Szent István University

Postgraduate School of Veterinary Science

Epizootic investigations of tularemia and the comparative characterization of *Francisella tularensis* strains

Ph.D. dissertation

Miklós Gyuranecz

2011

Supervisors and consultants:

.....
Professor László Fodor, C.Sc.
Department of Microbiology and Infectious Diseases
Faculty of Veterinary Science
Szent István University
supervisor

.....
László Makrai, Ph.D.
Department of Microbiology and Infectious Diseases
Faculty of Veterinary Science
Szent István University
co-supervisor

Ádám Dán, Ph.D.
Department of Molecular Biology
Veterinary Diagnostic Directorate
Central Agriculture Office
consultant

Béla Dénes, C.Sc.
Department of Immunology
Veterinary Diagnostic Directorate
Central Agriculture Office
consultant

Károly Erdélyi, Ph.D.
Department of Mammalian Pathology
Veterinary Diagnostic Directorate
Central Agriculture Office
consultant

Gábor Földvári, Ph. D.
Department of Parasitology and Zoology
Faculty of Veterinary Science
Szent István University
consultant

Professor Paul S. Keim, Ph.D.
Center for Microbial Genetics and Genomics
Northern Arizona University
consultant

Levente Szeredi, Ph.D.
Department of Mammalian Pathology
Veterinary Diagnostic Directorate
Central Agriculture Office
consultant

Copy ….. of eight.

…….
Miklós Gyuranecz

Table of contents

Abbreviations

16S rRNA gene	16S ribosomal ribonucleic acid gene
B.Br	type B (*F. tularensis* ssp. *holarctica*), branch
bp	base pair
canSNP	canonical SNP
CFU	colony forming unit
Ct	cycle threshold
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
fopA gene	outer membrane protein gene
GN2	Gram-negative 2
GN-FAS	Gram-negative, fastidious
HE	hematoxylin and eosin
IHC	immunohistochemistry
LD_{50}	lethal dose 50
LPS	lipopolysaccharide
MAMA	mismatch amplification mutation assay
MLVA	multi-locus variable-number tandem repeat analysis
NMRI	Naval Medical Research Institute
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
ssp	subspecies
Tm	melting temperature
Tris-EDTA	Tris-ethylenediaminetetraacetic acid
tul4 gene	17 kDa major membrane protein precursor gene
VNTR	variable-number tandem repeat
WG	whole genome

1. Summary

Francisella tularensis is the etiological agent of tularemia, a lethal zoonotic disease and a potential biological warfare agent. In the last decades, several emergences or re-emergences of tularemia were seen all over the world which has focused the attention on this disease. The first human tularemia cases were observed in Hungary in 1951 and the disease has been diagnosed every year ever since. In most cases tick bite, close contact with European brown hares (*Lepus europaeus*), hamsters (*Cricetus cricetus*) or rats (*Rattus* spp.) were found in the anamnesis. Furthermore, in efforts to replenish game populations for sporting purposes, thousands of brown hares are annually translocated from Hungary to France and Italy which is a significant income for the country. The tularemia free status of the exported hares is crucial to maintain this export.

Through retrospective data collection it was shown that the number of human cases in Hungary ranged between 20 and 148 per year during the past two decades (1984-2009). At the same time the prevalence of tularemia among hares, captured for live animal export (2.8-40 thousand exported hares/year) ranged between 0.31% and 20.2%.

A one-year study of the ecological cycle of *F. tularensis* was performed in an enzootic area during an inter-epizootic period. The study was based on multiple sampling of all major elements of the disease cycle. Seroprevalence of tularemia in the European brown hare population was 5.1% (10/197) with low titers (1/10 and 1/20) and *F. tularensis* ssp. *holarctica* was isolated from four hares. Based on these results the modification of the diagnostic tube agglutination titer 1/80 was presumed. *F. tularensis* was not detected by real-time polymerase chain reaction in any of the trapped 38 common voles (*Microtus arvalis*), 110 yellow-necked mice (*Apodemus flavicollis*), 15 stripped field mice (*Apodemus agrarius*) and a by-catch of 8 Eurasian pygmy shrews (*Sorex minutus*) and 6 common shrews (*Sorex araneus*). A total of 1106 *Ixodes ricinus* and 476 *Haemaphysalis concinna* ticks were collected from vegetation and 404 *I. ricinus*, 28 *H. concinna* ticks and 15 *Ctenophtalmus assimilis* and 10 *Nosopsyllus fasciatus* fleas were combed off small mammals. One *H. concinna* female and one nymph collected from the vegetation were infected with *F. tularensis* ssp. *holarctica* thus resulting a 0.42% (2/476) prevalence. *F. tularensis* was not detected in environmental water samples and the examined 100 sheep, 50 cows and 50 buffaloes, grazed in the study area, were all found seronegative. It can be hypothesized that during interepizootic periods *F. tularensis* ssp. *holarctica* persists only in the European brown hare – *H. concinna* cycle. *H. concinna* may not serve exclusively as an arthropod vector but it might also harbor bacteria for three to four years through multiple life stages and act as an important reservoir of *F. tularensis*. Rodent species probably do not play as true reservoir hosts of *F. tularensis*.

The role of the common hamster in the natural cycle of *F. tularensis* was examined using serologic methods on 900 hamsters and real-time polymerase chain reaction on 100 hamsters in an endemic agricultural area. 374 *Ixodes acuminatus* ticks were collected from the hamsters and tested by real-time polymerase chain reaction. The results of all tests were negative. To examine clinical signs, pathology and histopathology of acute tularemia infection similar to the natural infection, two hamsters were infected with a large dose of a wild strain of *F. tularensis* ssp. *holarctica.* After a short period of apathy, the animals died on the 8th and 9th days postinfection. The pathological, histopathological and immunohistochemical examination contributed to the diagnosis of septicemia in both cases. The results confirmed previous findings that common hamsters are highly sensitive to *F. tularensis*. It was concluded that although septicemic hamsters could pose substantial risk to humans during tularemia outbreaks, hamsters in interepizootic periods do not act as a significant reservoir of *F. tularensis*.

Lesions induced by *F. tularensis* were examined in 50 cases of naturally infected, seropositive European brown hares. Gross pathological examination revealed scant to numerous, grayish-white foci with a diameter of 0.1-1 cm in single (24 cases) or multiple organs (20 cases) in a total of 44/50 (88%) cases. These lesions were proven to be areas of granulomatous inflammation, frequently encompassing necrosis. *F. tularensis* antigen was detected with immunohistochemistry in a total of 46/50 (92%) cases, while *F. tularensis* ssp. *holarctica* was isolated by culture and identified by polymerase chain reaction from 35/50 cases (70%). Infection by respiratory route was presumed by the presence of tissue lesions in the thoracic organs in 44/50 (88%) cases. These results emphasize the importance of the European brown hare as a reservoir of *F. tularensis*.

Generalized tularemia were diagnosed in a patas monkey (*Erythrocebus patas*) and a vervet monkey (*Chlorocebus aethiops*), which both died suddenly in Szeged Zoo, Hungary. Macroscopic lesions in each animal included disseminated, grayish-white foci in the lungs, lymph nodes, spleen, liver, and kidney. All focal lesions were characterized microscopically as purulent to pyogranulomatous to granulomatous inflammation with necrosis. *F. tularensis* ssp. *holarctica* strains were isolated from tissue samples and identified by a commercial carbon-source utilization test and polymerase chain reaction.

A *F. tularensis* ssp. *holarctica* strain collection was established in Hungary. Sixty-three strains were isolated from European brown hares originating from different parts of Hungary and two strains from Austria. Two further strains were isolated from the patas monkey and the vervet monkey from Szeged Zoo.

Utilisation of carbon sources of 15 *F. tularensis* strains was characterised with the Biolog system. The system was already able to identify the strains after 4 hours of incubation, instead of the standard 24 hours. After the analysis and comparison of the metabolic profiles

of our strains with the Biolog database, it was concluded that not all carbon sources indicated in the database were utilized by our isolates. The Biolog software failed to distinguish the highly virulent *F. tularensis* ssp. *tularensis* and the moderately virulent *F. tularensis* ssp. *holarctica.* Still the Biolog microplates could be manually read to differentiate the two subspecies based on glycerol source utilisation. As none of the studied strains was able to use glycerol they could be identified as *F. tularensis* ssp. *holarctica*. The dendrogram based on the metabolic relationship of the strains showed that the isolates are very similar to each other, which correlates with the conservative genetic character of *F. tularensis* ssp. *holarctica.* The whole genome of a Hungarian *F. tularensis* ssp. *holarctica* isolate was sequenced and was compared to 5 other complete genomes. The phylogenetic characterization of 19 *F. tularensis* isolates from Hungary and Italy was also performed. *F. tularensis* isolates from Hungary belonged to the B.Br.013 lineage and descended from a diverse set of minor subclades comprised of strains found throughout Central Europe, Scandinavia and Russia. *F. tularensis* isolates native to Italy belonged to the B.Br.FTNF002-00 subclade, a distinct genetic group comprising isolates from France, Spain, Switzerland and parts of Germany. The results on the genetic differences of the strains enabled us to contradict the hypothesis that Central Europe is the direct source of Western European (e.g. France, Italy) *F. tularensis* strains through hare importation. Important additions to the European phylogeographic model of *F. tularensis* were also provided and a set of powerful molecular tools for investigating *F. tularensis* dispersal throughout Europe was presented.

2. Introduction

Tularemia is a zoonotic disease caused by the small, Gram-negative bacterium, *Francisella tularensis*, one of the most infectious bacteria known, with <10 organisms capable of causing severe disease in both humans and animals (Ellis et al., 2002).

It is reported from most countries in the northern hemisphere, although its occurrence varies widely from one region to another and it recently emerged in areas with no previously known risk (Petersen and Schriefer, 2005). Differences in *F. tularensis* virulence and geographic distribution are highly correlated with their genetic designation which is structured into subspecies and subclades (Keim et al., 2007).

F. tularensis has a remarkably broad host range, probably the broadest of all zoonotic agents. However, tularemia is primarily a disease of the genera *Lagomorpha* and *Rodentia* while haematophagous arthropods serve as vectors for transmission (Mörner and Addison, 2001). The pathology of tularemia differs considerably among different animal species. Generally, in acute cases, the most characteristic necropsy finding is the enlarged spleen, while multiple, granulomatous foci of coagulation necrosis are found in several organs in a more chronic form of the disease. Diagnosis of tularemia is based on the combined results of necropsy findings and the detection of *F. tularensis* from the samples or tissues using isolation, molecular tools or serological tests (OIE, 2008).

Humans are highly susceptible to *F. tularensis*. Infections in humans are not contagious and most often transmitted to humans by arthropod bites, by direct contact with infected animals, infectious animal tissues or fluids, by ingestion of contaminated water or food or by inhalation of infective aerosols (Dennis et al., 2001). In addition to its natural occurrence, *F. tularensis* causes great concern as a potential bioterrorism agent. It is on the list of Class A biothreat agents (Dennis et al., 2001).

In Hungary, the first human *F. tularensis* infections were detected in 1951 and the disease has been observed every year ever since. Generally, tick bites, close contact with European brown hares (*Lepus europaeus*), hamsters (*Cricetus cricetus*) or rats (*Rattus* spp.) were found in the anamnesis (Epinfo). Thousands of brown hares are annually translocated from Hungary to France and Italy to replenish game populations for sporting purposes. This is a significant income for the country (Somogyi, 2006) but the tularemia free status of the exported hares is crucial for the export.

3. Review of the literature

3.1. History

F. tularensis, the etiological agent of tularemia, was first isolated and characterized by McCoy and Chapin in 1912 during an outbreak of a “plague-like” disease in ground squirrels in Tulare County, California, United States (McCoy and Chapin, 1912). They named the infectious agent “*Bacterium tularense*”. Two years later, the first human illness attributed to *F. tularensis* was described by Wherry and Lamb in Ohio, United States, who isolated the bacterium from two patients with confirmed wild rabbit contact (Wherry and Lamb, 1914). Subsequently, Edward Francis (Figure 1), after whom the genus is named, confirmed that several clinical syndromes throughout the United States were caused by *F. tularensis* and proposed the name “tularemia” to describe them (Francis, 1921, Francis et al., 1922). Both the species and disease name are derive from Tulare County.

Figure 1. Portrait of Edward Francis (1872–1957) (University of Montana, MO).

Until 1925, it was widely believed that tularemia was a disease with a risk limited to the United States. In the Union of Soviet Socialist Republics, in 1928, *F. tularensis* was recognized as the causative agent of an illness acquired by trappers who skinned water-rats for their pelts (Olsufjev et al., 1959). Ohara et al. (1935), studying hare diseases in Japan, recognized a similar disease to tularemia and sent specimens to Francis, who confirmed the presence of *F. tularensis*. Soon thereafter, tularemia was also reported in Norway (1929), Canada (1930), Sweden (1931) and Austria (1935) (Ellis et al., 2002). Historically it was known as plague-like disease of rodents, hare plague (Sweden), leemands soet (lemming fever – Norway), sibiriskaia iazva (Siberian ulcer – Russia), deerfly fever (USA), or yato-byo (hare disease – Japan) (Friend, 2006).

The first human tularemia cases were recorded in Hungary in 1951 and *F. tularensis* infection has been diagnosed every year ever since. The last scientific data were published in this field in Hungary in the 1960's and 1970's (Füzi and Kemenes, 1972; Kemenes, 1976; Kemenes et al., 1965; Kocsis, 1964; Münnich and Lakatos, 1979) except two small reports; one about the *F. tularensis* infection of children (Dittrich and Decsi, 1999) and the other about the clinical experiences of tularemia treatment from the Pándy Kálmán hospital of Békés county (Bányai and Martyin, 2006).

3.2. Taxonomy and geographic distribution

Phylogenetically, *F. tularensis* is not closely related to any other pathogenic bacteria. It belongs to a group of intracellular bacteria. The *Francisella* genus is the sole member of Francisellaceae family, a member of the gamma-subclass of Proteobacteria (Sjöstedt, 2005). This genus comprises five species; *F. hispaniensis, F. noatunensis, F. piscicida, F. philomiragia* and *F. tularensis* (DSMZ, 2010). Four subspecies of *F. tularensis* are recognized: the highly virulent *F. tularensis* ssp. *tularensis* (Type A), the moderately virulent *F. tularensis* ssp. *holarctica* (Type B) and *F. tularensis* ssp. *mediasiatica* and the low virulent *F. tularensis* ssp. *novicida* (Figure 2) (DSMZ, 2010; Keim et al., 2007).

Tularemia occurs mainly in the northern hemisphere. *F. tularensis* ssp. *tularensis* has almost been exclusively found in North America (Keim et al., 2007), however, several isolates of this subspecies were obtained in the 1980's from mites from Slovakia (Gurycová, 1998). *F. tularensis* ssp. *tularensis* has two subgroups A.I. and A.II. They have distinct geographic distributions: A.I. is found primarily in the eastern United States but also in California, whereas A.II. is found in the Rocky Mountain region of the western United States (Keim et al., 2007).

F. tularensis ssp. *holarctica* is found throughout Europe, Asia and North America (Keim et al., 2007). It occurs throughout much of Europe except the United Kingdom, Ireland and Iceland. It is typically a disease of Northern and Central Europe and the countries of the former Soviet Union (Ellis et al., 2002; Hubalek et al., 1998; Tärnvik et al., 2004). Further subdivisions within *F. tularensis* ssp. *holarctica* have been proposed but have not been accepted so far by international taxonomic committees. Three biovars of *F. tularensis* ssp. *holarctica* have been suggested; biovar I (erythromycin sensitive), biovar II (erythromycin resistant), and biovar japonica (Olsufjev and Meshcheryakova, 1983).

F. tularensis ssp. *mediasiatica* has been isolated only in Kazakhstan and Turkmenistan. *F. tularensis* ssp. *novicida* has been linked to waterborne transmission in Australia and the United States (Hollis et al., 1989; Whipp et al., 2003). The isolate from Australia is the only *F. tularensis* strain originating from the southern hemisphere to date.

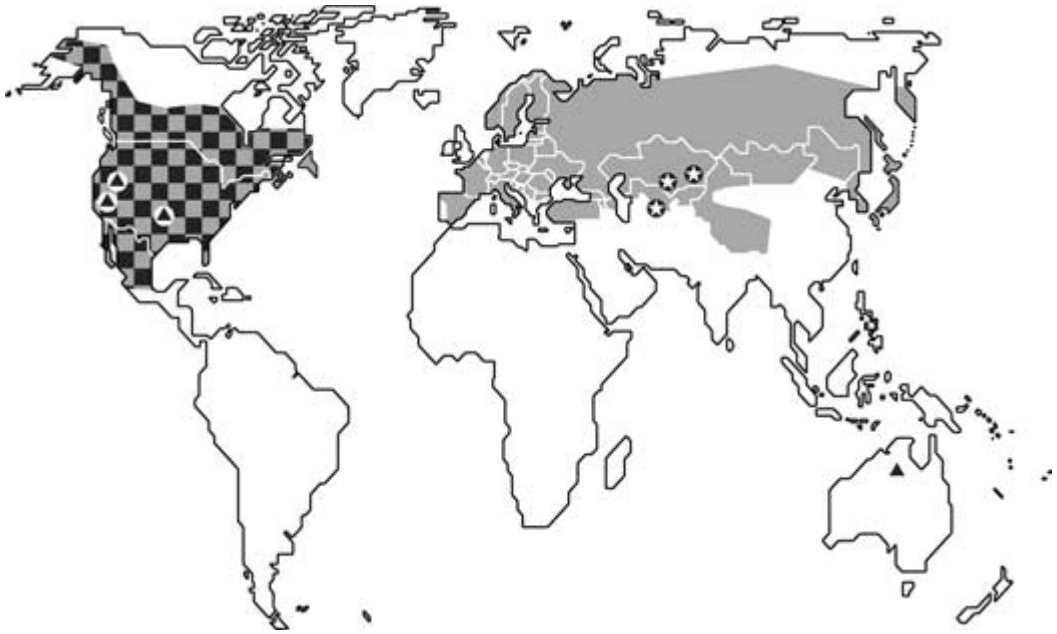


Figure 2. Worldwide distribution of tularemia. (The checkered pattern in North America indicates the range of *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica*. Europe and northern areas of Asia are colored gray to indicate the occurrence of *F. tularensis* ssp. *holarctica*. Triangles indicate the occurrence of *F. tularensis* ssp. *novicida*. Stars indicate the isolations of *F. tularensis* ssp. *mediasiatica*.) (Keim et al., 2007)

Based on 16S rRNA gene sequence analysis, several tick endosymbionts are closely related to *F. tularensis* and have been preliminarily placed in the *Francisella* group (Scoles, 2004; Sréter-Lancz et al., 2009; Sun et al., 2000).

In the last two decades, several emergences or re-emergences of tularemia were seen all over the world which has focused the attention on this disease (Petersen and Schriefer, 2005). Tularemia appeared in 1997 in Spain (Pérez-Castrillón et al., 2001), in 2000 in Kosovo (Reintjes et al., 2002), in 2004 in Germany (Kaysser et al., 2008) and it emerged in an unexpected way in the United States where a large outbreak was recognised in prairie dogs in 2002 (Avashia et al., 2004; Petersen et al., 2004).

3.3. *F. tularensis* ssp. *holarctica* evolution, phylogenetics

Understanding the phylogenetic structure of pathogens provides the means for inferring how they evolved, dispersed, and became ecologically established in the environment (Keim and Wagner, 2009). Phylogenetic knowledge also provides insight into epidemiological tracking of an organism at different evolutionary scales, from within a single patient (Smith et al., 2006) to across the Globe (Holt et al., 2008; Nubel et al., 2008; Van Ert et al., 2007).

F. tularenis has a ~1.9 mega base pair (bp) size genome (*F. tularensis* ssp. *holarctica* LVS NC_007880; *F. tularensis* ssp. *tularensis* Schu S4 NC_006570). It is a highly clonal bacterium, which means it inherits deoxyribonucleic acid (DNA) in a vertical manner and does not transfer DNA laterally between cells (Vogler et al., 2009a). Subspecies *holarctica* reflects the characteristics of a pathogen that recently emerged from a genetic bottleneck during which the common ancestor of this clone possessed some adaptive advantages that permitted rapid dispersal across the entire northern hemisphere (Keim et al., 2007; Keim and Wagner, 2009). Therefore *F. tularensis* ssp. *holarctica* population is genetically very homogeneous around the globe.

The cost and time requirements for sequencing an entire genome have significantly decreased since the first genomes of *Mycoplasma genitalium* (Fraser et al., 1995) and *Haemophilus influenzae* (Fleischmann et al., 1995) were sequenced; therefore phylogenetic studies using whole genome (WG) sequence now include single species phylogenies, whereas previously they were dominated by attempts to determine how species were related to each other (Pearson et al., 2009). WG single nucleotide polymorphism (SNP), rare mutations that primarily arise from unrepaired DNA replication errors (Keim et al., 2004), are effective genetic markers for reconstructing the evolutionary history of clonal bacterial populations (Achtman et al., 2004; Foster et al., 2009; Keim et al., 2004; Vogler et al., 2009a) as long as SNP discovery bias is considered (Pearson et al., 2004; Pearson et al., 2009). (Discovery bias: When two WG sequences are aligned and examined for SNPs, only mutations that occurred along the evolutionary pathway that directly connects the discovery strains will be found. Branches on a tree are defined by the mutations that occurred in that

lineage. As no SNPs can be discovered that are specific to lineages that are outside the evolutionary pathway that links the discovery strains; all such branches will collapse, leaving the samples that stay in such ‘‘branch points’’ as specific positions in the lineage where a new evolutionary branch can be defined by an additional WG sequence.) SNPs have relatively low mutation rates and are thus evolutionarily stable and have effectively been used for determining broad patterns of evolution (Pearson et al., 2009). SNPs can theoretically occur at any nucleotide throughout a genome. Therefore, if entire genomes are compared and examined for SNPs, a sufficient number may be found to provide resolution at even among very recently emerged genetically homogeneous organisms (Keim et al., 2004; Pearson et al., 2004; Pearson et al., 2009). Once an accurate population pylogenetic structure has been defined using the WG-SNPs, canonicalSNPs (canSNP), which define each branch in the phylogeny, either species specific, major lineage specific ones, or strain specific, can be selected (Keim et al., 2004; Pearson et al., 2009; Vogler et al., 2009a). Phylogenetic trees can be drawn using principles of maximum parsimony analysis. Although such trees are highly accurate, they are unlike typical phylogenetic trees because they do not contain any secondary branching due to the discovery bias (Pearson et al., 2009).

Multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) provides further discrimination within each SNP group (Vogler et al., 2009a; Vogler et al., 2009b). This MLVA consists of a series of VNTR loci that are polymerase chain reaction (PCR) amplified by flanking primer sites and then examined for size variation with electrophoresis. Differences in amplicon size at individual loci are assumed to be due to the variation in repeat copy numbers at that locus. MLVA could be used to follow local epidemics as it has a great discriminatory power among highly related strains (Pearson et al., 2009; Vogler et al., 2009a; Vogler et al., 2009b). Nevertheless the highly mutable VNTR markers can be compromised for larger phylogenetic analyses due to the likelihood of convergent evolution and the resulting homoplasy (character state similarity due to independent evolution) (Pearson et al., 2009).

