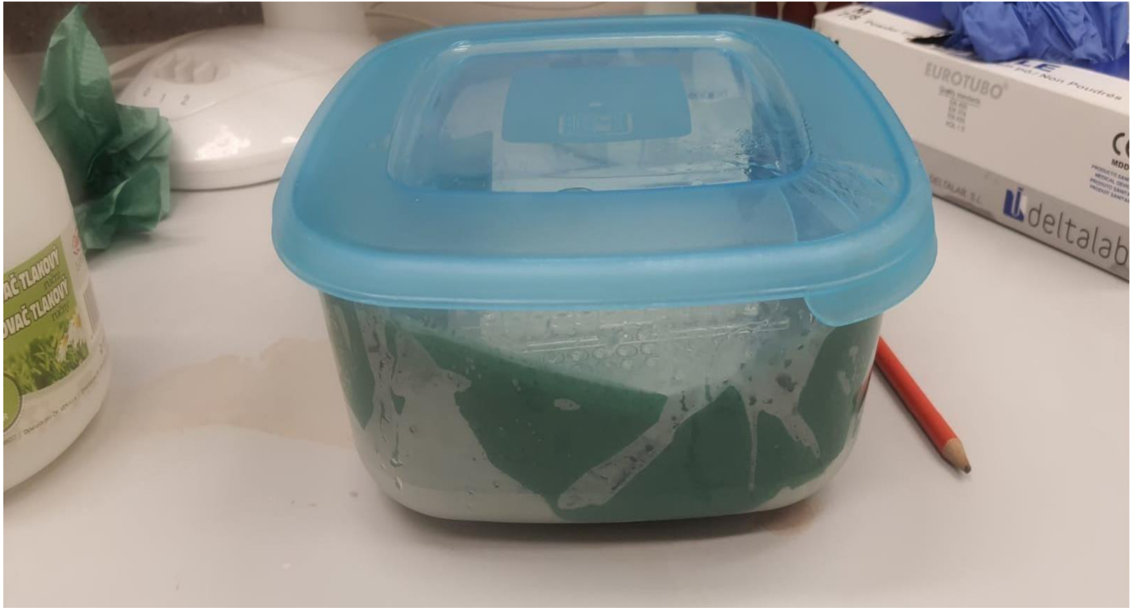


Figure 11. Picture of the Improvised Humid Chamber.

3.2 Statistical Analysis

Average optical densities for each serum sample were calculated as a mean from three independent measurements. These three independent measurements served also as a base to calculate standard deviations. Samples were considered positive when their average OD value was 2 times higher than OD of the negative control.

Seroprevalence was calculated for all collected samples from the whole Czech Republic as well as for individual sampled regions. For seroprevalence calculation following formula was used:

$$\text{Calculation of Prevalence\%} = \frac{\text{Number of positive samples}}{\text{Total number of samples collected in each region.}}$$

4.0 Results

Fifty-seven (57) samples were collected from three areas of the Czech Republic (South Moravian – n = 46, Central Bohemia – n = 8, and Zlin Regions – n = 3). Basically, I was able to detect *Francisella tularensis* antibodies only in hare sera from the South Moravian Region. Eighteen samples (18) out of 46 collected in Březí u Mikolova were positive for *Francisella tularensis* antibodies (Tab. 3, Fig. 12) which depicts that the seroprevalence of *Francisella tularensis* antibodies in Southern Moravia region is 39.1% (Tab. 4, Fig. 14). No samples collected in the Central Bohemia Region and Zlin Regions were positive (Tab. 3, Fig. 12).