In summary the WG sequences can be aligned and compared to discover genome-wide SNPs that define a basic tree. Then combining the SNP and MLVA markers in progressive hierarchical resolving assays can provide highly accurate and highly discriminating phylogenetic analyses for *F. tularensis* where deeper phylogenetic relationships can be defined by canSNP markers and strain discrimination within each canSNP group is provided by MLVA (Figure 3) (Keim et al., 2004; Pearson et al., 2009; Vogler et al., 2009a).

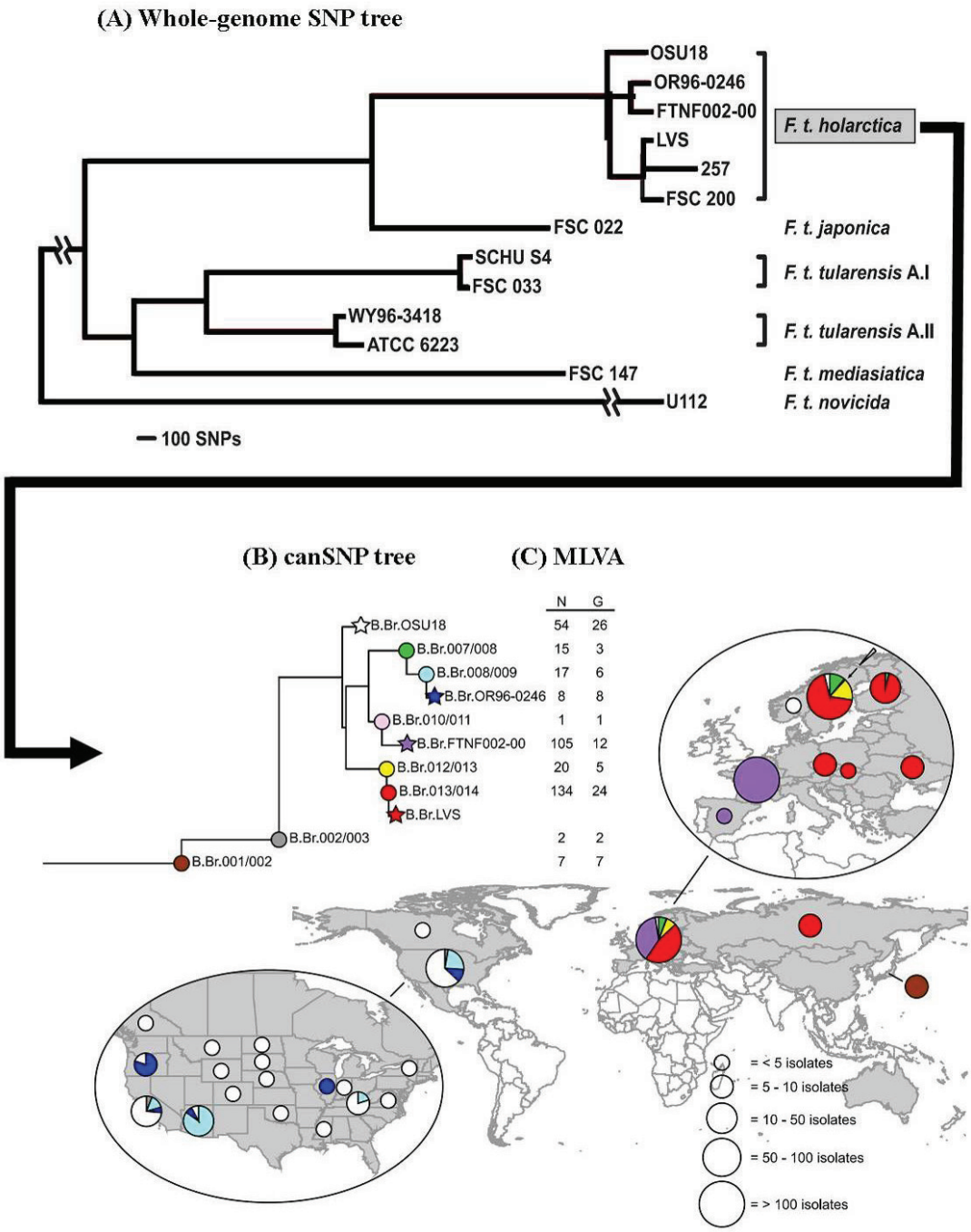


Figure 3. Hierarchical approach for *F. tularensis* ssp. *holarctica*. (A) WG SNP phylogeny (maximum parsimony) of 13 *F. tularensis* strains was used to design clade and subclade specific canSNPs. (B) Only the *F. tularensis* ssp. *holarctica* portion of the canSNP phylogeny is presented along with a map indicating the frequencies and geographic distribution of *F. tularensis* ssp. *holarctica* subclades throughout the world. Stars indicate terminal subclades defined by one genome used for SNP discovery while circles represent collapsed branch points along the lineages that contain subgroups of isolates. (C) The number of isolates (N) and number of distinct MLVA genotypes (G) within each subclade are indicated (Vogler et al., 2009a).

As mentioned above, very little genetic diversity has been identified within *F. tularensis* ssp. *holarctica* indicating that this subspecies only recently emerged through a genetic bottleneck and spread to its current distribution. Only 10 subclades are discovered within *F. tularensis* ssp. *holarctica* all around the world (Vogler et al., 2009a). The geographic origin is debatable wheater it was in Asia or North America. It is presumed that *F. tularensis* ssp. *holarctica* lineage diverged within North America and eventually gave rise to the highly successful *F. tularensis* ssp. *holarctica* clade that was spread around the northern hemisphere (Vogler et al., 2009a). Scandinavia may be the point of origin for many of the Eurasian subclades, as indicated by the canSNP diversity observed among the Scandinavian isolates. Within the European populations, subclades B.Br.FTNF002-00 ("purple group") and B.Br.013/014 ("red group") dominate the continent in a segregated pattern (Figure 3) (Pilo et al., 2009; Svensson et al., 2009; Vogler et al., 2009a). Isolates of B.Br.FTNF002-00 subclade are dispersed throughout the Western European countries like Spain, France, Switzerland and parts of Germany. The lack of MLVA diversity among these isolates indicates that the spread of this clade was likely a very recent event. Isolates of B.Br.013/014 subclade are dispersed throughout Central and Eastern Europe from Germany to Russia with significant amount of MLVA diversity, which indicates the discovery of several additional canSNP groups and could reveal additional geographic patterns.

Isolates from several European countries, like Hungary and Italy, are not represented in this phylogeographic model (Pilo et al., 2009; Svensson et al., 2009; Vogler et al., 2009a), thereby limiting our ability to understand tularemia dispersal in the continent wheather caused by nature or by the practice of large scale transcontinental relocation of the European brown hare.

3.4. Properties of the agent

F. tularensis is an obligate aerobe, small (0.2-0.7 μm × 0.2-1.7 μm), Gram-negative, non-motile, pleomorphic coccobacillus, covered by a carbohydrate-rich capsule. *F. tularensis* is oxidase-negative, weakly catalase-positive and cysteine is required for its growth. Utilisation of glycerol is an effective tool to differentiate *F. tularensis* ssp. *tularensis* (glycerol positive) and *F. tularensis* ssp. *holarctica* (glycerol negative) (Barrow and Feltham, 1993; Nano 1998; Sjöstedt, 2005). Furhter discriminating characteristics of *F. tularensis* subspecies are presented in Table 1 (WHO, 2007).

Table 1. Discriminating characteristics of *F. tularensis* subspecies. (WHO, 2007)

Characteristic	*F. tularensis* subspecies			
	tularensis	***holarctica***	***mediasiatica***	***novicida***
cysteine/cystine requirement	+	+	+	–
maltose fermentation	+	+	–	weak
sucrose fermentation	–	–	–	+
D-glucose fermentation	+	+	–	+
glycerol fermentation	+	–	+	weak
citrulline ureidase production	+	–	+	+
oxidase production	–	–	–	–
H_2S production	+	NE*	NE*	NE*
cell size (µm)	0.2-0.7x0.2	0.2-0.7x0.2	0.2-0.7x0.2	0.7x1.7

*Not examined

Survival of *F. tularensis* in nature is dependent upon a variety of factors such as temperature (1 hour at 60 ºC) or direct exposure to sunlight (3 hours at 29 ºC) (Friend, 2006). General survival in carcass tissues is 3 to 4 weeks and 4 months in water at 4-6 ºC (Friend, 2006). *F. tularensis* does not form resistant structures and is relatively sensitive to all standard inactivation procedures (WHO, 2007). Therefore the destruct cycle of inactivation used in autoclaves is suitable for the inactivation of *F. tularensis*. The bacterium is sensitive to hypochlorite and other commonly-used chemical decontaminants and is readily inactivated on exposure to ultra violet irradiation.

3.5. Hosts, vectors and ecology

Although *F. tularensis* is a potential biological warfare agent and several emergences or re-emergences of tularemia have recently been seen all over the world (Kaysser et al., 2008; Petersen and Schriefer, 2005), the ecology of the disease is still only partially understood, with many open questions about reservoirs and vectors (Keim et al., 2007). The current knowledge is primarily based on investigations performed during disease outbreaks (Gurycová et al., 2001; Kaysser et al., 2008).

F. tularensis naturally occurs in certain ecosystems. It has a remarkably broad host range probably the broadest of all zoonotic agents and the transmission routes among these various hosts are extremely diverse (Mörner, 1992). Natural infections with *F. tularensis* have been reported in a range of vertebrates including mammals, birds, amphibians, and fish, as well as in certain invertebrates (Mörner and Addison, 2001). Despite its broad host range, tularemia is primarily a disease of the genera *Lagomorpha* and *Rodentia* (Friend, 2006). In the New World, the cottontail rabbit (*Sylvilagus* spp.), the black-tailed jackrabbit (*Lepus californicus*) and the snowshoe hare (*Lepus americanus*) are important in the ecology of tularemia (Friend, 2006). The European brown hare (*Lepus europaeus*) is a common host of *F. tularensis* in Central Europe where it is also an important game species, which causes a public health problem (Hopla, 1974; Keim et al., 2007; Mörner and Addison, 2001; Pikula et al., 2004; Strauss and Pohlmeyer, 2001; Sztojkov, 2006). Tularemia occurs frequently in mountain hares (*Lepus timidus*) in Scandinavia and Russia, in some of these regions, both species of hare are affected (Mörner and Addison, 2001). The Japanese hare (*Lepus brachyurus*) is the third lagomorph associated with tularemia in the Old World (Friend, 2006). The European wild rabbit (*Oryctolagus cuniculus*) and thus the domestic rabbit are both relatively resistant to *F. tularensis* (Bell, 1980). Rodents are of great importance for maintaining enzootic foci of tularemia. Voles (*Microtus* spp., *Arvicola amphibius, Myodes glareolus*) are most frequently involved in tularemia epizootics but other rodent species (*Ondatra zibethicus*, *Castor* spp., *Lemmus* spp., *Rattus rattus, Mus musculus, Apodemus flavicollis, Tamias sibiricus, Sciurus vulgaris,* etc.) are also found to be infected (Mörner and Addison, 2001). The common hamster (*Cricetus cricetus*) is a species of hamster native in Western, Central and Eastern Europe, Central Russia, and Kazakhstan (Nechay, 2000). Trapping of hamsters for pest control and fur collection for sale is a widespread practice in eastern Hungary (Bihari and Arany, 2001). Trappers, who skin more than half a million hamsters a year (Bihari, 2003), regularly become infected with *F. tularensis* in this area (Münnich and Lakatos, 1979). *Cricetus* spp. are highly sensitive to *F. tularensis* (Olsufjev and Dunayeva, 1970; WHO, 2007). Haematophagous arthropods have substantial role both maintaining of *F. tularensis* in the nature, and in disease transmission. Ticks are believed to be the most important arthropods in the ecology of tularemia. They are both mechanical and biological vectors, the latter by amplifying the number of bacteria contributing to retransmission and by maintaining the bacterium throughout its multiple life stages (Hopla and Hopla, 1994). Ticks are true reservoir hosts that may perpetuate specific enzootic foci during inter-epizootic periods. The tick *Dermacentor reticulatus* plays an important role in maintaining and transmission of *F. tularensis* among small and medium sized mammals in Central Europe (Gurycová et al., 1995; Gurycová et al., 2001; Hubálek et al., 1998). Other ticks, such as *Ixodes ricinus*, *I. persulcatus*, *D. marginatus*, *Rhipicephalus rossicus* and *Haemaphysalis concinna,* have

also been found to be naturally infected with *F. tularensis* in Europe and *D. variabilis*, *D. andersoni* and *Amblyomma americanum* in North America (Hopla and Hopla, 1994; Keim et al., 2007). Other blood-sucking arthropods transmit *F. tularensis* mechanically. Mosquitoes belonging to the genera *Aedes*, *Culex* and *Anopheles* are historic vectors in the northern boreal forest of Scandinavia and Russia (Petersen and Schriefer, 2005). Horse-flies (Tabanidae, e.g. *Chrysops discalis*) have also been observed to serve as a route of infection in the former Soviet Union and in the United States (Petersen and Schriefer, 2005). *F. tularensis* has been frequently isolated from haemotophagic Gamasid mites (Gamasidae, e.g. genera *Laelaps*, *Haemogamasus*, *Haemolelaps*) collected from rodents in Europe. Fleas (*Siphonaptera*) are considered of minor importance for transmission and maintenance of *F. tularensis* (Keim et al., 2007; Parker and Johnson, 1957).

There are two known cycles of tularemia; the terrestrial and the aquatic (Figure 4) (Petersen and Schriefer, 2005). In the terrestrial cycle, hares and rodents are the most important mammalian hosts, while haematophagous arthropods play a role as vectors. The European brown hare is moderately sensitive to *F. tularensis* infection and can possibly maintain tularemia for a longer time than the mountain hare, acting as a reservoir (Mörner, 1994). Infection in mountain hares is often fatal, in them the alimentary route of transmission seems to be important in Scandinavia in winter (Mörner et al., 1988). The water vole (*Arvicola amphibius*) and the common vole (*Microtus arvalis*) in addition to being highly susceptible to *F. tularensis*, may also become chronically infected and thereby serve as disease reservoirs during periods between epizootics (Bell and Stewart, 1983; Mörner and Addison, 2001; Olsufjev et al., 1984). Voles are hosts for immature stages of several important tick species as well. Mouse species (*Mus musculus*, *Apodemus* spp.), because of their high susceptibility and sensitivity to *F. tularensis* (Bandouchova et al., 2009), are probably not important reservoir hosts (Friend, 2006). Stress-related aggression can facilitate transmission whereas cannibalism can be a route of transmission among voles, especially in populations of high density (Friend, 2006). Hares and rodents can contaminate the environment through their body discharges. In the aquatic cycle, voles and maybe muskrats and beavers serve as the main host species, shedding live bacteria into the environment (Pérez-Castrillón et al., 2001; Petersen and Schriefer, 2005).

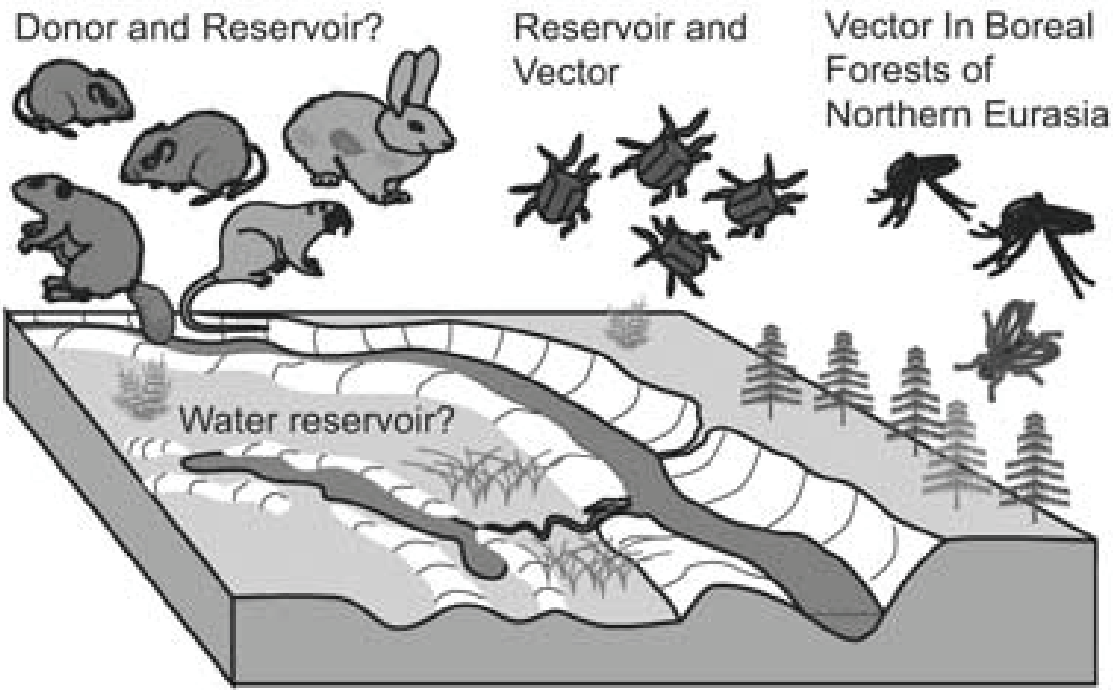


Figure 4. Host and vector associations of *F. tularensis* (Keim et al., 2007).

Carcasses of infected animals can further contaminate the water (Friend, 2006). It was found that a protozoon species (*Acanthamoeba castellanii*) can be an important aquatic-environment reservoir of *F. tularensis* (Abd et al., 2003). *F. tularensis* can persist for a long time in watercourses, and at low temperatures in the terrestrial environment (Friend, 2006).

Carnivores and scavengers are not considered to have a major role in the maintenance of *F. tularensis* in nature; their high seroprevalence indicates former exposure and a probable ability to survive infection (Friend, 2006). Birds are not regarded as important components of the ecology of tularemia (Friend, 2006). Potentially, their most significant role is the transport of infected arthropod vectors to new areas. At the same time they indicate tularemia activity in their prey species, and contaminate surface waters through body discharges.

Tularemia rarely occurs among domestic animals or in zoological collections. Among domestic animals, sheep and cats are the ones that are infected most frequently (Friend, 2006). Outbreaks generally occur among sheep in spring, in the lambing season (O'Toole et al., 2008). Companion animals such as dogs and cats are infected when exposed to a variety of habitats (Friend, 2006; Valentine et al., 2004). The result of tularemia infection of dogs and cats varies from subclinical infection to death (Woods et al., 1998). These pet animals can be involved in the transmission of tularemia by bringing infected ticks into the household. Mechanical transfer of *F. tularensis* to people occurs from the contaminated mouths and claws of these pets that have recently fed on diseased rodents or rabbits (Capellan and Fong, 1993). Zoo primates hunt and consume small prey species such as rodents that enter their enclosures and may contract tularemia from their prey. Tularemia of primates is uncommon in

European zoos. An outbreak of tularemia affected 18 cynomolgus monkeys (*Macaca fascicularis*) in the German Primate Center in 2005 (Mätz-Rensing et al., 2007), and solitary cases were recorded in a golden-headed lion tamarin (*Leontopithecus chrysomelas*) (Hoelzle et al., 2004) and a common marmoset (*Callithrix jacchus*) (Posthaus et al., 1998) in Switzerland. As *F. tularensis* is a potential biowarfare agent experimental studies are conducted on primates as well (Twenhafel et al., 2009).

The environmental conditions that favour outbreaks in mammals and man are poorly understood. One study performed in the United States suggested that there is a subtle shift in geographical distribution of tularemia with climate change. A warmer climate 1965-2003 was associated with a northward movement of tularemia (Nakazawa et al., 2007). Illustrating the complexity of tularemia ecology, the geographical trend of tularemia reports in Europe is less consistent with a northward movement (Rydén et al., 2009). Large epidemics have continued to be reported in the north (e.g. Sweden and Finland), while some reports claim the disease has only recently been established in the South (e.g. Spain).

3.6. Pathogenesis

F. tularensis is a highly infectious agent. It can enter the body several ways: via inoculation by haematophagous arthropods or wounds, across the conjunctiva, by inhalation of infected aerosols, by ingestion of contaminated meat (cannibalism) or by water. As low as 10 lethal dose 50 (LD_{50}) colony forming unit (CFU) of *F. tularensis* ssp. *tularensis* is enough to cause fatal infection in mice, guinea pigs or rabbits and a similarly small dose is enough to induce a severe or sometimes fatal infection in humans. *F. tularensis* ssp. *holarctica* causes lethal infection in mice and guinea pigs at a similarly small inoculation dose, however, a higher dose is needed to induce the disease in rabbits (LD_{50}: $>10^6$ CFU) or humans (LD_{50}: $>10^3$ CFU) (Ellis et al., 2002).

After entering the body, the bacteria multiply locally causing ulceration and necrosis and then invade the blood and lymph vessels and spread to the lymph nodes and organs such as liver, spleen, lung, kidney, serosal membranes and bone marrow, causing multiple foci of coagulation necrosis (Mörner and Addison, 2001). *F. tularensis* is a typical intracellular pathogen with a high predilection to growing in macrophages but can infect many other cell types, such as epithelial cells, hepatocytes, muscle cells and neutrophils (Mörner, 1994).

Little is known about the immune response of the host to *F. tularensis*. Cell-mediated immunity has long been believed to be crutial for protection. The importance of humoral immunity has recently been recognised. Synergy between antibodies, T cell-derived cytokines, and phagocytes appears to be critical to achieving immunity against *F. tularensis*

and clearing infection (Kirimanjeswara et al., 2008). In humans, the antibody response is measurable by the second week post-infection. Antibody levels are highest during the second month after infection and decline gradually thereafter (WHO, 2007).

F. tularensis septicemia occurs at the end stage of the disease, when the bacteria invade the blood vessels without lesions indicative to tissue response. Only this septicaemic form is seen in highly sensitive species and animals, which die within 2-10 days (Mörner and Addison, 2001; OIE, 2008). Relatively resistant animals can survive the infection and develop protective immunity (Friend, 2006; OIE, 2008).

3.7. Clinical signs and pathological lesions

Clinical cases of tularemia are infrequently observed in free-ranging wildlife as infected animals are usually found moribund or dead (Friend, 2006). Non-specific signs include depression and pyrexia. Local inflammation or ulceration at the portal of entry and enlargement of the regional lymph nodes may be observed (Mörner and Addison, 2001). Highly sensitive animals develop fatal septicemia and may be non-responsive before death. In hares, depression, stupor, loss of body weight and lack of fear, facilitating capture, are observed in the late stages of the disease (Friend, 2006).

The pathology of tularemia differs considerably between different animal species. In Scandinavia, acute forms of tularemia have been described in mountain hares (Mörner, 1988), while in Central Europe infection of European brown hares has apparently a more chronic course (Mörner, 1994; Kemenes, 1976). In mountain hare, the most characteristic necropsy finding is the enlarged spleen. Multiple white foci of coagulative necrosis can be seen in the spleen, liver and bone marrow in some cases. During wintertime hemorrhagic enteritis and typhlitis can be found as well. The mucosa in the jejunum and caecum is congested and occasionally necrotic. The crypts and villi of the intestine may show focal necrosis. Histologically, the focal lesions are initially characterized by apoptosis, often absence of inflammatory cell and thrombosis of small vessels (Mörner et al., 1988; Mörner, 1994). Little is known about the pathology of tularemia in European brown hare. It is described as a more chronic form with granulomas with central necrosis, particularly in the lungs and kidneys (Kemenes, 1976; Sterba and Krul, 1986) and occasionally in the liver, spleen, bone marrow and lymph nodes. The granulomas contain heterophils, macrophages and giant cells, as well as calcified areas.

The pathology of tularemia, in rodents, depends on the sensitivity of the species. The usual macroscopic finding is the enlarged spleen and, less frequently, the liver. Pinpoint white foci can be seen in these organs. Microscopically, multifocal coagulation necrosis is

characteristically found in the spleen, liver, lymph nodes, bone marrow and lungs. Karyolysis, pyknosis and the presence of inflammatory cells such as macrophages and heterophils in less acute cases is observed (Mörner and Addison, 2001).

Tularemia can cause late-term abortions or listlessness and death in lambs and, to a lesser extent, ewes. Lesions are multifocal pinpoint necrotic foci in tissues, particularly spleen, liver, and lung (O'Toole et al., 2008). There is a description about ulceroglandular tularemia at the ventral cervical region of a cat. The histopathologic diagnosis was severe locally extensive pyogranulomatous and necrotizing cellulitis in this case (Valentine et al., 2004).

In primates, the clinical signs include increased body temperature, heart rate, peak cardiac pressure and mean blood pressure following an air-borne infection (Twenhafel et al., 2009) or an ulceroglandular syndrome with local lymphadenopathy, gingivostomatitis after alimentary tract infection (Mätz-Rensing et al., 2007). Prominent gross changes after both air-borne and alimentary tract infection included numerous, well-demarcated, necrotic foci present in the lungs, mediastinal lymph nodes and spleen but also seen in the heart, mediastinum, diaphragm, liver, urinary bladder, urethra and mesentery. Histologic changes consisted of well-delineated foci of necrosis and neutrophilic and histiocytic inflammation, with varying amounts of hemorrhage, edema, fibrin, and vasculitis (Mätz-Rensing et al., 2007; Twenhafel et al., 2009).

3.8. Diagnosis and differential diagnosis

Field examination of carcasses is not recommended when tularemia is suspected because of the potential for human exposure and the risk of contaminating the environment (Friend, 2006; OIE, 2008). There is a high risk of direct infection of humans by direct contact with *F. tularensis*. Special precautions, including wearing gloves, masks and eyeshields are recommended when handling infective materials. Procedures should be performed within secure biosafety containment facilities (biosafety level 2 or 3) (OIE, 2008; Sewell, 2003). Diagnosis is based on the combined results of necropsy findings and the demonstration of *F. tularensis* from the samples or tissues.

F. tularensis appear as numerous Gram-negative small-sized bacteria in impression smears or in histological sections of spleen, liver, lung, kidney, bone marrow and lymph node as well as in blood smears. *F. tularensis* can be detected specifically by direct or indirect fluorescent antibody tests (Karlsson et al., 1970; Mörner, 1981). Immunohistochemical (IHC) assay is a very useful and sensitive method for the detection of *F. tularensis* in domestic and wild animals (Twenhafel et al., 2009; Valentine et al., 2004; Zeidner et al., 2004). Plentiful bacterial

antigen can be observed, often extracellularly, in foci of necrosis. Intracellular *F. tularensis* is found in macrophages and giant cells and less frequently in other cell types.

F. tularensis can be identified by culture. The most adequate samples for culture are, in acute cases, heart blood, spleen, liver or bone marrow whereas in chronic cases, the granulomatous lesions. Due to the highly fastidious culture requirements of *F. tularensis,* isolation can be difficult as it grows poorly in conventional culture media. Francis medium (peptone agar containing 0.1% cystine/cysteine, 1% glucose, 8–10% defribrinated rabbit, horse or human blood), McCoy and Chapin medium (60g egg yolk and 40ml normal saline solution mixed and coagulated by heating to 75 ºC) or Modified Thayer-Martin agar (glucose cysteine agar-medium base supplemented with haemoglobin and Iso VitaleX /Becton Dickinson Inc., NJ/) are recommended (OIE, 2008). Isolation of *F. tularensis* from carcasses may be difficult due to overgrowth of other bacteria. Penicillin, polymyxin B and cycloheximide can be added to prepare selective culture media (OIE, 2008). If it is difficult to isolate *F. tularensis* on primary culture, it may be isolated following inoculation of tissue suspension from suspect cases into laboratory animals such as mice or guinea pigs. All routes of administration in mice, such as subcutaneous, percutaneous, or intravenous, will lead to an infection that is invariably fatal within 7-10 days (OIE, 2008). The colonies of *F. tularensis* are small, round and do not appear within the first 48 hours of incubation at 37 ºC. Since *F. tularensis* ssp. *tularensis* and *holarctica* differ in citrulline ureidase activity and glycerol fermentation conventional biochemical assays can be utilized for biochemical differentiation based on glycerol fermentation or citrulline ureidase activity (Sandström et al., 1992). Alternatively, the automated system, Biolog (MicroLog™ MicroStation™ System, GN2 Microplates; Biolog Inc., Hayward, CA), may also be used to detect glycerol fermentation but little is known about the carbon source utilization pattern of *F. tularensis* strains and whether it is a reliable method of characterization and identification of different isolates (Petersen et al., 2004).

A variety of PCR methods have been described for the detection of *F. tularensis* DNA in both clinical and environmental specimens. The gel-based PCR assays target the 16S ribosomal ribonucleic acid (rRNA) gene and genes encoding the outer membrane proteins, *fopA* or *tul4*, show good specificity and allow rapid detection of *F. tularensis* in specimens (Forsman et al., 1994; Sjöstedt et al., 1997). However, *Francisella*-like endosymbionts of ticks can produce non-specific positive results in these assays (Sréter-Lancz et al., 2009; Versage et al., 2003). Real-time PCR methods show no evidence of cross-reactivity with non *F. tularensis* bacteria (environmental bacteria and vector-borne organisms) and the detection limit of very low numbers of organisms increases the likelihood of detecting *F. tularensis* in environmental samples in which the number of organisms is low (Escudero et al., 2008; Fujita et al., 2006; Kugeler et al., 2005; Versage et al., 2003). Molecular methods are also applied to cultured *F.*

tularensis to provide resolution among the *Francisella* species, subspecies and within-subspecies strains (see above) (Keim et al., 2007; Kugeler et al., 2006).

Serology can be carried out for investigating exposure to *F. tularensis* in species which are relatively resistant to the disease, such as European brown hare, sheep, cattle, pig, elk, dog, cat or birds (Mörner et al., 1988; OIE, 2008). Whole blood, sera or saline extract of lung tissue can be used for seroepidemiologic surveys (Mörner et al., 1988). A slide agglutination test, using one drop of stained bacteria and one drop of whole blood, is a widely used field method for screening the European brown hare populations in Central Europe. The standard serologic test is the tube agglutination test (OIE, 2008). Possible cross-reaction with *Brucella abortus*, *B. melitensis*, *B. suis*, *Legionella* spp. and *Yersinia* spp. may occur. The enzyme-linked immunosorbent assay allows an early diagnosis of tularemia (Carlsson et al., 1979; OIE, 2008). Western blotting, microagglutination, indirect immunofluorescence assay and flow cytometry have also been assessed for the serological diagnosis of tularemia (Porsch-Özcürümez et al., 2004).

The differential diagnosis of tularemia include plague due to *Y. pestis*, pseudotuberculosis due to *Y. pseudotuberculosis*, brucellosis, mycobacterial infection, staphylococcal infection, salmonellosis, Tyzzer-disease, systemic herpesvirus infection, and parasites such as *Capillaria hepatica*, ascarid nematodes or larval cestodes which may encyst in the liver (Mörner and Addison, 2001)

3.9. Management, control and treatment

F. tularensis has an extremely broad host range and very complex ecological transmission cycles, therefore it is difficult to control (Friend, 2006). Monitoring and surveillance of wildlife, arthropod vectors, and surface water for tularemia activity in enzootic areas provide useful information for wildlife managers and public health authorities (Friend, 2006; WHO, 2007). Monitoring focuses only on a small number of primary species despite the broad host range. These surveys could be achieved by systematic and directed investigation of susceptible mammals (lagomorphs and rodents) and arthropods in a region of interest; searching and testing carcasses and desiccated remnants (skin, bones) of dead animals; and examining water and mud samples collected close to places with dead animals or evident rodent activity (WHO, 2007). At present, there are no European Union regulations specifically for reporting tularemia but it is, however, a notifiable disease on the World Animal Health Information Database (WAHID) (OIE, 2008).

Translocating hares can introduce *F. tularensis* into so far untouched areas. In efforts to replenish the population for sporting purposes, thousands of hares are annually translocated

from Central European countries, like Hungary, to France and Italy (Somogyi, 2006). Screening the hares before they are exported is necessary but the regulations about this vary and are usually based only on bilateral agreements between countries. The quarantine of individual hares for a week and their screening with, at a minimum, the slide agglutination test at the beginning and at the end of the quarantine period would detect most infected animals with a view to preventing the introduction of *F. tularensis* into new areas. Despite very strict pre-export screening protocols, the potential of introducing Central European *F. tularensis* strains into non-native regions due to the release of infected hares is probable and has important consequences on public health. *F. tularensis* is a category A biothreat agent and therefore a critical goal of any investigation of a human tularemia case is to determine if the infection source originated from a natural outbreak (local environment) or a nefarious event. Outbreaks from non-native *F. tularensis* strains introduced from imported game could confound investigative efforts or, even worse, could trigger false alarm of a nefarious event.
The lack of any licensed vaccine and the very broad host range and complex disease transmission routes of *F. tularensis* make the vaccination more as a theoretical solution than a management tool for wildlife.

3.10. Public health concern

Humans are highly susceptible to *F. tularensis,* which is on the list of Class A biothreat agents, as a potential biological warfare cause. The virulence of the strain, dose, and route of exposure are all important factors influencing the clinical form and severity of the disease in humans. People can be infected by several routes such as bites from infected arthropods; handling of infectious animal tissues or fluids; wounds, small cuts, direct contact, with or ingestion of contaminated water or food, through the conjunctiva and inhalation of infective aerosols (Dennis et al., 2001; Hauri et al., 2010). Human infection often occurs during hunting, trapping or skinning infected wildlife (Amoss and Sprunt, 1936; Mörner and Addison, 2001). Hay, grain and water supplies contaminated by rodents have been the source for numerous human cases (Gill and Cunha, 1997; Greco et al., 1987). Outdoor activities expose humans to infected animals, bites by infected arthropods or contact with contaminated surface waters (Friend, 2006). Laboratory-work poses a significant risk of contracting tularemia, for example by aerosol exposure.

The most frequent clinical signs in humans are inflammation and later ulceration at the primary site of infection (Figure 5), with swelling of regional lymph nodes (Figure 6), which may become abscessed. Generally, the course of the clinical disease includes sudden onset of fever, generalized aches, inflammation of the upper respiratory tract with nasal discharge, vomiting, malaise, and anorexia. Seven clinicopathological forms of tularemia have been described in human medicine: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septicemic (Dennis et al., 2001).

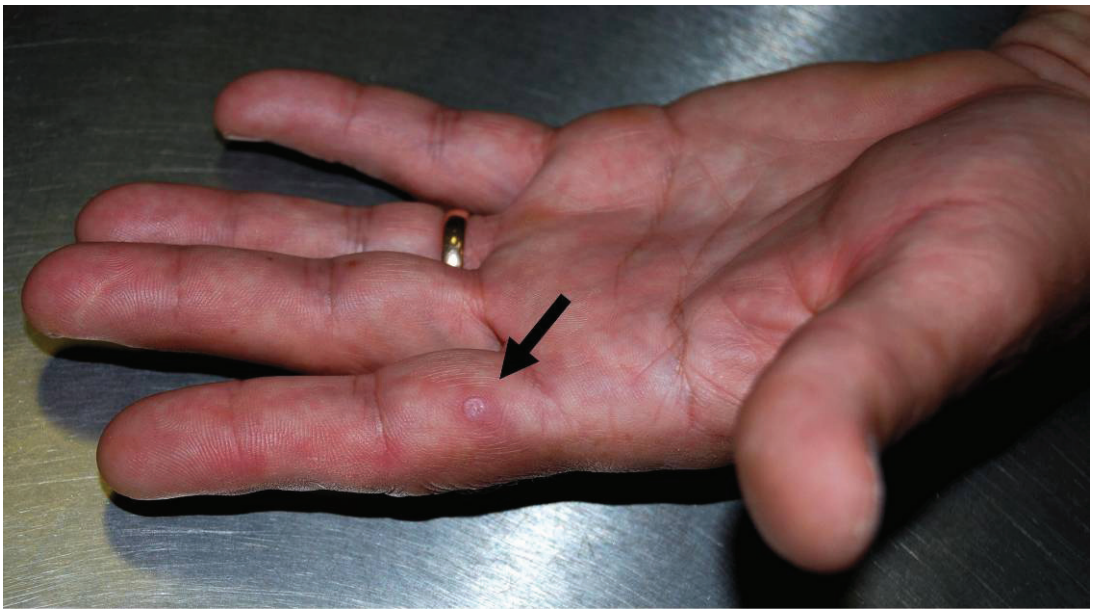


Figure 5. Healing ulcus, due to *F. tularensis* infection, is on the finger.

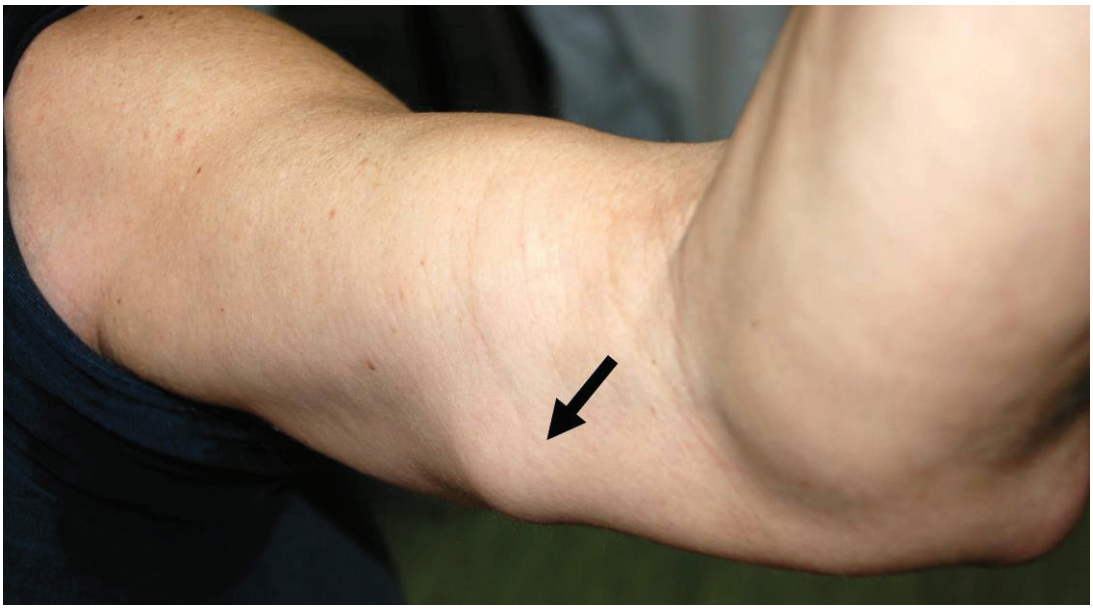


Figure 6. Enlarged cubital lymph node due to *F. tularensis* infection.

Infection of humans by *F. tularensis* can be treated with antibiotics such as streptomycin, gentamicin, ciprofloxacin, levofloxacin, doxycycline, tetracycline and rifampicin (Bányai and Martyin, 2006; Brown et al., 2004; Füzi and Kemenes, 1972; Tomaso et al, 2005). People can minimize their potential exposure to *F. tularensis*. Publication of epizootics and providing information on treatment and protection are important. To prevent contact transmission rubber gloves should be worn by trappers or hunters when skinning those species commonly associated with tularemia. As for arthropod transmitted infection, the use of insect repellent, protective clothing and frequent body searches with prompt removal of ticks can greatly reduce the risk of infection. Meat from potentially infected animals should be well cooked. Untreated water from lakes and streams should not be consumed. Diagnostic laboratory procedures should be performed within secure biosafety containment facilities (biosafety level 2 or 3) and wearing appropriate protective clothing (WHO, 2007).

Vaccination has generally not been widely applied but they have been used for high risk situations, typically for laboratory researchers. Many vaccine candidates including acellular subunit, killed whole cell and live attenuated vaccines have been developed in the recent years but none of them has been licensed yet (Barry et al., 2009).

4. Aims of the study

Aims of the study were:

Ad 1. to obtain retrospective data about the tularemia situation of Hungary in the way of collecting data about the annual percentage of *F. tularensis* seropositive hares and about the annual absolute number of human cases about the time frame of 1984-2009.

Ad 2. to study the direct and indirect detection of *F. tularensis* in potential animal reservoirs, domestic animals, arthropod vectors and natural waters in order to obtain data about the ecological cycle of *F. tularensis* in an enzootic area during an inter-epizootic period.

Ad 3. to investigate the role of hamsters in the natural cycle of *F. tularensis* and to examine clinical signs, pathology and histopathology of acute tularemia of two trapped hamsters.

Ad 4. to identify the gross and histological lesions characteristic for *F. tularensis* infection of European brown hares.

Ad 5. to summarize the postmortem lesions and the results of the bacteriological examination of a patas monkey (*Erythrocebus patas*) and a vervet monkey (*Chlorocebus aethiops*) that died suddenly due to tularemia at Szeged Zoo (Csongrád County, Hungary).

Ad 6. to collect *F. tularensis* strains from different parts of Hungary from different host species to establish a Hungarian *F. tularensis* strain collection.

Ad 7. to characterize representative *F. tularensis* strains isolated from different host species and locations in Hungary and to examine their metabolic fingerprinting based on the utilization of 95 carbon sources.

Ad 8. to sequence the WG of a Hungarian isolate, compare it to 5 other compleate genomes, to phylogenetically characterize 19 *F. tularensis* isolates from Hungary and Italy, to provide important complementary data to the European phylogeographic model and to present a set of powerful molecular tools for investigating tularemia dispersal throughout Europe.

5. Materials and Methods

5.1. Retrospective data collection

Retrospective data were collected from the databases of the local veterinary authorities (Jász-Nagykun-Szolnok County Agriculture Office, Heves County Agriculture Office) and live hare export stations (Euroharex kft., Vadex Zrt., Medo kft.) about the annual number of exported live hares from the different hunting areas of Hungary and the number of *F. tularensis* seropositive (screened by slide agglutination test) animals found between 1984 and 2009. The annual numbers of Hungarian human cases with their suspected exposure sites were obtained from the National Center for Epidemiology for the same time period. By this analysis, the annual mean percentage of *F. tularensis* seropositive hares was counted, combined with the annual absolute number of human cases and visualized on a graph.

5.2. Slide and tube agglutination tests

Tests were performed according to the manufacturer's instructions of the used diagnostic kit (Bioveta Inc., Ivanovice na Hané, Czech Republic) listed in the World Health Organization Guidelines (WHO, 2007) utilizing inactivated bacteria.

In the slide agglutination test a drop of whole blood (approx. 0.04 ml) was mixed with 5 drops (approx. 0.2 ml) of antigen and the reaction was considered positive if flakes appeared within 1-3 minutes at 20-25 ºC (Figure 7).

The tube agglutination test was performed with 0.5 ml aliquots of serial dilutions (from 1:10 to 1:160) of sera mixed with 0.5 ml of diluted (1:4) antigen. The test was considered positive if visible agglutination with clear supernatant fluid took place after 20 hours of incubation at 37 ºC and 1 hour at room temperature (Figure 8). According to the manufacturer's directions agglutination at dilutions of 1:80 or higher should be considered as a positive result, while at 1:40 it is still ambiguous. The positive control serum was provided by the manufacturer.

Crystal violet powder added to a final concentration of 0.25% makes the agglutination more visuable (OIE, 2008) like it used to be in the case of the Sanofi-Phylaxia Inc. (Budapest, Hungary) tularemia antigen (Figure 7).

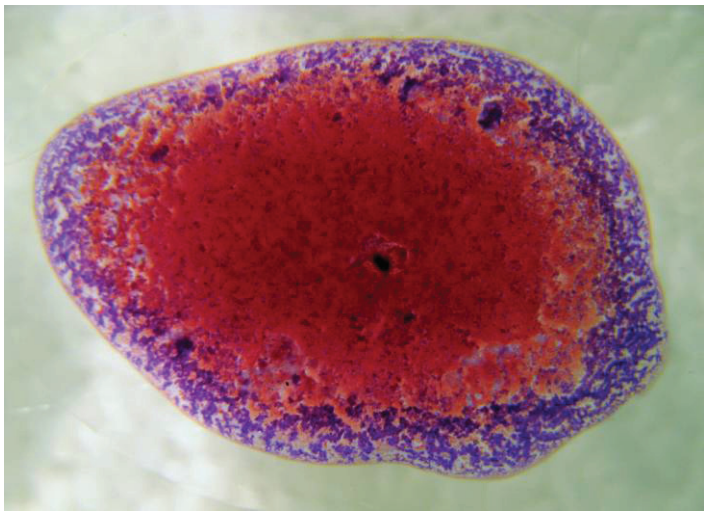


Figure 7. Positive reaction in the slide agglutination test using the stained Sanofi-Phylaxia Inc. antigen (Budapest, Hungary).

Figure 8. Negative and positive reaction in tube agglutination test using the non-stained Bioveta Inc. antigen (Ivanovice na Hané, Czech Republic).

5.3. Pathological methods

5.3.1. Sample collection from European brown hares

European brown hares, collected at different locations in Hungary during live hare export events over two winter hunting seasons (2007-2008 and 2008-2009), were screened by the slide agglutination test. Forty-seven animals (16 males, 31 females, 32 adults, 15 juveniles) were found to be seropositive and were used for laboratory examinations. Carcasses of three dead seropositive adult, male hares submitted for diagnostic examination were also included in the study, so a total of 50 seropositive animals were examined. Tissue samples of 20 seronegative hares were collected and served as negative control. Animals were categorized as same year juveniles and older, based on the so called Stroh-mark (Pintur et al., 2006). The body condition was estimated using a simplified, categorical (good, moderate, weak) version of the kidney/fat index (Pintur et al., 2006). Tissue samples (heart, pericardium, lung, liver, spleen, kidney, small and large intestine and bone marrow from 50 animals, testicle and epididymis from 19 animals, ovarium and mammary gland from 31 animals and mediastinal lymph node from 35 animals) were collected for histology. The same tissue samples of 20 seronegative hares served as negative control.