Table 3. Results from the ELISA Plate**.**

SN	Location	Mean	S.DS	Well 1	Well 2	Well 3	Interp.
1	Mëstce Králove	0,367017	0,107898	0,30359	0,30586	0,4916	Negative
2	Mëstce Králove	0,27976	0,020503	0,29231	0,29087	0,2561	Negative
3	Mëstce Králove	0,29184	0,004946	0,29672	0,29197	0,28683	Negative
4	Mëstce Králove	0,313973	0,052503	0,27493	0,29333	0,37366	Negative
5	Mëstce Králove	0,49796	0,008055	0,49278	0,50724	0,49386	Negative
6	Mëstce Králove	0,396793	0,028369	0,42803	0,38972	0,37263	Negative
7	Mëstce Králove	0,77279	0,028216	0,74048	0,78531	0,79258	Positive
8	Mëstce Králove	0,358713	0,018317	0,33863	0,36301	0,3745	Negative
9	Brezi u Mikulova	0,90445	0,031816	0,86969	0,93213	0,91153	Negative
10	Brezi u Mikulova	0,381757	0,008733	0,38334	0,37234	0,38959	Negative
11	Brezi u Mikulova	0,31516	0,027456	0,28785	0,34276	0,31487	Negative
12	Brezi u Mikulova	0,357617	0,022982	0,33553	0,3814	0,35592	Negative
13	Brezi u Mikulova	0,906393	0,007603	0,90699	0,91368	0,89851	Positive
14	Brezi u Mikulova	1,0609	0,027372	1,0676	1,0308	1,0843	Positive
15	Brezi u Mikulova	0,382293	0,012823	0,36799	0,39276	0,38613	Negative
16	Brezi u Mikulova	0,451793	0,032222	0,48702	0,44455	0,42381	Negative
17	Brezi u Mikulova	0,994217	0,046764	0,94125	1,0298	1,0116	Positive
18	Brezi u Mikulova	0,278383	0,029115	0,27053	0,254	0,31062	Negative
19	Brezi u Mikulova	0,887497	0,015658	0,89129	0,87029	0,90091	Positive
20	Brezi u Mikulova	0,381417	0,014335	0,39119	0,36496	0,3881	Negative
21	Brezi u Mikulova	0,348243	0,011626	0,34282	0,34032	0,36159	Negative
22	Brezi u Mikulova	0,317773	0,010536	0,31053	0,32986	0,31293	Negative
23	Brezi u Mikulova	0,86845	0,005561	0,86207	0,87227	0,87101	Positive
24	Brezi u Mikulova	0,987103	0,032143	0,96604	1,0241	0,97117	Positive

25	Brezi u Mikulova	0,411963	0,028189	0,41389	0,43914	0,38286	Negative
26	Brezi u Mikulova	0,41678	0,016777	0,39741	0,42619	0,42674	Negative
27	Brezi u Mikulova	0,91899	0,015376	0,93428	0,90353	0,91916	Positive
28	Brezi u Mikulova	0,902217	0,00371	0,89915	0,90634	0,90116	Positive
29	Brezi u Mikulova	0,32694	0,0151	0,31154	0,32756	0,34172	Negative
30	Brezi u Mikulova	1,042357	0,067712	1,0636	0,96657	1,0969	Positive
31	Brezi u Mikulova	0,97034	0,046748	0,91689	1,0036	0,99053	Positive
32	Brezi u Mikulova	0,348863	0,011338	0,34593	0,33928	0,36138	Negative
33	Brezi u Mikulova	0,923377	0,04117	0,90474	0,97057	0,89482	Positive
34	Brezi u Mikulova	0,503267	0,018622	0,48566	0,50138	0,52276	Negative
35	Brezi u Mikulova	0,47801	0,032523	0,47273	0,51285	0,44845	Negative
36	Brezi u Mikulova	0,306153	0,019435	0,30613	0,28673	0,3256	Negative
37	Brezi u Mikulova	1,04933	0,023496	1,1745	0,89919	1,0743	Positive

38	Brezi u Mikulova	0,857467	0,199196	0,82242	0,84244	0,90754	Positive
39	Brezi u Mikulova	0,397637	0,014245	0,34305	0,45569	0,39417	Negative
40	Brezi u Mikulova	0,55764	0,139343	0,53431	0,55643	0,58218	Negative
41	Brezi u Mikulova	0,783413	0,044505	1,0081	0,63928	0,70286	Positive
42	Brezi u Mikulova	0,35939	0,0564	0,35372	0,35991	0,36454	Negative
43	Brezi u Mikulova	1,234967	0,023958	1,265	1,1956	1,2443	Positive
44	Brezi u Mikulova	1,142167	0,197164	1,1617	1,1344	1,1304	Positive
45	Brezi u Mikulova	0,36185	0,005429	0,37667	0,37384	0,33504	Negative
46	Brezi u Mikulova	0,644717	0,035629	0,65653	0,63716	0,64046	Negative
47	Brezi u Mikulova	1,03725	0,017034	0,99595	1,0585	1,0573	Positive
48	Brezi u Mikulova	0,77699	0,023261	0,77438	0,77313	0,78346	Positive
49	Brezi u Mikulova	1,1192	0,010363	1,1115	1,1193	1,1268	Positive
50	Brezi u Mikulova	0,428133	0,035772	0,39617	0,50112	0,38711	Negative
51	Brezi u Mikulova	1,182867	0,005638	1,2142	1,213	1,1214	Positive
52	Brezi u Mikulova	0,639917	0,00765	0,58603	0,64398	0,68974	Negative
53	Brezi u Mikulova	0,603	0,06337	0,50188	0,51957	0,78755	Negative
54	Brezi u Mikulova	1,031067	0,053235	1,0191	1,0331	1,041	Positive
55	Kladeruby	0,39853	0,051974	0,39107	0,37967	0,42485	Negative
58	Blank	0,481933	0,131614	0,36781	0,62591	0,45208	Blank
59	Negative	0,45014	0,086057	0,41036	0,54889	0,39117	**Control**

Figure 12. Bar plots representing mean of O.D values.