5.3.2. Histology

Tissue samples were fixed in 10% buffered formalin. Four µm thick sections of formalin-fixed and paraffin-embedded tissue samples were stained with hematoxylin and eosin (HE), and examined by light microscopy.

5.3.3. Immunohistochemistry

Immunohistochemistry was applied for the demonstration of *F. tularensis* lipopolysaccharide (LPS) antigen in tissue sections. After deparaffinization and antigen retrieval (in a microwave oven at 750 W, for 20 min in citrate buffer, pH 6.0) the sections were incubated in 3% H_2O_2 solution for 10 min and then in a 2% solution of skimmed milk powder for 20 min. The samples were incubated overnight at 37 ºC with a 1 in 6,000 dilution of *F. tularensis* LPS-specific mouse monoclonal antibodies (clones FB11 and T14, MAB8267; Chemicon International Inc., Southhampton, UK). Antibody binding was detected by a horseradish peroxidase-labelled polymer (EnVision™+ Kit; Dako Inc., Glostrup, Denmark). A serial section incubated with phosphate buffer solution was used as a negative control.

5.4. *F. tularensis* isolation

5.4.1. Sample collection for *F. tularensis* isolation

F. tularensis seropositive European brown hares identified by the slide agglutination test were collected from live hare export stations (Euroharex kft., Vadex Zrt., Medo kft.), from hunting events at different locations in Hungary and game slaughter houses (Vadex Zrt. – Austrian import hares) during three winter hunting/export seasons (2007-2008, 2008-2009, 2009-2010). A patas monkey and a vervet monkey died of tularemia in Szeged Zoo during the fall of 2003 were also submitted for strain isolation. Carcasses were necropsied under appropriate biosafety conditions at the Veterinary Diagnostic Directorate of the Central Agriculture Office, Budapest on the day of collection.

5.4.2. *F. tularensis* isolation method

Tularemic foci in parenchymal organs were excised and about 1 g of each tissue sample was homogenized individually and suspended in 2 ml of normal saline. Mice (Naval Medical Research Institute [NMRI] mouse, approximately 20g) were injected subcutaneously with 1 ml of suspension. Mice were checked three times a day. Diseased animals died after 7-10 days of injection. Heart blood and bone-marrow samples were inoculated on modified Francis agar plates (chocolate agar plate containing 1% glucose and 0.1% cysteine) on the day of their death. Culture plates were then transferred to incubator at 37 ºC for 5 days in an atmosphere containing 6.5% CO_2 and growth of *F. tularensis* was checked daily. Each strain was subcultured three times to obtain pure cultures. Culture, morphological and biochemical characteristics were examined using standard methods (Barrow and Feltham, 1993).

5.5. Isolates used in the carbon source utilization and molecular phylogenetic characterization studies

Fifteen, representative, Hungarian *F. tularensis* ssp. *holarctica* strains from different host species and geographic locations (Table 2 and Figure 9) were selected for carbon source utilization characterization. These and further four Italian strains (native isolates: human-2001, European brown hare-2006, water-2008 and imported isolate: collected in Italy from an imported hare of Central European origin) were used for the molecular phylogenetic characterization study.

Table 2. Year of isolation, host species and geographic origin of the 15 Hungarian *F. tularensis* ssp. *holarctica* strain used in the carbon source utilization and molecular phylogenetic characterization studies.

ID number	Host	Origin	Year of isolation
TUL M1	patas monkey	Szeged Zoo	2003
TUL M2	vervet monkey	Szeged Zoo	2003
TUL1	European brown hare	Alattyán	2007
TUL3	European brown hare	Kengyel	2007
TUL4	European brown hare	Békés	2007
TUL6	European brown hare	Battonya	2007
TUL7	European brown hare	Szarvas	2007
TUL11	European brown hare	Kőrösladány	2007
TUL12	European brown hare	Kőröstarcsa	2007
TUL13	European brown hare	Csökmő	2007
TUL14	European brown hare	Jászberény	2007
TUL17	European brown hare	Kecel	2008
TUL19	European brown hare	Jászárokszállás	2008
TUL21	European brown hare	Hegyeshalom	2008
TUL22	European brown hare	Mosonszentjános	2008

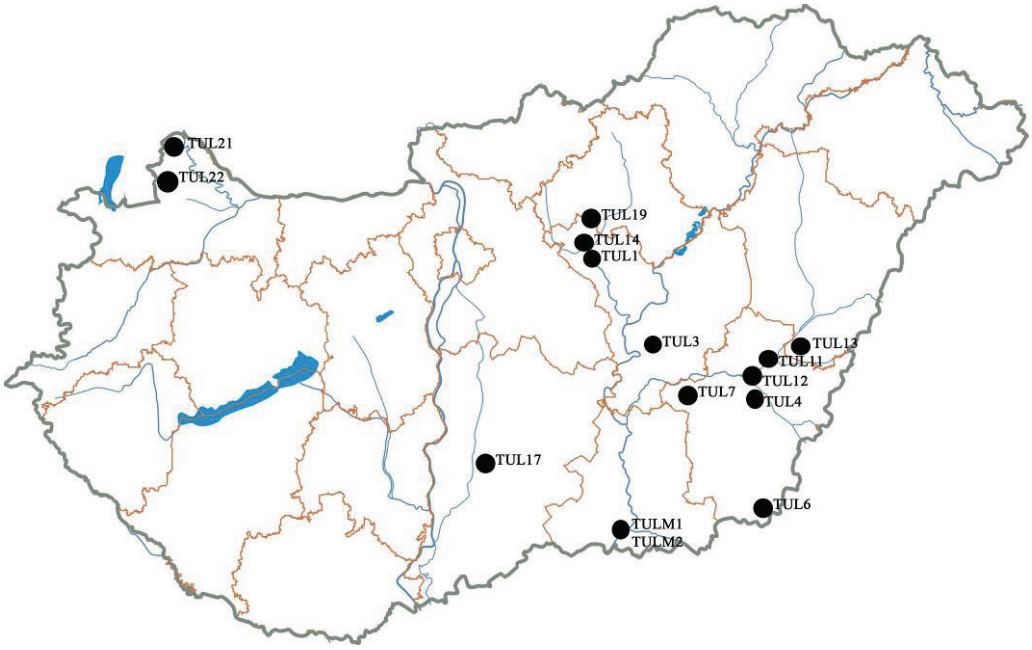


Figure 9. Geographic source of the 15 Hungarian *F. tularensis* ssp. *holarcitca* strain used in the carbon source utilization and molecular phylogenetic characterization studies.

5.6. Carbon source utilization

A 96-well automated MicroLog MicroStation System with GN2 Microplates (Biolog) was used for the characterization of carbon source utilization. Microplates were set up and analyzed with minor modification of the manufacturer's instructions. Single, pure colonies of *F. tularensis* were subcultured on modified Francis agar plates three times. Two pure colonies from the third subculture of each strain were subcultured onto 2 modified Francis plates evenly covering the whole surface of the plate. Plates were cultured at 37 ºC in 6.5% CO_2. After 24 h incubation a thin and confluent layer of *F. tularensis* colonies was formed on the surface of the plate. Bacteria were collected with a wooden stick and suspended in 18 ml inoculation fluid to obtain a homogeneous mixture. The turbidity of bacterial suspensions was set to 20 ± 2% using the Biolog Turbidimeter. A GN2 MicroPlate was inoculated with 150 µl bacterial suspension per well and incubated at 37 ºC in 6.5% CO_2. Metabolic activity was determined by visual reading of the plates after 4 and 24 hours. Positive wells were entered manually into the Biolog MicroLog2 (release 5.20) software, along with choosing the plate type (GN2), strain type (GN-FAS) and incubation time (4-6 h and 16-24 h). Further identification of the bacterial strain was performed by the software. The Biolog database includes *F. tularensis* and *F. philomiragia*, but it is not able to identify *F. novicida* and to differentiate between the three *F. tularensis* subspecies. Dendrogram showing the metabolic relationships between the strains was created with a modified unweighted pair group method with arithmetic mean analysis (Biolog software).

5.7. Molecular methods

5.7.1. DNA extraction from *F. tularensis* isolates

DNA was extracted from one distinct colony of each strain with the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations for Gram-negative bacteria.

5.7.2. DNA extraction from tissue and insect samples

Tissue or tick pools were homogenized in Tris-ethylenediaminetetraacetic acid (Tris-EDTA; pH 8.0) buffer using the TissueLyser high-throughput disruption instrument (Qiagen) according to the manufacturer's recommendations. Homogenized pools were centrifuged at 12.000 g for 5 min at 4 ºC. A 100 µl supernatant from each sample was used for DNA extraction conducted on an X-tractor Gene automated nucleic acid extraction robot (Corbett Robotics Pty. Ltd., Queensland, Australia) using the Total RNA Isolation Kit, Nucleospin 96 RNA (Macherey-Nagel GmbH. & Co., Düren, Germany) according to the manufacturer's instructions, except for the DNase incubation step. DNA was eluted in 50 µl elution buffer.

5.7.3. 16S rRNA gene based PCR

A 1000 bp large part of the 16S rRNA gene was amplified using a PCR system to identify the isolated *F. tularensis* strains. The system utilised the following primer pair: 91E: 5'-TCAAAGGAATTGACGGGG-3'; 13B: 5'-CCGGGAACGTATTCACCG-3' (Relman, 1993). PCR was performed in 50 µl total volume, containing 1 µl target DNA, 5 µl Taq Buffer ($/NH_4/_2SO_4$; Fermentas Inc., Burlington, Canada), 4 µl $MgCl_2$ (25 mM; Fermentas), 5 µl deoxyribonucleotide triphosphate (dNTP, 2 mM; Fermentas), 1 µl each primer (25 pmol/µl) and 0.33 µl Taq Polymerase (5 unit/µl; Fermentas). The PCR was performed in a Biometra – T Personal thermal cycler (Biometra Inc., Göttingen, Germany). The PCR consisted of initial denaturation for 3 min at 95 ºC followed by 39 amplification cycles of denaturation for 30 sec at 93 ºC, primer annealing at 49 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension step was performed for 5 min at 72 ºC. After amplification, 10 µl of each reaction mixture was subjected to electrophoresis in 1% agarose gel, and the amplified gene products were visualized with ultra violet light after ethidium bromide staining.

5.7.4. 17 kDa major membrane protein (*tul4*) precursor gene based TaqMan real-time PCR

A 100 bp large fragment of the *tul4* gene was amplified using a real-time TaqMan PCR system as described previously for screening tissue, water and insect samples with the following primers and probe: Tul4F: 5'-ATTACAATGGCAGGCTCCAGA-3', Tul4R: 5'-TGCCCAAGTTTTATCGTTCTTCT-3' and Tul4P: FAM-5'-TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAG-3'-BHQ (Versage et al., 2003). Probe was synthesized with a 6-carboxy-fluorescein reporter molecule attached to the 5'end and a Black Hole Quencher attached to the 3'end. All PCR were performed in 25 µl total volume, containing 2 µl target DNA, 12.5 µl commercially purified water (Millipore Co., Billerica, MA), 2.5 µl TaqGold buffer (Applied Biosystems Inc., Foster City, CA), 4 µl $MgCl_2$ (25 mM; Fermentas), 0.7 µl dNTP (10 mM; Fermentas), 1 µl of each primer and probe (10 pmol/µl), and 0.3 µl TaqGold polymerase (5 unit/µl; Applied Biosystems). PCRs were set up with the help of CAS-1200 Robotic Liquid Handling System (Corbett Robotics). PCR amplifications were performed on a Rotor-Gene™ 6000 real time instrument (Corbett Robotics). The PCR consisted of initial denaturation for 10 min at 95 ºC followed by 45 amplification cycles of denaturation for 15 sec at 95 ºC, primer annealing at 60 ºC for 30 sec, and extension at 72 ºC for 20 sec. According to the original description the detection limit of this assay is 1 CFU, while the validation procedure during the adaptation of the test determined a 10 CFU detection threshold (10 CFU: cycle threshold /Ct/ 34). A liver sample (about 50 mg) from an experimentally infected mouse (NMRI mouse, approximately 20g) served as the positive control both for DNA extraction and PCR.

5.7.5. 17 kDa major membrane protein (*tul4*) precursor gene based conventional PCR

A 400 bp large sequence of the *tul4* gene was amplified using a conventional PCR system as described earlier with the following primer pair: TUL4-435: 5'-GCTGTATCATCATTTAATAAACTGCTG-3' and TUL4-863: 5'-TTGGGAAGCTTGTATCATGGCACT-3' (Sjöstedt et al., 1997). This system was used on the real-time PCR positive insect samples in order to amplify a larger part of the *tul4* gene for further sequencing. PCRs were performed in 50 µl total volume, containing 10-100 ng target DNA diluted in 1 µl MilliQ water (Millipore), 10 µl 5X Green GoTaq Flexi Buffer (Fermentas), 5 µl $MgCl_2$ (25 mM; Fermentas), 1,5 µl dNTP (10 mM; Fermentas), 4 µl each primer (10 pmol/µl) and 0.4 µl GoTaq DNA polymerase (5 unit/µl; Fermentas). The PCR was performed in a Biometra – T Personal thermal cycler (Biometra) and consisted of initial denaturation for 5 min at 95 ºC followed by 35 amplification cycles of denaturation for 30 sec at 94 ºC, primer

annealing at 65 ºC for 60 sec and extension at 72 ºC for 1 min. The final extension step was performed for 5 min at 72 ºC. After amplification, 10 µl of each reaction mixture was subjected to electrophoresis in 1% agarose gel, and the amplified gene products were visualized with ultra violet light after ethidium bromide staining.

5.7.6. Direct cycle sequencing

PCR products were isolated from agarose gel with the QIAquick Gel Extraction Kit (Qiagen) and direct cycle sequencing, using the Big Dye Terminator V3.0 sequencing kit (Applied Biosystems) according to the manufacturer's recommendations, was performed with the same primers used in PCR in an ABI 373A automated DNA sequencer (Applied Biosystems).

5.7.7. Whole genome sequencing and analyses

To resolve the phylogenetic structure of Hungarian isolates, we sequenced the WG of a single Hungarian strain (TUL07/2007, SRP003185.3) through Solexa (Illumina Inc., San Diego, CA) next-generation sequencing technology. The library for this sample was prepared using sonication of 5 µg of genomic DNA into an average fragment size of 350 bp, followed by sample preparation and cluster generation protocols for paired-end reads from Illumina. The library was quantified using SYBR green-based (Applied Biosystems) quantitative PCR and primers modified from the adaptor sequence. The library was then run in two lanes of the flow cell to allow for sufficient coverage average. Read lengths were ca. 40 bp, thus we generated a 32 giga bp of sequence. Image analysis for base calling and alignments followed previosly published methods (Craig et al., 2008).

5.7.8. SNP discovery and analysis

SNPs of the Hungarian WG were identified in a comparison to the WG SNPs of 4 other strains, classified in the B.Br.013 lineage (LVS NC_007880, FSC 257 NZ_AAUD00000000, FSC 200 NZ_AASP00000000, RC503 http://www.hgsc.bcm.tmc.edu/microbial-detail.xsp?project_id=144) and to more distantly related OSU18 (NC_008369) which was used as an outgroup. LVS is an attenuated strain from Russia used for the development of vaccine while FSC 200 is a virulent Russian strain genetically close to LVS. FSC 257 was obtained from Moscow isolated from a tick *D. pictus* in 1949. RC503 is a virulent strain used for challenge studies in murine by researchers in Russia (Vogler et al., 2009a). SNPs were identified using an in-house bioinformatics pipeline (Auerbach, 2006; Vogler et al., 2009a). The in-house pipeline uses both Perl and Java scripts for sequence comparisons and data

parsing. Briefly, the pipeline compares genome sequences in a pairwise fashion for sequence alignment using MUMmer 3.0 system (Sourceforge.net) (Kurtz et al., 2004), and then groups the SNPs by shared location for comparisons across all taxa. A sliding window for comparison of regions was used, with the potential SNP flanked by 100 bases on each side. The repeated regions and paralogous genes from analysis were excluded. A matrix was generated which included the SNP state for each genome, referenced to the LVS sequence. Apparent tri-states SNPs and SNPs with mismatch cutoff of 1 on each side of the sequence were removed from analysis based on the assumption that they were from sequencing errors. Homoplastic SNPs were included in the analysis and actual numbers were very low therefore reflecting the clonal nature of *F. tularensis* species. A maximum-parsimony WG SNP tree was constructed using the software package PAUP 4.0b10 (Sinauer Associates Inc., Sunderland, MA).

5.7.9. Canonical SNP discovery and analysis

We genotyped 19 unknown *F. tularensis* isolates from Hungary and Italy using previously published canSNP assays known to be phylogenetically diagnostic for existing phylogroups (Svensson et al., 2009; Vogler et al., 2009a). The genome locations, primer sequences, and annealing temperatures for the previously published canSNP assays can be found in Table S1.

We further resolved the phylogenetic structure of the Hungarian lineage (B.Br.TUL07/2007) by designing high-throughput SNP assays for the 20 SNPs specific to the Hungarian strain as previously described (Papp et al., 2003). The SNP genotyping assay used mismatch amplification mutation assay (MAMA) based on allele-specific primers that are SNP specific at the 3'end (Cha et al., 1992). A single base mismatch at the ante-penultimate (-3) position of each allele-specific primer enhanced the SNP discrimination capacity of these assays (Papp et al., 2003). Allele-specific MAMA primers were designed using Primer Express 3.0 software (Applied Biosystems). The derived allele-specific primer possessed an additional 19 bp GC-clamp at the 5'end with a sequence of CGGGG and the other allele primer had no additional sequence. The GC-clamp increased the melting temperature (Tm) of the resulting PCR product by 3-5 ºC, a shift that is detectable by SYBR Green dye (Applied Biosystems) on a real-time PCR platform (Melt-MAMA). Each 10 µl Melt-MAMA reaction mixture contained 1xSYBR green PCR master mix (Applied Biosystems), derived and ancestral allele-specific primers, a common reverse primer, and 1 µl of DNA template at ~1ng/µl. Melt-MAMA assays were performed using ABI 7900HT fast real-time PCR system with SDS v2.3 software (Applied Biosystems). Reactions were first raised to 50 ºC for 2 min to activate the uracil glycolase, then raised to 95 ºC for 10 min to denature the DNA, and then cycled at 95 ºC for 15 sec and 55 ºC or 60 ºC for 1 min for 50 cycles. Immediate after the completion of the PCR

cycle, amplicon melt dissociation was measured by ramping from 60 to 95 ºC in 0.2 ºC/min increment and the fluorescent intensity was recorded.

These assays were screened across 100 geographically diverse set of European isolates belonging to the B.Br.020/021 subclade (including the 14 Hungarian and the 1 imported Italian isolates). This screening effort resulted in the identification of 6 new phylogenetic subclades. The genome locations, primer sequences, and annealing temperatures for these canSNP assays can be found in Table 3.

Table 3. The genome locations, SNP state, primer sequences and thermal cycler parameters for the 6 new canSNP assays from our genome comparisons.

SNP	LVS position	Genome SNP state (D/A)*	Melt-MAMA primer†	Melt- MAMA primer sequences‡	Primer concentration (µM)	Annealing temperature (°C)	Melting T_m (°C)
B.Br.033	1716449	A/G	A D C	ATTGCTACTTCTATTTACGCCAAcAg ggggcggggcggggcATTGCTACTTCTATTTACGCCAAgAa TGTGAACAACCAAGTTGGCTT	0.200 0.200 0.200	60	74.3 79.0
B.Br.034	1302760	T/C	A D C	GTAGCGAGCATTATTTGCTGGtTc ggggcggggcggggcTAGCGAGCATTATTTGCTGGgTt ATAAAACTATAAATTTACATAAAATGAAAACTTCTC	0.400 0.200 0.200	60	69.2 78.6
B.Br.035	164258	A/C	A D C	GCCTTAATCTAGTATTTTCGCTTATCtCc ggggcggggcggggcGCCTTAATCTAGTATTTTCGCTTATCaCa CGGGCTCTAAAATAAGATTTAAGTTAGTAAGT	0.400 0.200 0.200	60	70.3 75.5
B.Br.036	554148	T/G	A D C	TATTATAGTTTCTAAAAACAGTCTAATTAATTtTg ggggcggggcggggcTATTATAGTTTCTAAAAACAGTCTAATTAATTgTt GTTCGACCATGACTACAGTGTTG	0.600 0.200 0.200	60	69.0 73.9
B.Br.037	215319	T/C	A D C	AACATTTTAGGAACTCTACGATGATAAACTTaAc ggggcggggcggggcCATTTTAGGAACTCTACGATGATAAACTTgAt GAAATATCTCAATGAAATCTAATTTAACTAAAATCAC	0.200 0.200 0.200	60	69.7 75.9
B.Br. TUL07/2007	1669446	G/A	A D C	ATGCCATCAGCCATTTACTACTCaCa ggggcggggcggggcCCATCAGCCATTTACTACTCcCg CTTCCCTGATTTTCTAAGTTCTGCTTG	0.200 0.200 0.200	60	73.7 80.1

*SNP states are presented according to their orientation in the LVS reference genome (NC_007880); D: Derived SNP state; A: Ancestral SNP state.

†D: Derived; A: Ancestral; C: Consensus.

‡Primer tails and antepenultimate mismatch bases are in lower case.

5.7.10. Multi-locus variable-number tandem repeat analysis

Fifteen Hungarian isolates were screened with an 11-marker MLVA system (Vogler et al., 2009b). This was done in order to determine the level of genetic diversity within each identified subclade.

PCR amplification of the 11 VNTR loci was performed in three multiplex and two singleplex (i.e. single-marker) PCRs that were subsequently pooled into three mixes for electrophoresis. Primer sets are presented in Table S2. All PCR mixes contained final concentrations of the following reagents: 1×PCR buffer without $MgCl_2$ (Invitrogen Inc., Carlsbad, CA), 2 mmol $l0^{-1}$ MgCl2 (Invitrogen), 200 µmol $l0^{-1}$ of each dNTPs (Invitrogen), 0.8 units of Platinum Taq DNA polymerase (Invitrogen), primers (IDT Inc., San Diego, CA; Applied Biosystems) (for concentrations see Table S2), 1 µl of template, and molecular grade water to a final volume of 10 µl. All PCRs were performed under the same thermocycling parameters in Bio-Rad 96-well DNA engines (Bio-Rad Laboratories Inc., Hercules, CA) equipped with hot bonnets. Reaction mixes were first raised to 94 ºC for 5 min to denature DNA and activate the polymerase, then cycled at 94 ºC for 30 sec, 58 ºC for 30 sec, and 72 ºC for 30 sec for 35 cycles, and, finally, incubated at 72 ºC for 5 min for a final polymerase extension step. MLVA PCR products were visualized on an ABI 3100 automated fluorescent capillary DNA sequencer (Applied Biosystems). Electrophoresis Mix 1 contained PCRs Mix 1A and Mix 1B, which were diluted in a ratio of 2:1:97 with molecular grade water. Electrophoresis Mix 3 contained PCRs Mix 3A and Mix 3B, which were diluted in a ratio of 2:1:97 with molecular grade water. Electrophoresis Mix 2 contained only one PCR (Mix 2) and did not require any pooling, but was diluted with molecular grade water in a ratio of 1:49. Half a microlitre of each diluted and pooled electrophoresis mix was then mixed with 9.5 µl of a HiDi formamide (Applied Biosystems) and size standard solution comprised of 9.45 µl HiDi formamide and 0.05 µl ROX-labelled MapMarker 50-1000 bp (BioVentures Co., Murfreesboro, TN) per sample. Amplicons were sized using Applied Biosystems GeneMapper software (Applied Biosystems).

5.8. Statistical methods

Formerly described formulas were used to calculate the specificity, sensitivity and predictive value of diagnostic tests (Moore et al., 1988):

$$\text{sensitivity} = \frac{TP}{TP + FN} \text{ x 100, in which TP = true positive, FN = fals negative;}$$

$$\text{specificity} = \frac{TN}{FP + TN} \text{ x 100, in which TN = true negative, FP = fals positive;}$$

$$\text{predictive value of a negative test} = \frac{TN}{TN + FN} \text{ x 100;}$$

$$\text{predictive value of a positive test} = \frac{TP}{TP + FP} \text{ x 100.}$$

5.9. Overview of methods used in ecological studies

5.9.1. Investigation of the ecology of *F. tularensis* ssp. *holarctica*

The study region was a 10 km^2, lowland scene situated in South-eastern Hungary (47º05'N, 20º56'E). Sodic steppes, grasslands, remnants of oak woods, marshlands and small water courses compose the landscape. The climate is continental with cold, cloudy and humid winters (-3 ºC mean temperature in January 2009) and warm to hot summers (23 ºC mean temperature in July 2009). Retrospective data about the tularemia situation of the region were obtained from the local veterinary authority and human health service. Samples for this study were collected during a one year period, from December 2008 until December 2009.

European brown hares shot during the annual hunting events, in December of 2008 and 2009, were screened with both slide and tube agglutination tests (OIE 2008; Treml et al., 2007). Necropsies, histological and immunohistochemical examinations, the isolation and identification of *F. tularensis* strains were carried out.

Small mammals were live-trapped during four sessions performed in April, July, October and November 2009, in two study habitats; an open grassland and an oak-wood area. Sherman traps laid out in 7 × 7 grids with 10 meter inter-trap intervals, were used for five nights and days for live trapping. Ectoparasites (ticks and fleas) were combed off the rodents' coats, identified on the basis of morphology and further examined individually. The blood of small mammals was screened by slide agglutination test as described above. Lung, liver, spleen and kidney tissue pools (a total of about 100 mg) from each small mammal were

homogenized in 1000 µl Tris-EDTA (pH 8.0) buffer. DNA was extracted and examined the *tul4* gene based TaqMan real-time PCR.

Ticks were collected by dragging from the vegetation in the grassland and the oak-wood near the Sherman traps for 3-3 hours in April, July and October, 2009. After identification, ticks were pooled (30 larvae, 15 nymphs, 10 or fewer males or females/pool), and homogenized in 500 µl Tris-EDTA (pH 8.0) buffer. DNA was extracted and analyzed with the *tul4* gene based TaqMan real-time PCR. Positive samples were further characterized by the sequencing of a 400 bp large sequence of the *tul4* gene amplified with the conventional PCR system.

250 ml water samples were taken from all the existing water sources located in the study area in April, July and October, 2009. Samples were filtered through 0.2 µm membrane filters (Seitz GmbH., Bad Kreuznach, Germany). Discs of 10 mm in diameter were excised from each membrane and homogenized in 750 µl Tris-EDTA (pH 8.0) buffer. DNA was extracted and analyzed by *tul4* gene based TaqMan real-time PCR. An additional 250 ml water sample collected from the lake in April was mixed with around 100 CFU *F. tularensis* ssp. *holarctica* isolate and served as a positive control to check for potential PCR inhibition.

Blood samples were collected from sheep (jugular vein), cows (caudal vein) and buffalos (caudal vein) in May, 2009. All animals belonged to extensively managed flocks of 100 sheep, and herds of 186 cattle and 92 buffalos grazed in the study area. Blood and sera were examined by slide and tube agglutination tests.

5.9.2. Effect of common hamster on the epizootiology of *F. tularensis* ssp. *holarctica*

The study site was an 80-km^2 agricultural area in Békés county, in Eastern Hungary. Retrospective data about the tularemia situation of the region were obtained from the local veterinary authority and human health service. Estimation of hamster population size in the study area was based on the number of active burrows/hectare (Bihari, 2003). Hamsters were trapped with kill traps and were screened with both slide and tube (1/10 dilution) agglutination tests using whole blood (sera) taken from the heart and thoracic cavity. Lung, liver, spleen, and kidney tissue pools (a total of about 100 mg) were collected from 50 hamsters trapped in May and from 50 animals trapped in October 2009. Ticks were also collected from 900 animals. After the identification to species, developmental stage, and sex, ticks were pooled (10 or fewer). DNA was extracted from the pools of ticks and organs and screened by *tul4* gene based TaqMan real-time PCR. Win Episcope 2.0 program was used for data analysis.

5.9.3. Susceptibility of the common hamster to *F. tularensis* ssp. *holarctica*

Two adult, male hamsters were trapped alive with box traps (Tomahawk Live Trap Co., Tomahawk, WI) and housed in individual cages (730x530x250 mm). In order to induce acute infections resembling natural cases both animals were infected in accordance with all applicable institutional and national guidelines with a wild *F. tularensis* ssp. *holarctica* strain isolated from a European brown hare in 2007. One animal was infected intramuscularly on its hind leg (case 1) with 10^3 CFU as ticks harbor high infectious doses of *F. tularensis* (Gurycová et al., 1995), and one orally with 10^5 CFU (case 2), a dose used in earlier studies in similar species in similar sensitivity, like voles (*Microtus pennsylvanicus* and *Microtus rossiaemeridionalis*) (Bell and Stewart, 1983; Olsufjev et al., 1984; Olsufjev and Dunayeva, 1970; WHO, 2007). Hamsters were checked three times a day to record clinical symptoms. The animals were necropsied right after natural death, and tissue samples (brain, heart, lung, liver, spleen, kidney, stomach, small and large intestine, submandibular, mediastinal and mesenterial lymph nodes, testicle, bone marrow and brain) were collected for routine histologic examination. Histologic and immunohistochemical examination was performed. Livers were used for reisolation of *F. tularensis* on a modified Francis agar plate.

6. Results

6.1. Retrospective data collection

The number of human cases in Hungary ranged between 20 and 148 per year during the past two decades. The percentage of *F. tularensis* seropositive hares, captured for live animal export (2.8-40 thousand exported hares/year) ranged between 0.31% and 20.2% (Table S3, Figure 10).

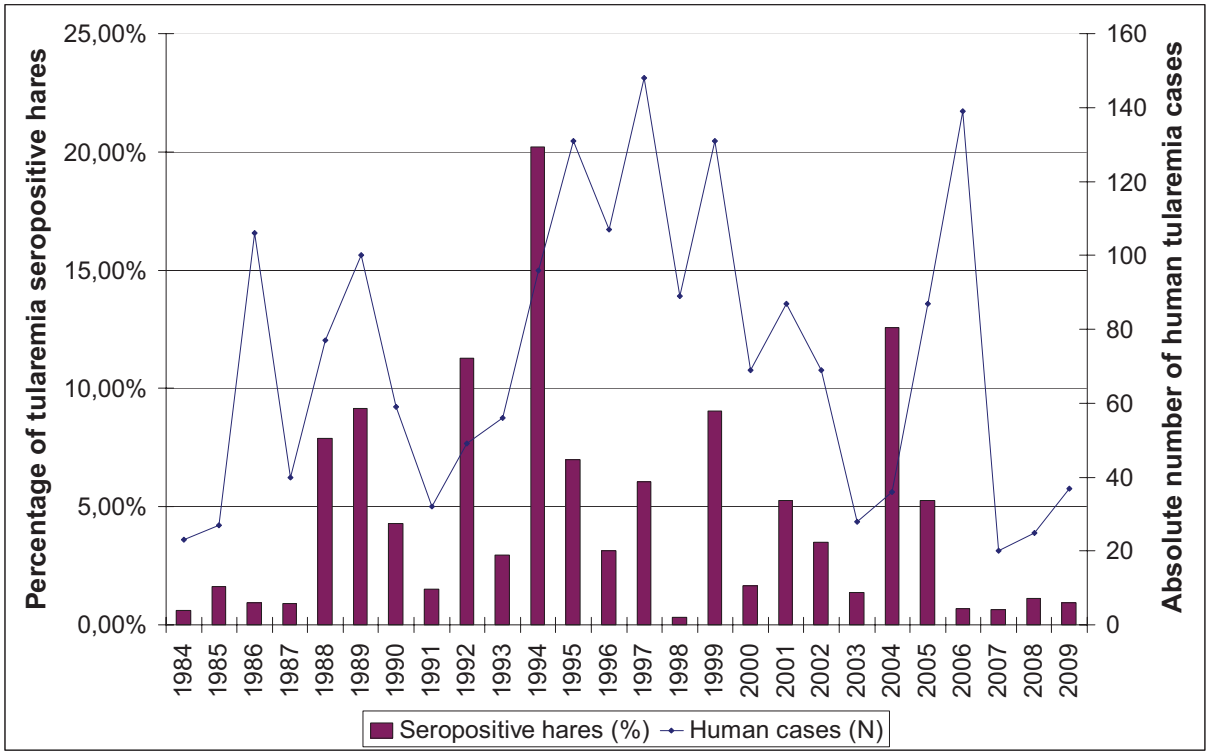


Figure 10. Percentage of *F. tularensis* seropositive hares, captured for live animal export (2.8 – 31 thousand exported hares/year) and the absolute number of human cases in Hungary between 1984 – 2009.

6.2. Investigation of the ecology of *F. tularensis* ssp. *holarctica*

The study area is considered to be a tularemia endemic region as the infection has repeatedly been detected in 5-7% of the local European brown hare population during the past 15 years (estimated from 3320 hares by slide agglutination test). In addition, 21 human clinical cases occurred in the surrounding towns (18000 inhabitants) during the same time period. The latest epizootics could be identified in 1997, 1999 and 2004 when seropositivities of 20.4%, 36.8% and 12.3% were recorded in the local hare population.

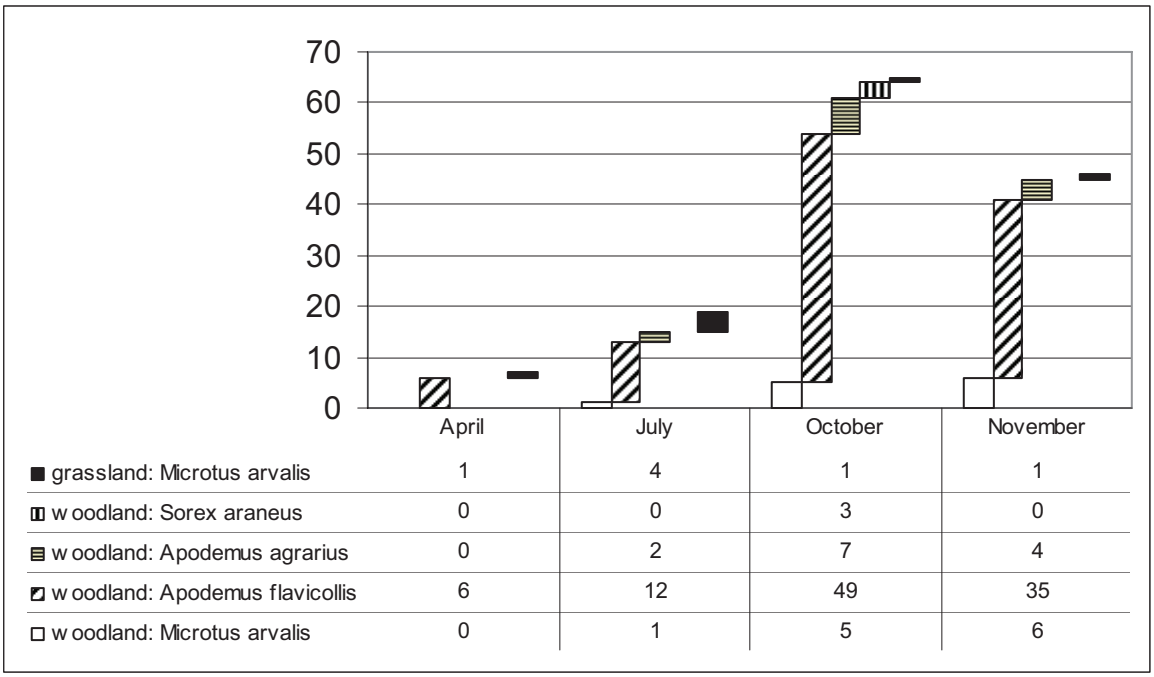
A total of 197 hares were shot during the annual hunting events in December of 2008 (75 hares) and 2009 (122 hares). The seroprevalence of tularemia was found to be 5.3 % (4/75) in 2008 and 4.9 % (6/122) in 2009 by the slide agglutination test. All animals seropositive by slide agglutination test (10 animals) exhibited tube agglutination titers of at least 1/10, even when evaluated strictly by the criteria (agglutinated sediment and clear supernatant fluid) recommended in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE 2008) (Table 4). The 1/40 cut off titer recommended by the manufacturer was identified in only two of these positive cases (one each year) and only a single animal reached the 1/80 antibody titer. The latter is the positive threshold value recommended by the manufacturer of the diagnostic kit. Six of the ten seropositive hares identified by slide agglutination test were necropsied and tularemic lesions demonstrating in-situ *F. tularensis* specific immunolabelling were identified in each individual. The most often affected organs were the lungs and the kidneys. *F. tularensis* ssp. *holarctica* was isolated from four of these six cases.

A total of 137 small mammals were trapped in the two study habitats during the year (Figure 11). Further 40 animals including 19 common voles, 8 yellow-necked mice (*Apodemus flavicollis*) 2 striped field mice (*Apodemus agrarius*) and a by-catch of 8 Eurasian pygmy shrews (*Sorex minutus*) and 3 common shrews (*Sorex araneus*) were trapped next to the oak wood in November. All these 177 small mammals were negative for *F. tularensis* infection with both slide agglutination test and real-time PCR. A total of 457 ectoparasite specimens were collected from the small mammals, comprising 404 *I. ricinus* (362 larvae, 41 nymphs, 1 female) and 28 *H. concinna* (27 larvae, 1 nymph) ticks and 15 *Ctenophtalmus assimilis* and 10 *Nosopsyllus fasciatus* fleas. All these ectoparasites were negative for *F. tularensis* by real-time PCR.

Table 4. Results of tube agglutination test, *F. tularensis* specific immunohistochemistry, and isolation from seropositive European brown hares identified by slide agglutination test.

	Slide agglutination	Tube agglutination					*F. t.* specific immunolabelling present in	*F. t.* isolation
		1/10	1/20	1/40	1/80	1/160		
1	+	+ + + +	+ +	–	–	–	lung	–
2	+	+ + + +	+ +	–	–	–	lung	+
3	+	+ + + +	+ + + +	+ + + +	+ + + +	++	lung, mammary gland	+
4	+	+ + + +	+ +	–	–	–	mediastinal lymph nodes	–
5	+	+	–	–	–	–	NE*	NE*
6	+	+ + + +	+ + +	+ +	–	–	kidney	+
7	+	+	–	–	–	–	NE*	NE*
8	+	+ + +	–	–	–	–	NE*	NE*
9	+	+ + + +	+ +	–	–	–	kidney	+
10	+	+ + + +	+ +	–	–	–	NE*	NE*

*NE=not examined



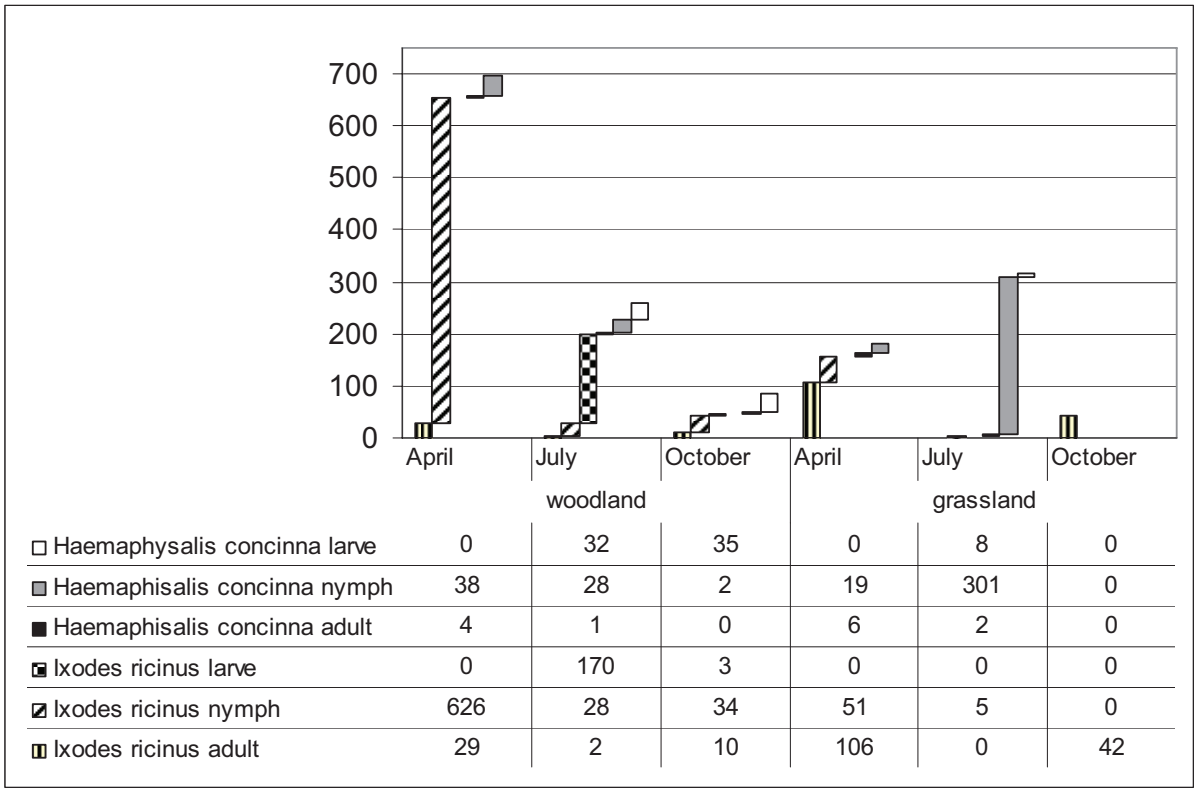
	April	July	October	November
grassland: Microtus arvalis	1	4	1	1
woodland: Sorex araneus	0	0	3	0
woodland: Apodemus agrarius	0	2	7	4
woodland: Apodemus flavicollis	6	12	49	35
woodland: Microtus arvalis	0	1	5	6

Figure 11. Number and distribution of small mammal species trapped in the grassland and in the oak-wood study habitat during the study year.

A total of 1582 *I. ricinus* and *H. concinna* ticks were collected from the vegetation in the grassland and in the oak-wood. Seventy percent of ticks were *I. ricinus* (1106) and 30% *H. concinna* (476). The two species exhibited different seasonal activity peaks during the year (Figure 12). One *H. concinna* female pool of 2 individuals and one *H. concinna* nymph pool of 15 ticks collected at the grassland area in July were found positive for *F. tularensis* by real-time PCR. The 400 bp large fragments of the *tul4* gene were sequenced and submitted to GenBank (HM014008, HM014009). These findings mean that *F. tularensis* ssp. *holarctica* was carried by at least two *H. concinna* ticks, indicating a 0.42% (2/476) minimum prevalence for the whole region and 0.59% (2/336) for the grassland habitat.

Water samples were taken from 6 ditches and one small lake located in the study area during April. In July and in October of 2009 samples were obtained only from the lake as the ditches dried up by then. *F. tularensis* was not detected with real-time PCR in any of the 9 water samples. The Ct value was 32 for the positive control water sample.

Blood samples were collected from 100 sheep, 50 cows and 50 buffalos grazed at the study area. The blood and sera of these 200 ruminants were also negative for *F. tularensis* antibodies with both slide and tube agglutination (agglutination titers <1:10) tests.



	April	July	October	April	July	October
		woodland			grassland	
Haemaphysalis concinna larve	0	32	35	0	8	0
Haemaphisalis concinna nymph	38	28	2	19	301	0
Haemaphisalis concinna adult	4	1	0	6	2	0
Ixodes ricinus larve	0	170	3	0	0	0
Ixodes ricinus nymph	626	28	34	51	5	0
Ixodes ricinus adult	29	2	10	106	0	42

Figure 12. Number and distribution of tick species and their development stages collected from the vegetation in the grassland and in the oak-wood habitat during the study year.

6.3. Susceptibility of the common hamster to *F. tularensis* ssp. *holarctica* and its effect on the epizootiology of tularemia

The estimated population size in the study area was 400,000 hamsters (50 burrows/hectare) in 2008 and 80,000 (10 burrows/hectare) in 2009. Overall 900 hamsters were trapped with kill traps in the study area: 250 in May 2008, 500 in May 2009, and 150 in October 2009. Thus, at an assumed 0.5% antibody prevalence the probability of diagnosing at least one positive animal was 100% from the 250 screened hamsters in 2008 and 96.2% from the 650 animals in 2009. The serologic testing of all 900 hamsters yielded negative results both with slide and tube agglutination. Only one tick species, *I. acuminatus* was found on the hamsters. Overall, 368 females and 6 nymphs were collected. *F. tularensis* DNA was not detected in any of the tick pools or organ pools of the 100 hamsters tested.

The two infected hamsters died on day 8 (case 1) and on day 9 (case 2) post infection. Clinical signs were observed on the day before death, when animals were found apathetic and quickly entered a moribund state. Both animals were antibody-negative by the slide agglutination test. Gross pathologic lesions were found only in the spleens, which were enlarged and congested. In case 1, the spleen presented several randomly distributed

pinpoint white necrotic foci on the serosal and cut surfaces. Histologically severe acute necrosis was found in almost the entire section of the spleens. Focal or multifocal acute necrosis was also evident in the livers (Figure 13), lymph nodes, and bone marrow. Additional findings of diffuse severe acute glomerulo- and tubulonephrosis, moderate lympho-histiocytic interstitial bronchopneumonia and acute multifocal hemorrhage in the lungs were observed in both cases. Bacterial emboli were found in the glomeruli and interstitial blood vessels of the kidney in both cases and in the blood vessels of the lung and in the sinusoids and blood vessels of the liver and spleen of case 1. *F. tularensis* antigen was found in large aggregates or small dots within the blood vessels or sinusoids of all organs examined (Figure 14).