Table 4. Showing location where samples were collected in Czech Republic.

Location OfSample Collection	Code	Total No. Of Samples	Annotation	Prevalence
Central Bohemia Knêzice u Mëstce Králové	ZIP 28902	8	0 Positive	0%
South Moravia Březí u Mikulova	ZIP 69181	46	18 Positive	39.1%
Zlín Region Kladeruby	ZIP 75643	3	0 Positive	0%

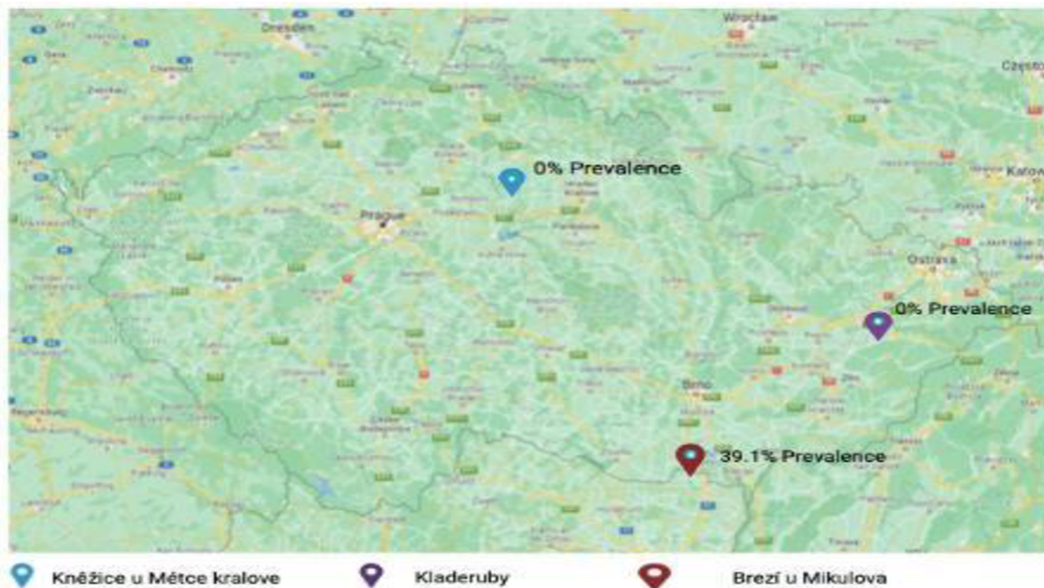
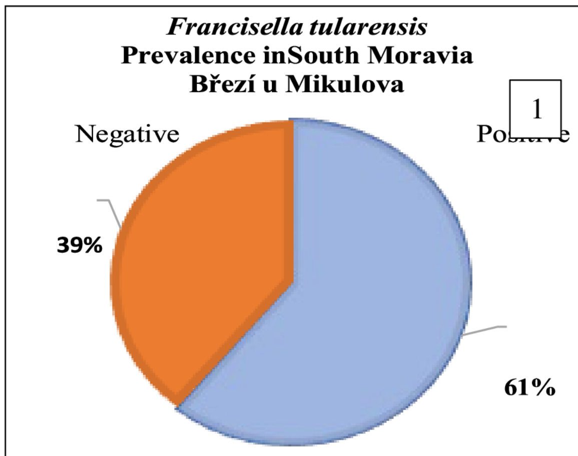
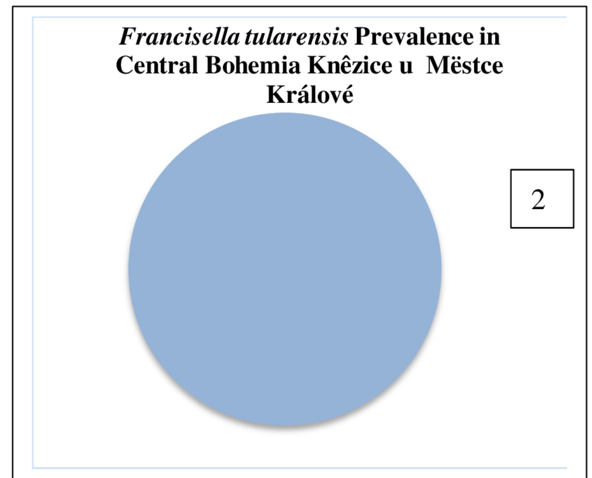
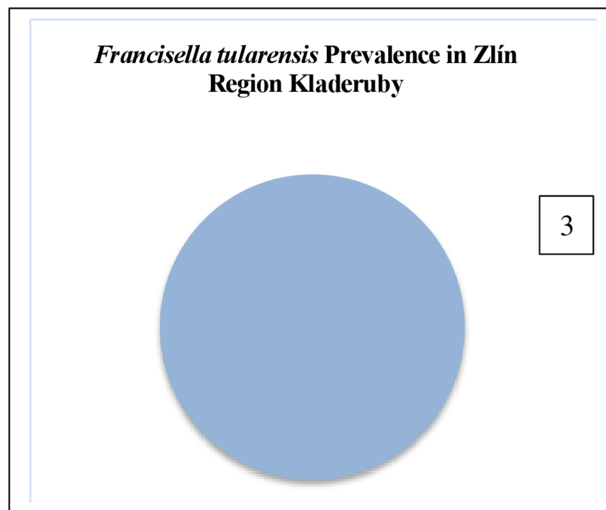