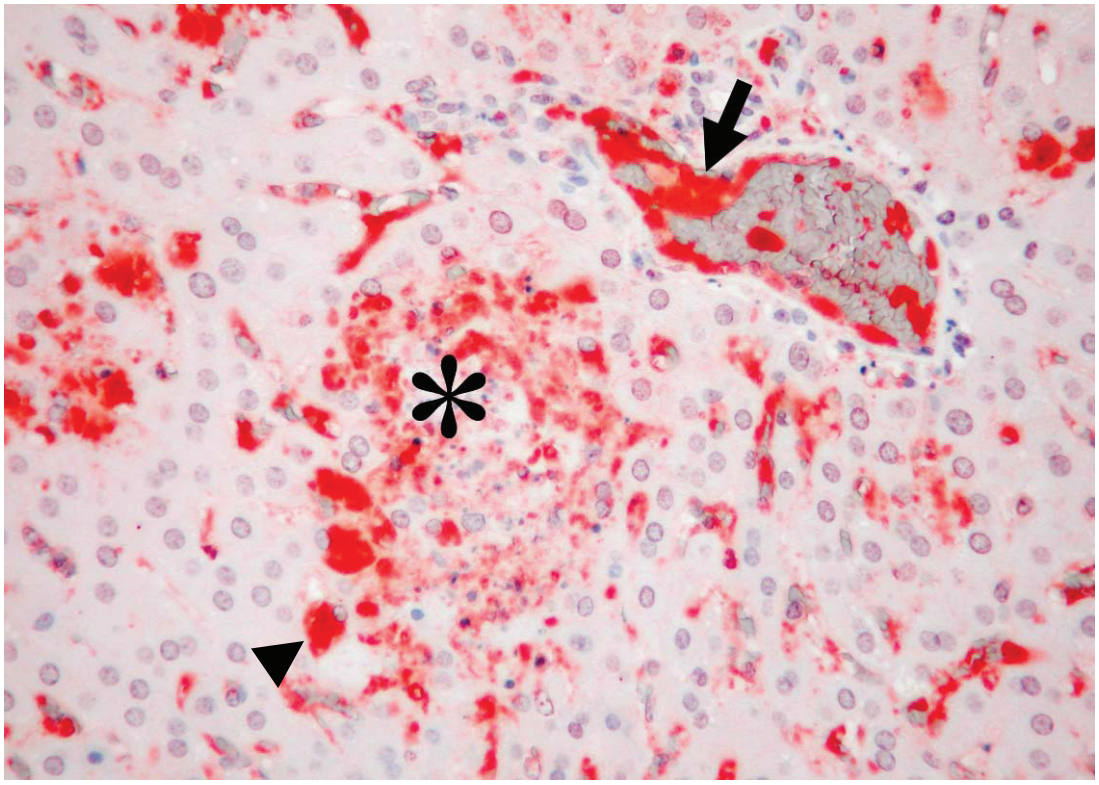


Figure 13. Liver; common hamster (case 1). Focal acute necrosis (asterisk) presenting significant amounts of *F. tularensis* antigen. There is strong immunoreactivity within a blood vessel (arrow), in sinusoids, endothelial cells, hepatocytes (arrow head) and in a few macrophages. Immunohistochemistry, mouse monoclonal antibody to *F. tularensis*, and hematoxylin counterstain.

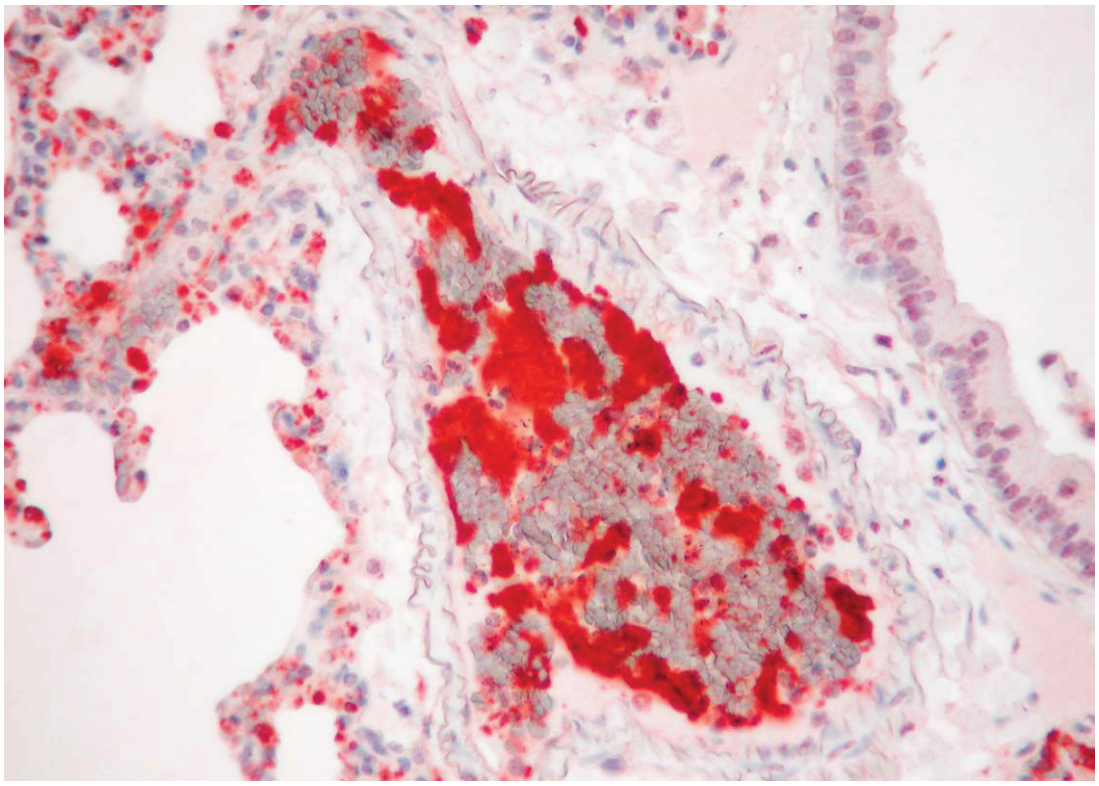


Figure 14. Lung; common hamster (case 1). Moderate lymphohistiocytic interstitial pneumonia and strong *F. tularensis* immunoreactivity within a blood vessel, in endothelial cells, pneumocytes and macrophages. Immunohistochemistry, mouse monoclonal antibody to *F. tularensis*, and hematoxylin counterstain.

Immunolabelling was more intensive and showed wider distribution in the organ samples of case 1. Intracytoplasmic bacterial antigen, visualized as small dots or diffuse granular staining, was frequently observed in macrophages, reticulocytes, endothelial cells, pneumocytes, enterocytes, hepatocytes, neurons, glial cells, epithelial cells of testis and stomach mucosa, and heart muscle cells. *F. tularensis* antigen accumulated in areas of necrosis. Bacteria were rarely found in the lumen of small intestine and seminiferous tubuli of the testes. *F. tularensis* was reisolated from both hamsters. The pathologic, immunohistochemical and bacteriologic results contributed to the diagnosis of septicemia.

6.4. Pathology of tularemia in European brown hares

Seropositive animals were in good, (12/50 cases, 24%) in moderate (11/50 cases, 22%) or in weak body condition (27/50 cases, 54%). The main gross pathological lesion of the disease was the presence of few to numerous necrotic foci in different organs in a total of 44/50 (88%) cases (Table 5). Necrotic foci were evident in single (24 cases), in two (13 cases) or in multiple organs (7 cases). Foci were randomly distributed, well demarcated, round, white, grayish-white or yellowish-white, with a diameter of 0.1-0.5 cm, and they were often surrounded by a dark, hyperemic ring.

Table 5. Number of cases with gross pathological lesions in the organs of seropositive European brown hares (n=50).

Organ	Number of positive cases/examined cases (%)
lung	40 (80%)
pericardia	14 (28%)
kidneys	10 (20%)
testicles	2 (4%)
bone marrow	2 (4%)
mammary gland	1 (2%)
liver	1 (2%)
Total	44 (88%)

The foci in the lungs and kidneys tended to coalesce to nodules of up to 1 cm in diameter in several cases. Foci were frequently observed on the serosal surface of the lungs (Figure 15) and kidneys (Figure 16). These foci and the foci in the pericardium (Figure 17) were raised, dome shaped, or flattened, their serosal surface was dry and granular, so the lesion corresponded to focal serositis. Affected testicles were enlarged, and several yellowish-white foci or nodules were evident on their cut surface (Figure 18). Low numbers of foci were also seen in the bone marrow and the mammary gland. Two of the three natural mortality cases were adult, male hares in weak body condition. They had swollen lungs, kidneys, livers and spleens. Petechial haemorrhages were observed in the lungs and large numbers of grayish-white or yellowish-white foci were seen in both lungs and pericardia as well as in the testicles of a single case. No lesions resembling tularemia or other infection were observed in seronegative hares.

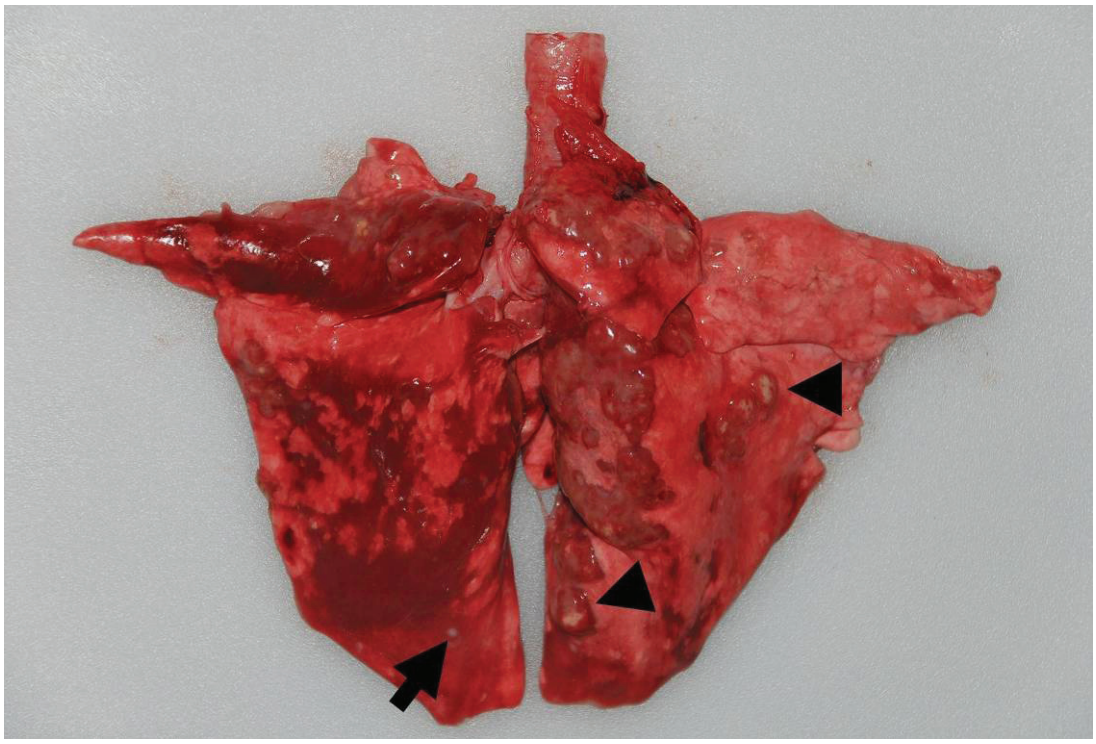


Figure 15. Lung; European brown hare. Numerous greyish-white foci (arrow) and nodules (arrowheads) surrounded by dark, hyperemic areas are scattered throughout the lungs.

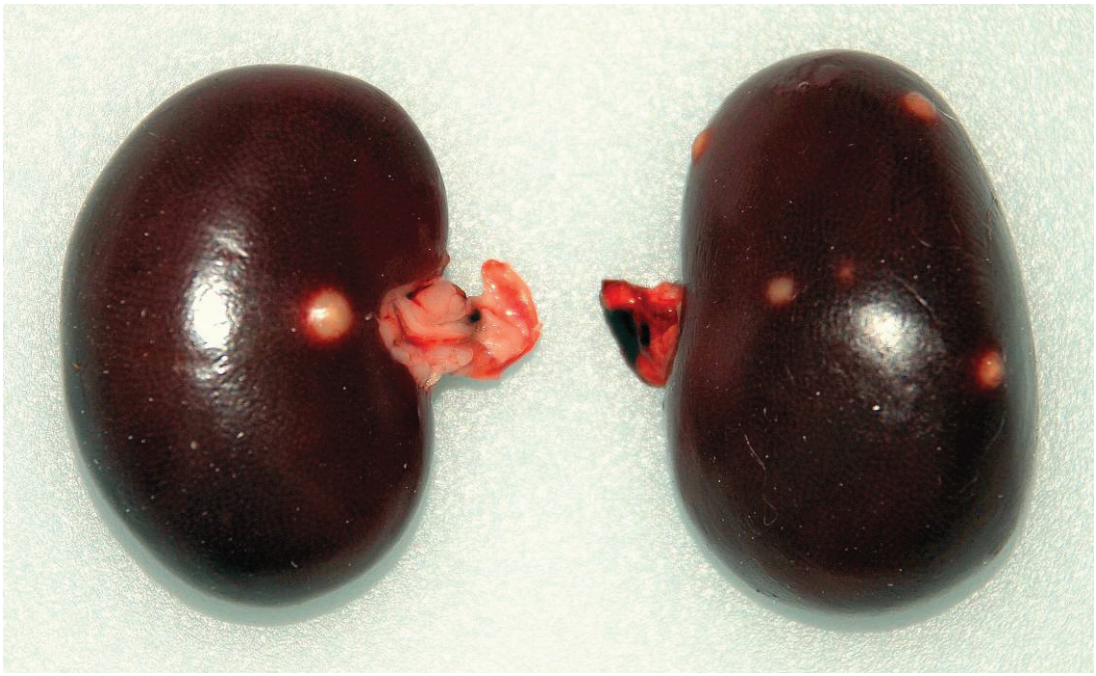


Figure 16. Kidneys; European brown hare. Greyish-white foci measuring 2–5 mm in diameter.

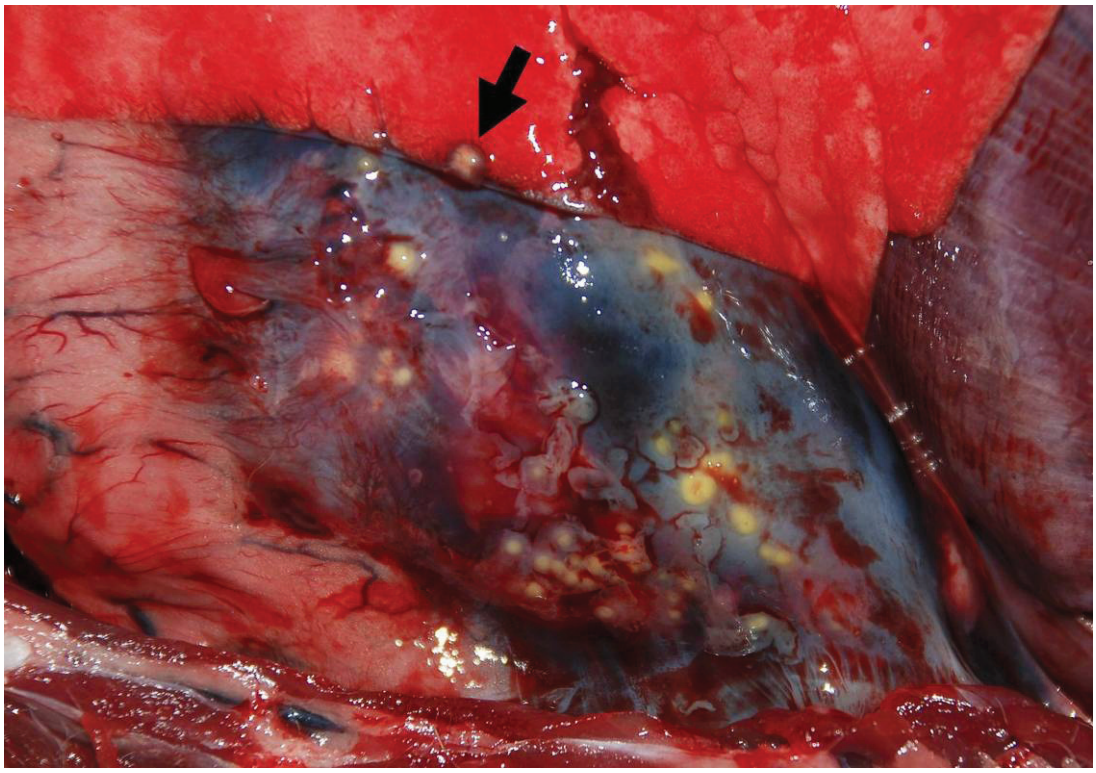


Figure 17. Pericardium and lung; European brown hare. Numerous yellowish-white, flattened foci of different size on the pericardium and one grayish-white nodule surrounded by a dark, hyperaemic area in the lung lobe (arrow).

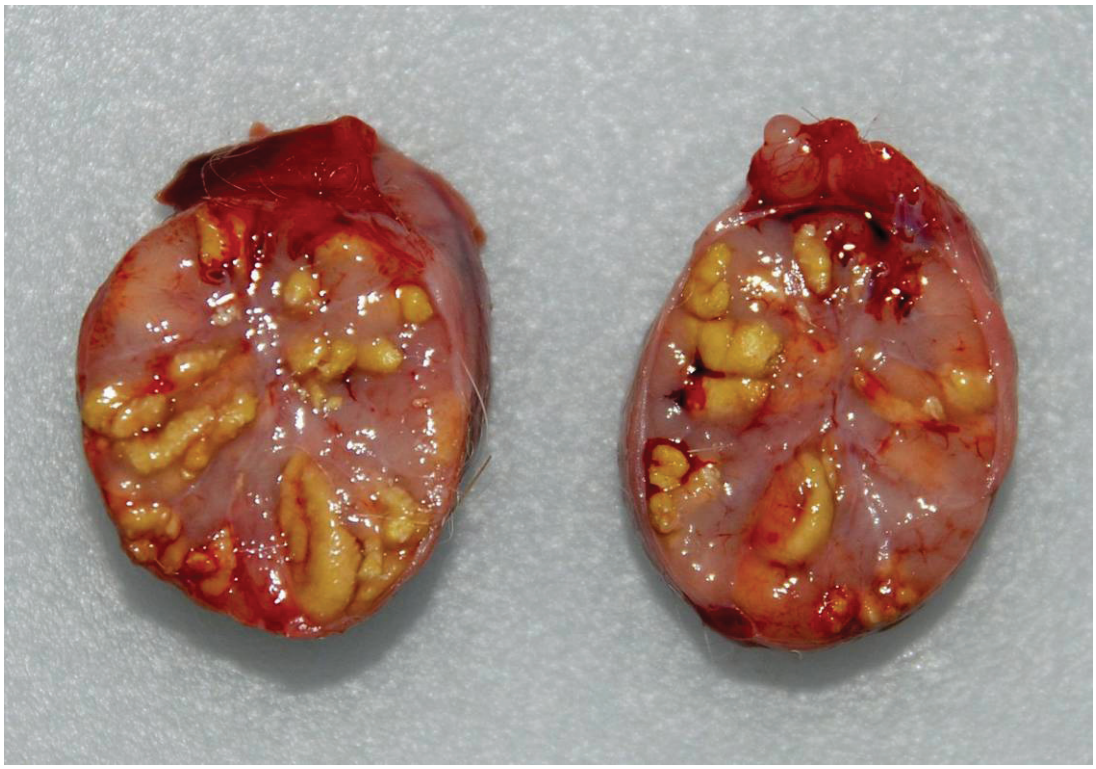


Figure 18. Testicle; European brown hare. Numerous yellowish-white foci and nodules on the cut surface.

The foci identified by gross pathological examination corresponded to focal or coalescing granulomatous inflammation, which completely replaced the normal tissue structure of the affected organs. These foci were randomly distributed in the organs, and serosal membranes were frequently involved. Macrophages were the dominant constituent cell type, but other cells including lymphocytes, heterophil granulocytes, multinucleated giant cells and fibrocytes were also found occasionally (Figure 19). Focal or multifocal necrosis was often observed in the centre of these lesions (Figure 20).

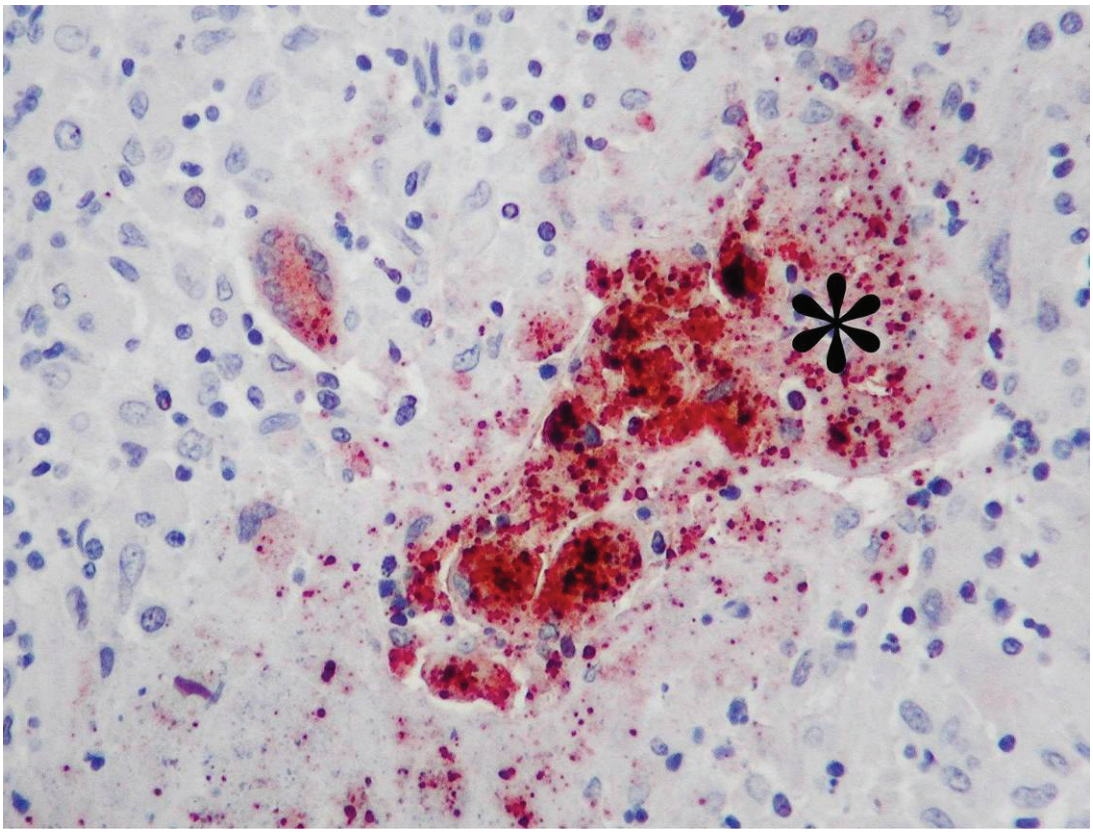


Figure 19. Lung; European brown hare. Focal granulomatous inflammation with central necrosis (asterisk). There is strong *F. tularensis* immunoreactivity within the necrotic foci, in macrophages and in a giant cell. Immunohistochemistry, mouse monoclonal antibody to *F. tularensis*, and hematoxylin counterstain. (Photo taken by L. Szeredi)

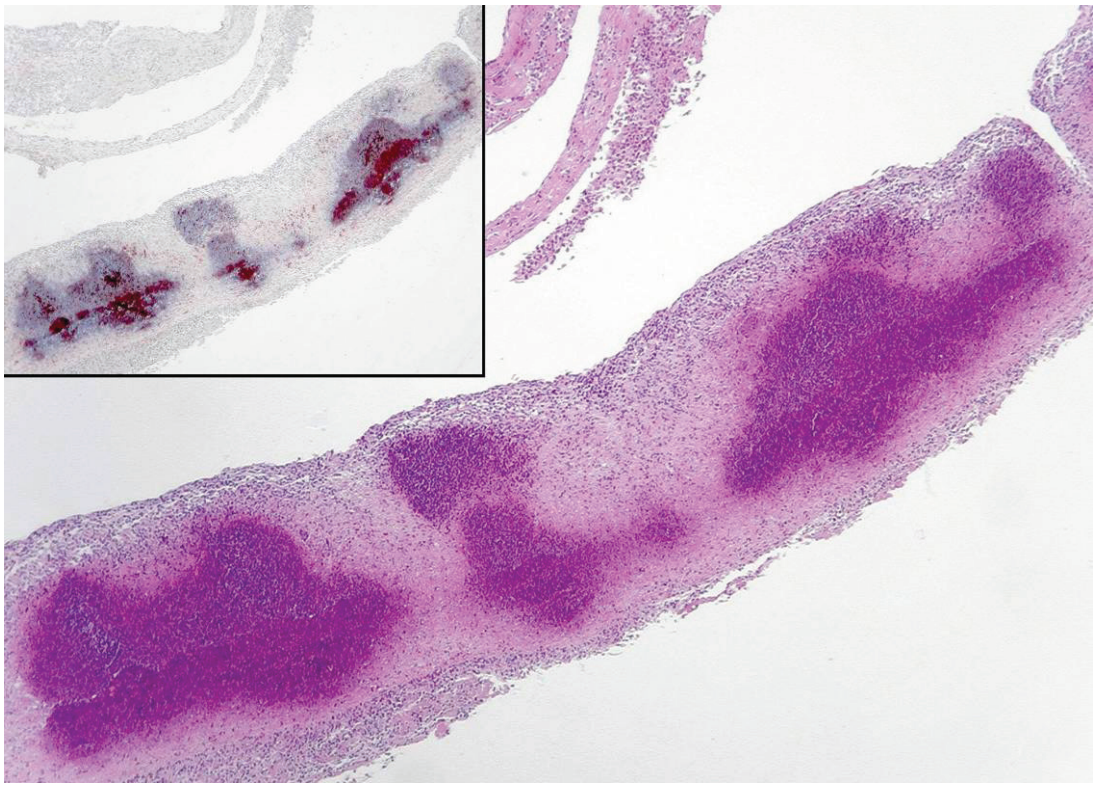


Figure 20. Pericardium; European brown hare. Coalescing necrotic foci in the pericardium completely replacing the normal tissue structure. HE. Insert: Strong *F. tularensis* immunoreactivity can be seen in the necrotic foci. Immunohistochemistry, mouse monoclonal antibody to *F. tularensis*, and hematoxylin counterstain.

Granulomatous inflammation was found with microscopic examination but not with gross pathological examination in the mediastinal lymph nodes of 12/35 cases, in the liver and spleen of 2/50 cases and in the bone marrow of 1/50 case. A total of 2/50 carcasses (4%) showed no visible gross lesions, but they exhibited microscopic changes indicative of the infection. Lesions were not found in 4/50 (8%) seropositive, and in the 20 seronegative control animals.

The coalescing granulomatous inflammation in the lungs contained no or only minor necrotic areas in several cases. Remnants of necrotic blood vessels and bronchioli were occasionally observed in affected areas. Inflammatory exudate and debris were found in several bronchioli not adjacent to the areas of granulomatous inflammation, and alveolar edema was also prominent in these areas. The foci in the pericardium showed excessive central necrosis, and fibrinous inflammation was obvious on adjacent serosal surfaces (Figure 20). Multiple foci of granulomatous inflammation were found both in the cortex and medulla of affected kidneys. The entrapped tubuli found in these areas, were partly or completely necrotic and filled with necrotic debris. Multifocal, granulomatous inflammation was also found in the suburothelial area of the renal pelvis, and adjacent inflammatory, necrotic exudate was present in the

lumen. In the testis and epididymis the granulomatous inflammation expanded the intertubular connective tissue lining over large areas. Several tubuli were partly or completely necrotic and their lumen was filled with necrotic inflammatory exudate (Figure 21). Foci of granulomatous inflammation with central necrosis were found in the liver, bone marrow, mammary gland, spleen, and mediastinal lymph nodes, too (Table 6).

Histological lesions were essentially the same in the 3 natural mortality cases and the shot animals. However, in two of the former cases numerous necrotic foci were evenly distributed in 4 or 5 different organs (Table 6). No lesions were found in heart muscle, ovaries and the intestines.

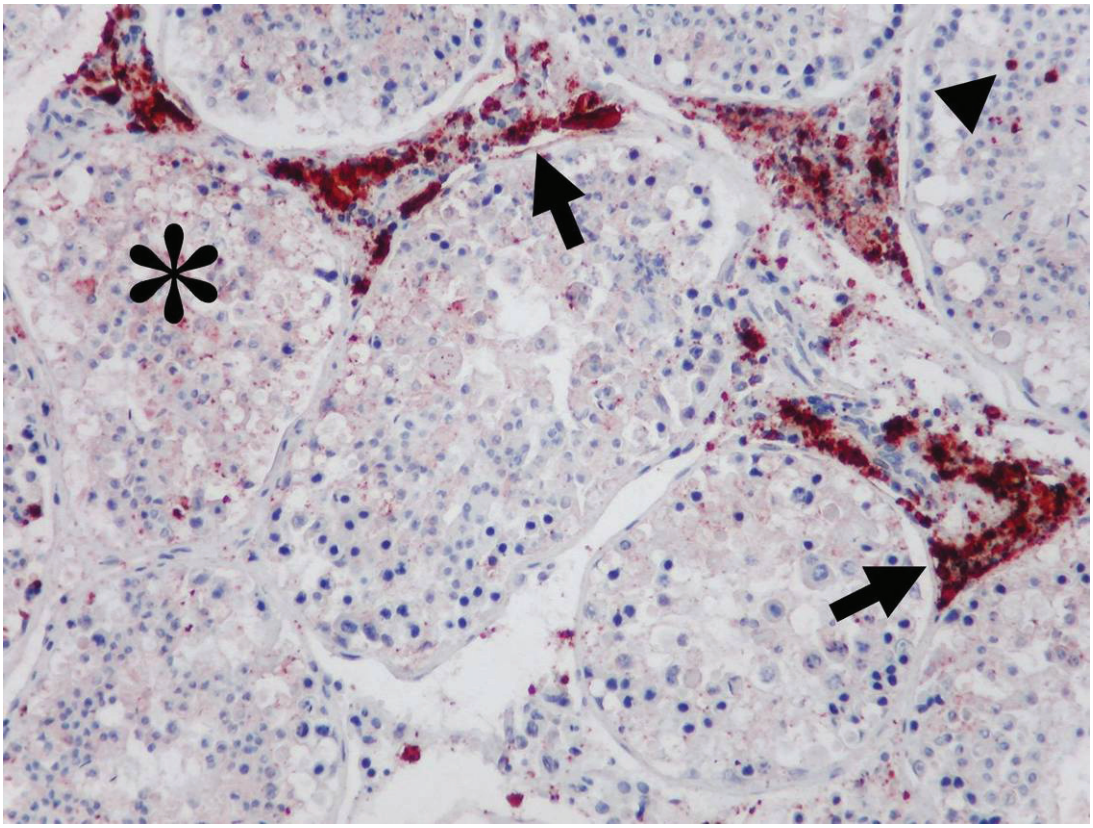


Figure 21. Testicle; European brown hare. There is strong *F. tularensis* immunoreactivity in the interstitium (arrows), while moderate labeling is present in the necrotic tubuli (asterisk). Strong immunoreactivity is also present in a few tubular epithelial cells (arrowhed). Immunohistochemistry, mouse monoclonal antibody to *F. tularensis*, and hematoxylin counterstain.

Table 6. Occurrence of histological lesions and *F. tularensis* LPS-antigen in the organs of seropositive European brown hares (n=50).

Number of affected organs	Number of cases (%)	Presence of histological lesions and *Francisella tularensis* LPS-antigen in organs (number of cases)
1	21 (42%)	• lung (15) • pericardium (2) • mediastinal lymph node (2) • kidney (1) • mammary gland (1)
2	15 (30%)	• lung and mediastinal lymph node (5) • lung and pericardium (5)** • lung and kidney (5)
3	6 (12%)	• lung, pericardium and mediastinal lymph node (2) • lung, pericardium and kidney (2) • lung, mediastinal lymph node and kidney (1) • lung, kidney, testicle (1)
4	2 (4%)	• lung, pericardium, mediastinal lymph node and bone marrow (1) • lung, liver, spleen, testicle (1)*
5	2 (4%)	• lung, pericardium, liver, spleen and bone marrow (2)**
No lesions	4 (8%)	

* natural mortality case; ** one of them natural mortality case

F. tularensis antigen was detected in all cases presenting tissue lesions (46/50, 92%) (Table 6). The bacterial antigen revealed granular or amorphous staining, and was clearly associated with histological lesions. *F. tularensis* antigen occurred in small quantities or was absent in some areas of granulomatous inflammation in several cases. In contrast, the bacterial antigen was observed in large amounts and mostly extracellularly in foci of tissue necrosis in all cases. Intracellular *F. tularensis* was found in macrophages and giant cells in the majority of cases, and less frequently in other cell types like tubular epithelial cells of the kidney, testis and epididimis, epithelial cells of the renal pelvis, hepatocytes, and bronchiolar epithelial cells. Extra- and intracellular labeling was present in the inflammatory exudate situated in the lumen

of airways, renal tubuli and pelvis, and tubuli of the testis and epididimis. Additionally, in the two severely affected natural mortality cases *F. tularensis* antigen was found in histologically unaffected tissue areas of lungs, livers and spleens. Bacterial antigen was visualized as fine, intracellular granular structure within intact alveolar epithelial cells, hepatocytes and intravascular macrophages while extracellular labeling was present in blood vessels. Seronegative animals and the negative control tissue sections remained negative. Four seropositive animals were negative with IHC. Slide agglutination test exhibited 100% sensitivity but only 83% specificity compared to the IHC assay. The predictive value of a negative test was 100% while the predictive value of a positive test was 92%.

F. tularensis ssp. *holarctica* strains were isolated in pure culture and identified from 35/50 cases (70%). All of these cases were also positive with IHC. Bacterial culture showed 100% specificity but only 76.1% sensitivity when compared to the IHC assay. The predictive value of a negative test was 68.6% and the predictive value of a positive test was 100%.

6.5. Generalized tularemia in a vervet monkey and a patas monkey

An adult, female, vervet monkey and a 2-year-old, male patas monkey were kept in the same large open-air enclosure with a closed shelter at Szeged Zoo. A total of 4 patas monkeys and 6 vervet monkeys were kept in this enclosure at the time. The 2 affected animals were born and raised in Szeged Zoo. After showing clinical signs of depression for 2 days, the vervet monkey died at the end of October 2003. The patas monkey died unexpectedly 10 days later and exhibited no prior clinical signs. The animals were not treated because of the mild nonspecific clinical signs or lack of clinical signs followed by sudden death. Other animals and in-contact humans did not show any clinical signs of concurrent illness and did not receive any prophylactic treatment.

In both monkeys, macroscopic postmortem findings included numerous, purulent, 1-5 mm, grayish-white foci surrounded by dark, hyperemic areas throughout the lung parenchyma (Figure 22). The lymph nodes were enlarged and contained several grayish-white foci measuring 1 mm in diameter. The spleens were dark, enlarged, and contained multifocal necrotic foci (Figure 23). The livers were friable and mildly enlarged. In addition, the liver of the vervet monkey also contained multiple grayish foci measuring 1 mm in diameter. Similar foci were found in the kidneys of the patas monkey.

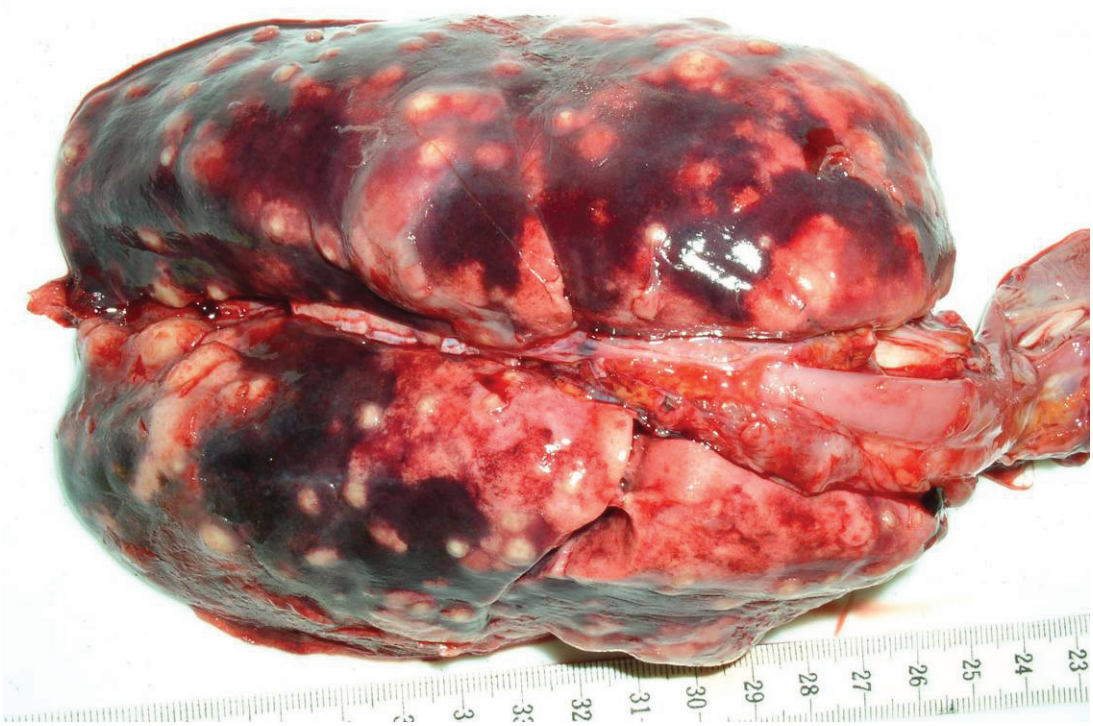


Figure 22. Lung; vervet monkey. Numerous greyish-white nodules are scattered throughout the lungs. (Photo taken by K. Erdélyi)

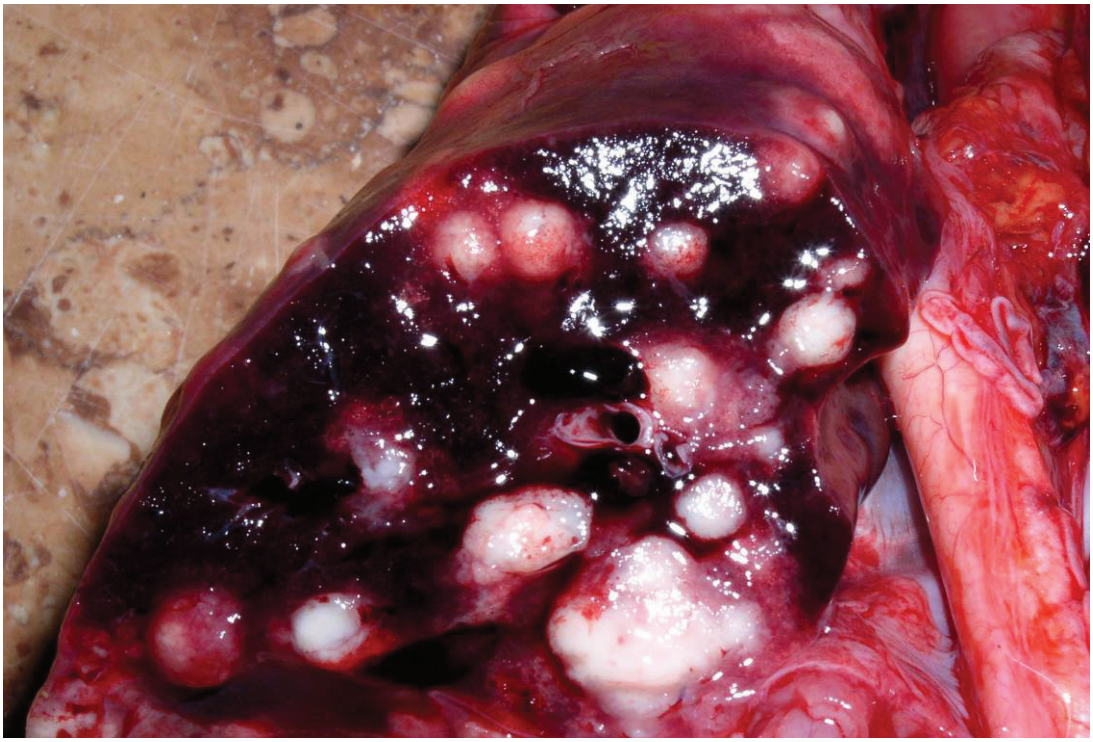


Figure 23. Spleen; patas monkey. Numerous greyish-white nodules on the cut surface. (Photo taken by K. Erdélyi)

Histopathological examination of the lung lesions revealed a severe multifocal purulent, pyogranulomatous, to granulomatous pneumonia throughout the lung parenchyma (Figures 24 and 25). Typical pyogranulomas were demarcated by connective tissue and consisted of a central necrotic area surrounded by hemorrhages and large numbers of histiocytes and fewer neutrophils (Figure 25). Spleens and lymph nodes of both monkeys had granulomatous inflammation with additional foci of necrosis in the lymph nodes. Granulomatous inflammation was also present in the liver of the vervet monkey, while a multifocal pyogranulomatous tubulonephritis was observed in the kidney of the patas monkey.

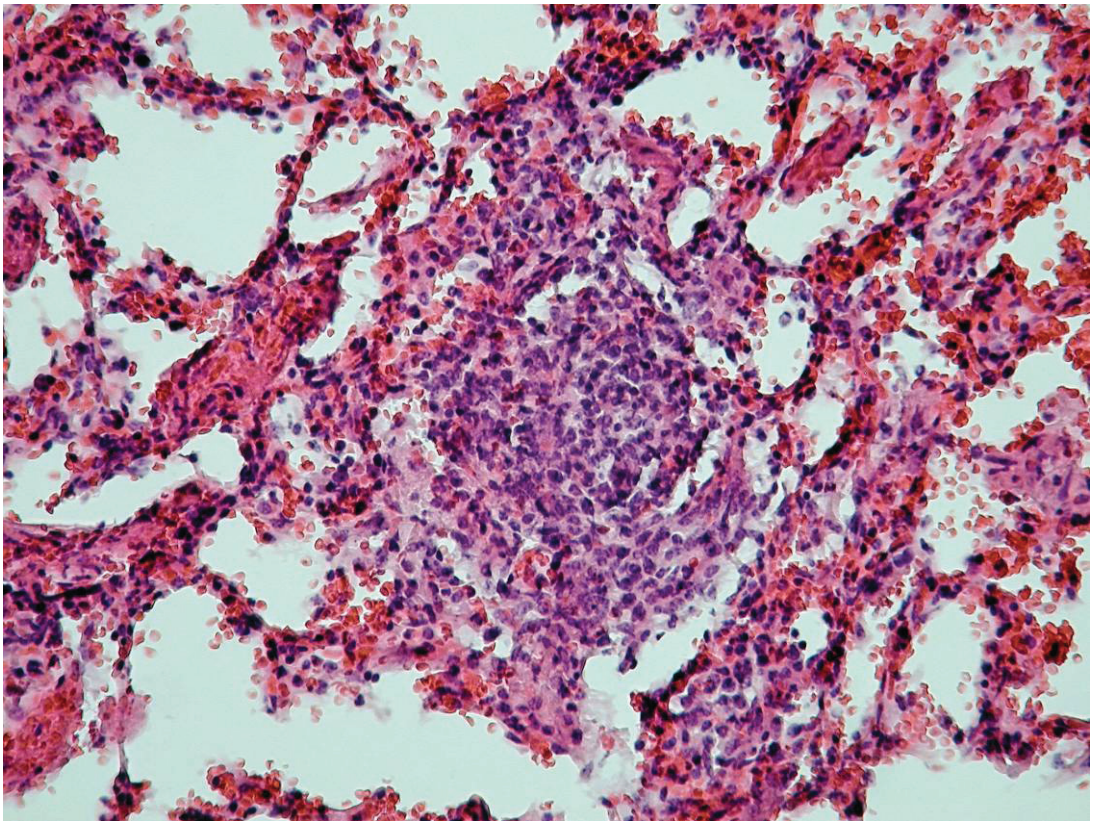


Figure 24. Lung; vervet monkey. Focal neutrophilic inflammation surrounded peripherally by fibroblasts and mononuclear cells. HE. (Photo taken by K. Erdélyi)

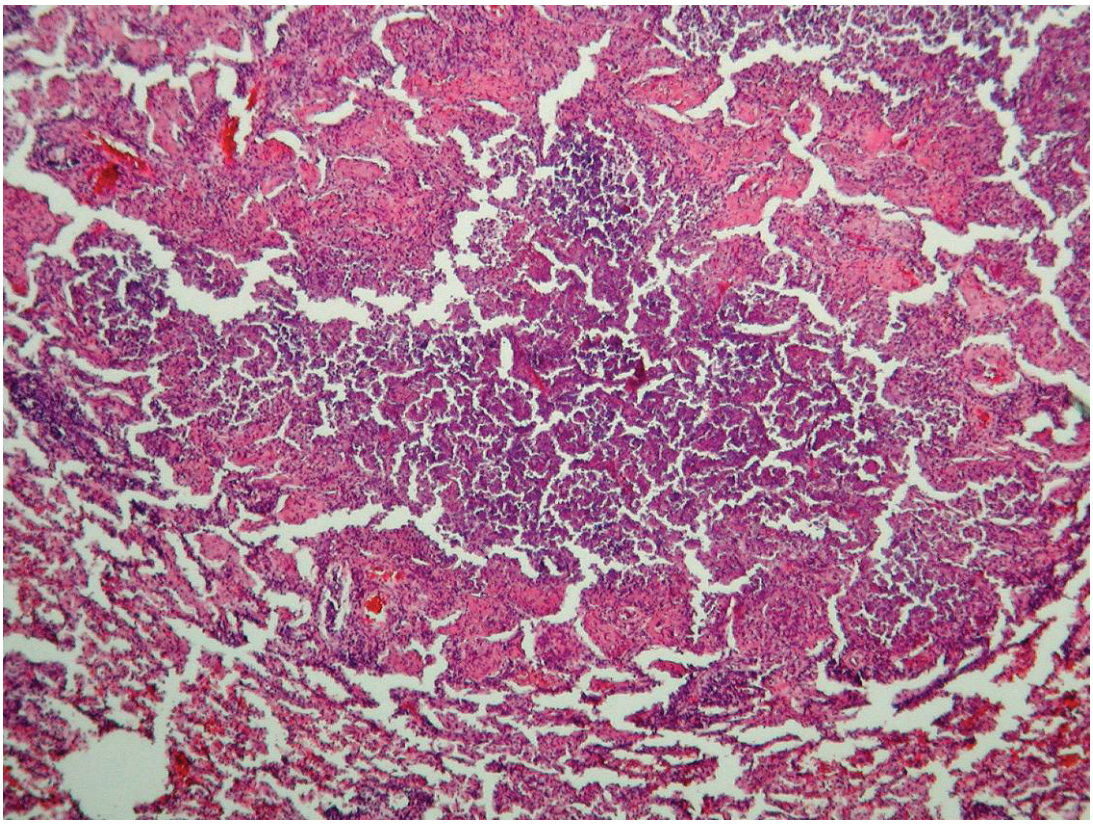


Figure 25. Lung; vervet monkey. A typical granuloma with a central area of inflammatory cells and caseous necrosis. HE.