Figure 13: Map Showing Area of Sample Collection with Prevalence (Total numberof positive samples) of *Francisella tularensis* in Central Bohemia Knêzice u MëstceKrálové, South Moravia Březí u Mikulova, Central Moravia Kladeruby.







Positive Samples

Negative Samples

Figure 14. Charts representation of prevalence of *Francisella tularensis* infection in hares in selected areas of Czech Republic.

The chart 1 depicts that the prevalence of t*ularensis* bacteria is 39.1% in South Moravian Region while its occurrence is 0% in both Zlin and Central Bohemia Region (chart 2 and 3).

5.0 Discussion

Serological analysis conducted in this thesis revealed that considerable number of hares in South Moravian Region (Březí u Mikulova) of the Czech Republic have antibodies against *Fransicella tularensis*. I was not able to detect any seropositive hares in the Central Bohemian Region (Městce Králové) and Zlín Region (Kladeruby).

This means that at some point the animals (hares) in the Mikulova area have been infected by *Fransicella tularensis*. When or how they got infected wasn't investigated. When animals or humans get infected by bacteria, there is production of antibodies (Immunoglobulins) basically the IgG and IgM. The antibodies are what is captured by the ELISA.

The ELISA method was adopted for this procedure as against the polymerase chain reaction because the disease detection window is substantially longer in ELISA, increasing the likelihood of determining whether an infection has occurred. It is possible to test positive for antibodies with ELISA long after the illness has left the herd or human, because it offers herd level information about previous infections unlike polymerase chain reaction (PCR) a diagnostic technique used to determine the cause of death or to confirm the clinical ailment by identifying the pathogen's RNA or DNA, PCR is made to identify the pathogen itself.

For the experiment, sera from three different localities which has been highlighted above were collected. This samples don't cover the whole Czech Republic. The samples were collected in 2021. Those samples were acquired and refined for the serologically analysis. An ELISA screening that proved to be effective for conducting epidemiological research in tularemia was carried out. A total of 57 samples were collected randomly in areas which includes Central Bohemian Region (Kněžice u Městce Králové), South Moravian Region (Březí u Mikulova) and Zlín Region (Kladeruby) and analyzed using ELISA test. During this procedure we ensured a reasonable degree of asepsis. The ELISA plates were labeled for easy identification.

In total, 18 of the samples collected showed the presence of antibodies against Francisella tularensis. The samples with optical density value of 0.90 and below is termed as negative. The samples which are positive have an optical density value of 0.90 and above. This is two times the value of the negative which we used as our standard (0.45).

The likelihood, that hares get infected by *Francisella tularensis* in South Moravian Region (Březí u Mikulova) is high with a seroprevalence of 31.6% and 18 positive samples out of total 46 samples gotten from there. This result is factual as it buttresses findings from other works of Cerny (2001) and Pikula (2004) showing that South Moravian Region is highly endemic for *Francisella tularensis*.

In the other two regions where samples were collected, no positive samples were detected. No positive sample was found out of the total 8 samples collected in Central Bohemian Region (Knězice u Městce Králové) thus the disease prevalence in that region is 0% and no positive sample was found in Zlín Region (Kladeruby) out of 3 samples collected which accounts for a prevalence of 0% (Table 3). Nevertheless, low number of samples gotten from these locations of the Czech Republic might be the reason why we have not detected tularemia prevalence in these two areas and therefore we cannot confirm absence of *Francisella tularensis* in these regions. It is also possible that the disease-causing organism *Francisella tularensis* isn't present in these localities due to the reduction of population of hares as result of increase in human activity e.g., advanced agriculture over the years or even the absence of arthropods transmitting the bacteria. (Miroslav 2002). Also, the geographical arrangement of Městce Králové and Kladeruby might not support the symbiotic host or vector relationship. Different other factors such as temperature, direct exposure to sunlight are physical factors that could affect survival of the microbes.

Looking at the results from the South Moravian Region, it is evident that hunters in this region (Březí u Mikulova) are at risk of contracting *Francisella tularensis.* Due to low number of samples from the last two regions, it is difficult to make any quantitative comparison and confirm the prevalence of *Francisella tularensis* in Central Bohemia (Kněžice u Městce Králové) and Zlín Region (Kladeruby).

The South Moravian Region (Březí u Mikulova) has favorable condition for *Francisella tularensis* such as annual air temperature of 8.1-10.0°C, precipitation of 450-700mm and sunshine duration of 1801-2200h (Pikula et al. 2004). Also, the South Moravian region is characterized by flat plains, dominated by fields, grassland used for hays and the remainders of riparian forest.

These attributes would encourage host and vector relationship thus *Francisella tularensis* causing disease tularemia would persist and thrive in such environment. This basic concept of landscapes epidemiology also gives a backing previous works by Pavlovsky (1964). Further, the European brown hare is adaptable and thrives in mixed farmland with open field and scattered bushes (Chapman et al. 1990) this isn't the geographical makeup in Kněžice u Hradce Králové nor Kladeruby. The lack of favorable conditions for hares to thrive might be the reason why there are less populations of hares to hunt which resulted to low sampling and finally no positives. Based on past scientific findings, there are two main regions where tularemia is prevalent in Czech Republic, they are basically the South Moravia and Central Bohemia. While the Southern Moravia is a well-known area for tularemia, the Central Bohemia hasn't become so known (Pikula et al. 2004).

This thus solidifies the claim our result which shows high prevalence (31.6%) (Fig 14) of tularemia cases in South Moravia. The possible reason for this high prevalence has I have earlier described is likely due to the geographical representation of this place. Tularemia in humans in Czech Republic cannot be overemphasized but not only humans (hunters) are affected species. Companion animals such as cats and dogs and wild animals contract this disease. Contracting this disease could be because of their feeding habit or social behaviors. In Europe and some other part of the world, dogs which are used in hunting often get infected with tularemia (Johnson 1944; Zidon 1964) when they come contact with either the ticks or infected hares.

When an infected hare is encountered in the woods, regardless of tularemia, it is always best to take only rabbits or other wild animals that show vigor and can be taken only with difficulty (Pikula et al. 2004). The typical infection as found in a rabbit can be recognized by presentation of tiny white spots on the liver and spleen. The white spots are abscesses caused by growth of the microorganism in those organs.

The infection is of relatively short duration in rabbits and hares, usually lasting about seven days. The enzyme-linked immunosorbent test (ELISA) has established itself as a trustworthy method for the identification of infections and the diagnosis of several dangerous infection.

Usually, 2 weeks after the commencement of the disease, the presence of an antibody response to *F. tularensis* confirms the diagnosis of tularemia patients (Koskela et al. 1985). Hunting is practically risk-free once cold weather has forced the ticks to abandon the rabbits and after an extra week has passed, it is expected that infected animal dies and decays (Green et al. 1943). This is an important recommendation that can be given to hunters. Also, laws that prevent indiscriminate transfer or movement of animals without prior test against tularemia should be enacted in other to protect the region such as Kněžice u Městce Králové and Kladeruby.

Finally, not all is negative about tularemia in the environment, the bacteria disease can also be put to use so far it can be controlled. The bacteria can be used to control rodent invasion (Bell et al. 1981; Jellison et al. 1958)

6.0 Conclusion

In the Laboratory for detection and characterization of pathogens, I was able to demonstrates the existence of antibodies against tularemia. In my result, I was able to find 18 positive sample in the total of 57 samples collected from hunters. The 18 positive samples which equals 31.6% of the total sample were gotten from South Moravia Březí u Mikulova.

This prevalence is highly considerably, and it means that hunters in the Czech Republic who come are in constant contact with hares are at risk on contract this infection. This experiment further emphasis previous work done in Czech Republic

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