The diagnosis of tularemia was confirmed by isolation of a strain showing typical cell and colony morphology, biochemical characteristics of *F. tularensis* ssp. *holarctica*. PCR amplification, and *F. tularensis* ssp. *holarctica* sequence data of the resulting amplicons confirmed the diagnosis.

6.6. Establishing of a *F. tularensis* strain collection

Sixty-three *F. tularensis* strains were isolated from European brown hares originating from different parts of Hungary and two strains from Austria (Table S4). Two further strains were isolated from a patas monkey and a vervet monkey. Greyish-white, 1 mm colonies could be seen after four days of incubation (Figure 26). On microscopic examination all isolates proved to be Gram-negative, small, <1 μm, coccoid, non motile rods. The culture, morphological and biochemical characteristics are shown in Table 7. Since the strains were unable to use glycerol as a sole carbon source, they were identified as *F. tularensis* ssp. *holarctica*. This result was confirmed by PCR and sequencing of the partial 16S rRNA gene fragments of all isolates. The amplified sequences were submitted for a BLAST search to the GenBank

nucleotide database where all the 67 isolates showed 100% identity with the *F. tularensis* ssp. *holarctica* (GenBank accession no. CP000803) and only 99% identity with the *F. tularensis* ssp. *tularensis* (GenBank accession no. CP000608) 16S rRNA sequences deposited in GenBank in correlation with previous findings (Keim et al., 2007).

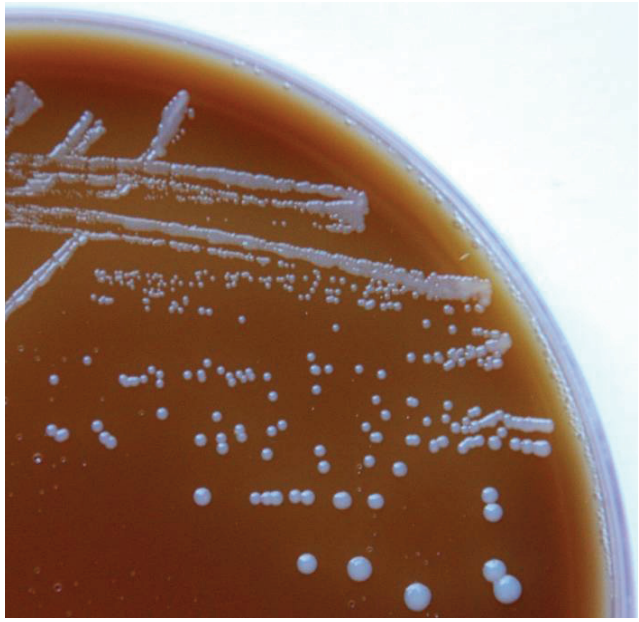


Figure 26. *F. tularensis* colonies on modified Francis agar plate.

Table 7. Characteristics of the *F. tularensis* strains isolated in Hungary and Austria.

Characteristic	Result
aerobic growth	+
anaerobic growth	−
haemolysis	−
Gram-staining	−
morphology	small rods, coccoid rods
size	<1 µm
motility	−
catalase-production	+
oxidase-production	−
glycerol-utilization	−

6.7. Carbon source utilization of *F. tularensis* ssp. *holarctica* strains

Using the Biolog system the metabolic pattern of all 15 *F. tularensis* ssp. *holarctica* strains was analysed (Table 8). After 4 hours of incubation two strains were exactly identified (100% probability, 0.837 similarity) by the Biolog system as *F. tularensis* while the other strains were suspected as *F. tularensis* (probability range: not measured, similarity range of 0.472 to 0.726). After 24 hours incubation all strains were exactly declared to be *F. tularensis* (probability range of 88% to 100%, similarity range of 0.649 to 0.792). According to the Biolog database *F. tularensis* strains have the ability to use 43 different carbon sources (Biolog standard) from the 95 available on the GN2 microplate (Table 8). All our strains were able to metabolise 13 carbon sources and 10-93% of our strains could utilise 5 further carbon sources within the first 4 hours of incubation. After 24-hour-long incubation 15 carbon sources were metabolised by all our strains and 17-93% of our isolates were able to metabolize 6 further sources (Table 8).

Unlike the Biolog standard, none of our strains used dextrin, glycogen, D-cellobiose, D-galactose, gentiobiose, maltose, β-methyl-D-glucoside, D-psicose, D-trehalose, turanose, xylitol, acetic acid, D-galacturonic acid, α-hydroxybutyric acid, α-ketoglutaric acid, succinic acid, glycyl-L-glutamic acid, D-serine, urocanic acid, inosine, glycerol, or D-glucose-6 phosphate after 24 hours of incubation.

All 15 isolates in the study were negative for glycerol fermentation based on both separate testing and the results of the H9 well on the GN2 microplate, thus identifying all isolates as *F. tularensis* ssp. *holarctica*.

The similarity of the analysed strains, as calculated from the metabolic profile data, is shown on a dendrogram (Figure 27). Despite the variety of both host species and geographic origin, all 15 isolates were similar to each other.

Table 8. Carbon source utilisation of the 15 *F. tularensis* ssp. *holarctica* strains (%).

Well	Carbon source	Biolog standard	4 hours of incubation	24 hours of incubation
A3	dextrin	46	0	0
A4	glycogen	20	0	0
A8	N-acetyl-D-glucosamin	73	100	100
A12	D-cellobiose	1	0	0
B2	D-fructose	99	100	100
B4	D-galactose	48	0	0
B5	gentiobiose	10	0	0
B6	α-D-glucose	99	100	100
B10	maltose	7	0	0
B12	D-mannose	96	100	100
C2	β-methyl-D-glucoside	1	0	0
C3	D-psicose	1	0	0
C8	D-trehalose	9	0	0
C9	turanose	14	0	0
C10	xylitol	1	0	0
C11	pyruvic acid methyl ester	99	100	100
C12	succinic acid mono-methyl ester	77	93	100
D1	acetic acid	36	0	0
D6	D-galacturonic acid	1	0	0
D10	α-hydroxybuyric acid	16	0	0
E3	α-ketobutyric acid	89	100	100
E4	α-ketoglutaric acid	30	0	0
E6	D,L-lactic acid	73	87	100
E12	succinic acid	5	0	0
F4	L-alaninamide	59	0	37
F5	D-alanine	67	10	17
F6	L-alanine	86	100	100
F7	L-alanyl-glycine	27	23	33
F8	L-asparagine	53	100	100
F9	L-aspartic acid	58	100	100
F10	L-glutamic acid	91	100	100
F12	glycyl-L-glutamic acid	6	0	0
G6	L-proline	53	13	90
G8	D-serine	3	0	0
G9	L-serine	98	100	100
G10	L-threonine	92	100	100
H1	urocanic acid	5	0	0
H2	inosine	2	0	0
H3	uridine	71	0	93
H4	thymidine	46	0	90
H9	glycerol	41	0	0
H10	D,L,α-glycerol phosphate	99	100	100
H12	D-glucose-6-phosphate	6	0	0

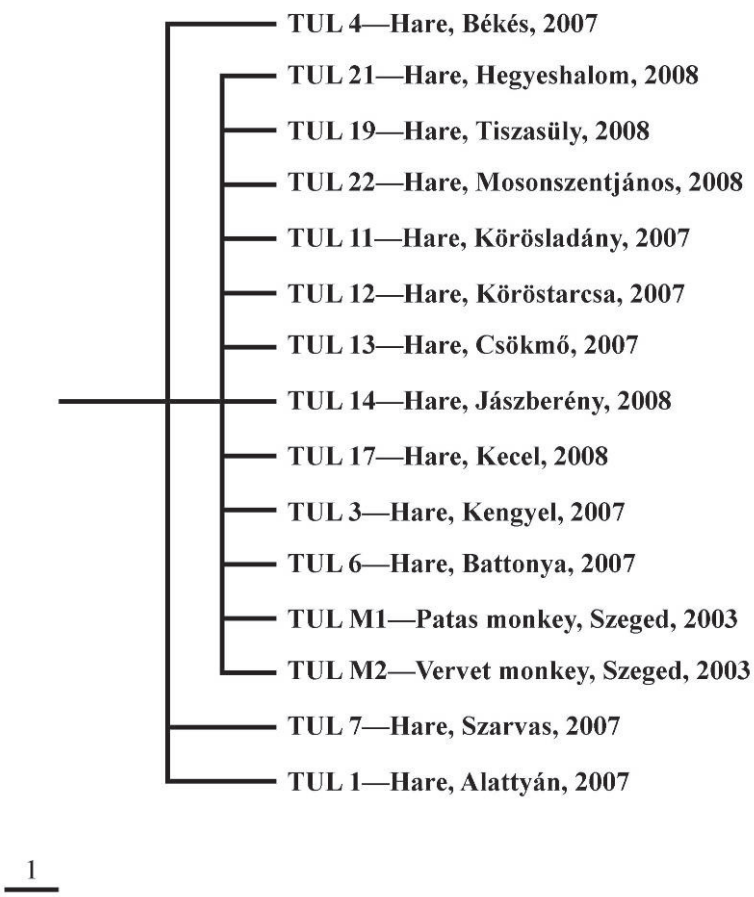


Figure 27. Dendrogram of metabolic similarity of the 15 *F. tularensis* ssp. *holarctica* strains after 24 hours of incubation.

6.8. Phylogenetic population structure of *F. tularensis* ssp. *holarctica* strains from Hungary

WG SNP comparisons among the 6 genomes resulted in the identification of ~820 putative SNPs with a very low homoplasy index (0.0164) and among these, 20 SNPs were specific to the Hungarian strain. The Hungarian strain grouped closely to the FSC 200 strain found in Sweden but differred from it by ~60 putative SNPs (Figure 28).

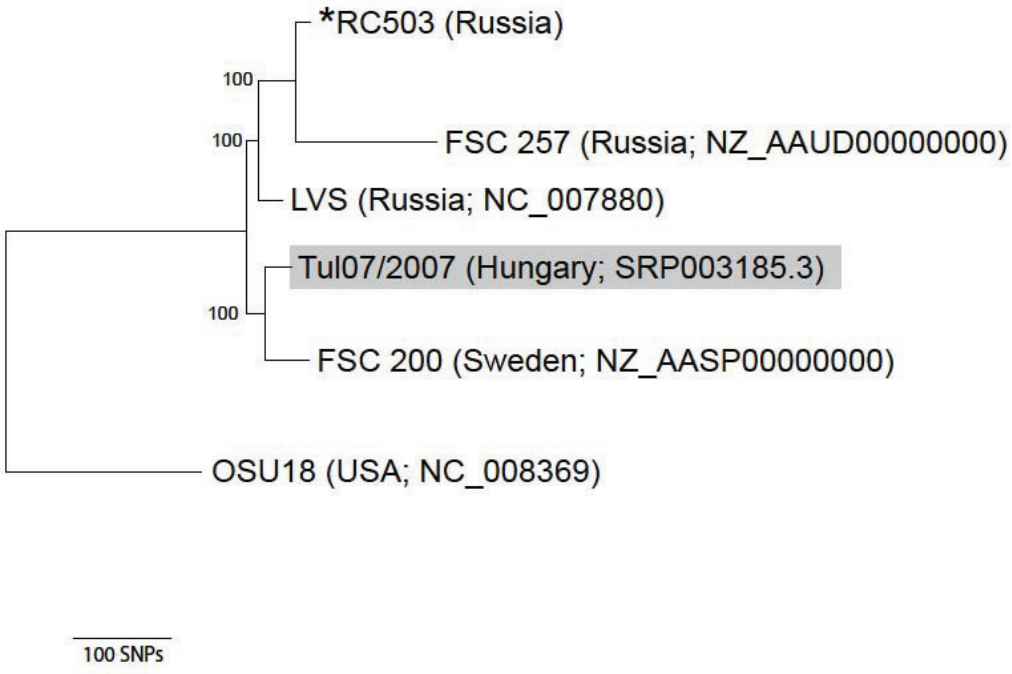


Figure 28. Maximum parsimony SNP phylogeny based upon putative SNPs discovered among 6 *F. tularensis* whole genome sequences. This phylogeny is rooted using OSU18 and has a homoplasy index of 0.0164. Bootstrap values are based on 1000 replicates in PAUP 4.0b10 with a heuristic search. * RC503 WG sequence can be found at http://www.hgsc.bcm.tmc.edu/microbial-detail.xsp?project_id=144.

All 15 geographically diverse Hungarian isolates (hares; n = 13, zoo monkeys; n =2, 2003-2009) belonged to the B.Br.013 lineage and clustered into two distinct subclades (Figure 29A). Fourteen isolates clustered in the subclade B.Br.020/021 and one isolate clustered in the B.Br.023/014/025 subclade (represented as “A” in Figure 29A). Among the four isolates collected in Italy, the three native isolates belonged to the B.Br.FTNF002-00 subclade (represented as “I” in Figure 29A) while the isolate collected in Italy from an imported hare of Central European origin belonged to the B.Br.013 lineage, specifically to the subclade B.Br.020/021. Thus, we conclusive classified our 19 isolates into existing phylogenetic groups using previously published canSNPs.

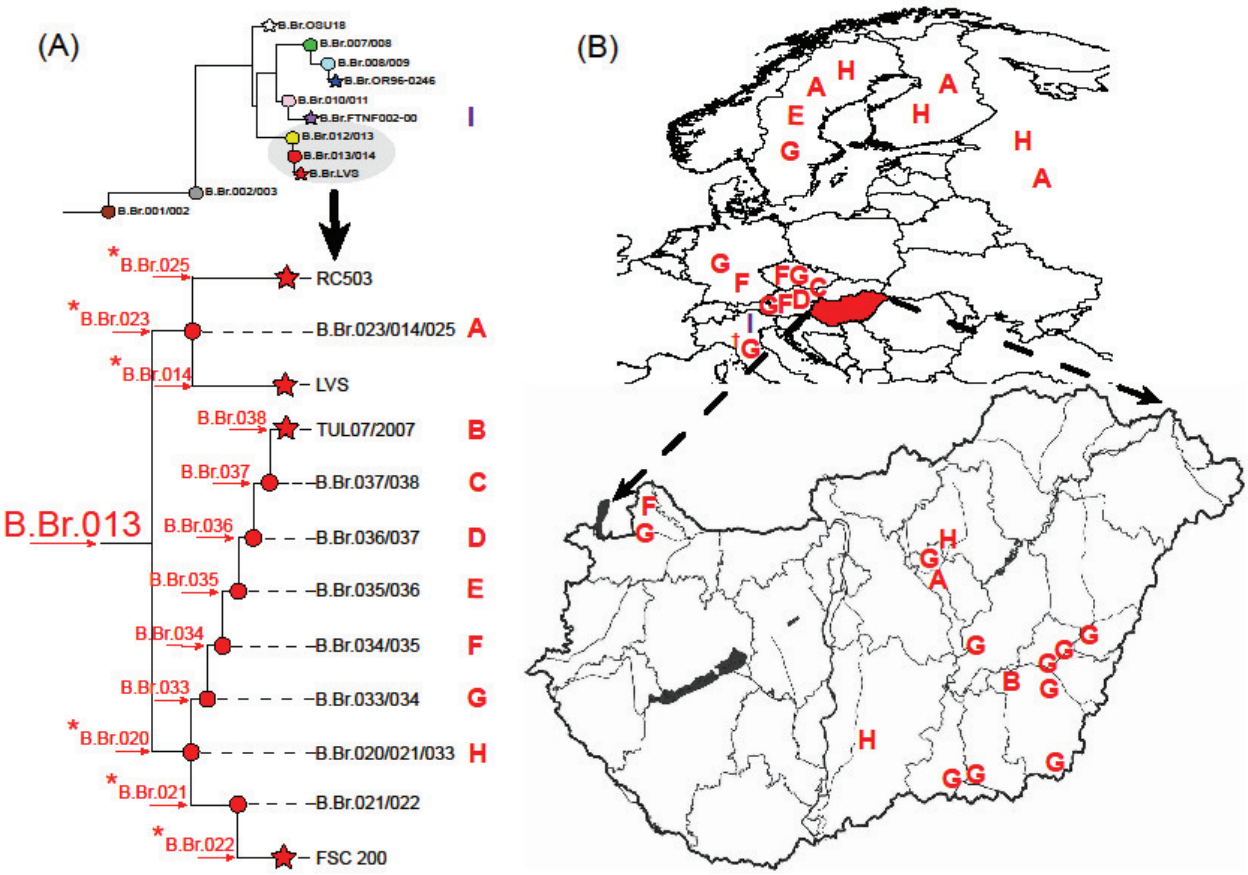


Figure 29. (A) A SNP phylogeny of *Francisella tularensis* ssp. *holarctica* subclades identified by Vogler et al. (2009a) with subclade Br.013/014 highlighted in grey (up) and an expanded subclade B.Br.013/014 phylogeny (down) depicting additional phylogenetic structure within this B.Br.013 lineage. Branches marked with an "*" were previously identified by Svensson et al. (2009). Terminal subclades representing sequenced strains are represented by stars and intervening nodes representing collapsed branches are indicated by circles. Letters A to H represent specific subclades within the B.Br.013 lineage that contained Hungarian and Italian isolates. Letter I represents the B.Br.FTNF002-00 lineage which contained Italian isolates (B) Geographic distribution of subclades (represented as A,C-I) in Europe (up). "†" indicates the isolate from an imported hare of Central European origin. The geographic distribution of fifthteen Hungarian isolates with subclade representation on the enlarged map of Hungary (lower) indicated as letters A to H.

We further resolved the phylogenetic structure of the Hungarian lineage (B.Br.TUL07/2007) by designing high-throughput SNP assays for the 20 SNPs specific to the Hungarian strain. These assays were screened across 100 geographically diverse set of European isolates belonging to the B.Br.020/021 subclade (including the 14 Hungarian and 1 Italian isolates). This screening effort resulted in the identification of 6 new phylogenetic subclades. B.Br.033-B.Br.TUL07/2007 labelled as isolates B-H (Figure 29). This indicates that the sequenced Hungarian strain (TUL07/2007) is a descendant of the genetic lineage shared by these six subclades. No Hungarian isolates belonged to subclades B.Br.035/036, B.Br.036/037, and B.Br.037/038. But isolates from these subclades were found in regions near Hungary and Sweden (Figure 29B). Among all European isolates examined, twelve isolates fell into the B.Br.020/021/033 subclade, thirty-five isolates fell into the B.Br.033/034 subclade, twelve isolates fell into the B.Br.034/035 subclade and a single isolate fell into the B.Br.035/036, B.Br.036/037, B.Br.037/038 subclades and B.Br.TUL07/2007 subclade.

MLVA with 11 VNTR markers showed 4 genotypes among the 10 Hungarian isolates that fell into the basal B.Br.033/034 subclade. This MLVA diversity suggests the existence of additional phylogenetic diversity among those Hungarian isolates, which would require WG sequencing to fully document. The Hungarian isolates in the other subclades either lacked MLVA diversity or contained only a single isolate.

7. Discussion

7.1. Retrospective data collection

The number of human cases documented by the National Center for Epidemiology are in agreement with the official reports of the human health servise (Epinfo). Contrary, the percentage of *F. tularensis* seropositive hares makes likely that the official reports (2002: 14 cases, 2003: 2 cases, 2004: 6 cases /WAHID/) are a gross under representation of the true incidence of tularemia. WAHID collects data on outbrakes of infectious diseses, number of cases and deaths. In our case we have data on clinically healthy, subclinically infected, seropositive animals and since they are not regarded ill, they are frequently not notified. These data are also higher than the 0.2 mean percentage recorded between 1966 and 1981 (Sztojkov, 2006). Despite this, the percentage of *F. tularensis* seropositive hares decreased to 0.6-1% in the past years. Because of the importance of tularemia and its public health aspects strengthening the notification discipline is very important.
The more detailed future analysis of these data (possible geographic patterns, case-control studies) should provide the opportunity for the better understanding of the epidemiology of this zoonotic disease in Hungary.

7.2. Investigation of the ecology of *F. tularensis* ssp. *holarctica*

The result of our study establishing 4.9-5.3% prevalence of tularemia in hares is in agreement with previous reports showing that the European brown hare is a common host of *F. tularensis* in Central Europe (Hopla, 1974; Treml et al., 2007). The public health importance of the brown hare is accentuated by being one of the most abundant and most significant European game species (Strauss and Pohlmeyer, 2001) and by the fact that direct contact with infected hares is a frequent mode of *F. tularensis* infection in humans (Mörner and Addison, 2001). Hares are moderately sensitive to *F. tularensis* infection, they seroconvert and potentially they can carry viable bacteria over a longer time span, and thus serve as a reservoir species. Therefore, serological tests are useful diagnostic aids and tools for epizootiological surveys of tularemia in European brown hares. In order to reduce the possibility of introducing tularemia into new areas, the tube agglutination test is commonly used for screening of trapped hares before translocation for sporting purposes, reintroduction and enhancement of existing populations. As shown in Table 4, the tube agglutination assay mainly demonstrated low

antibody titers and the diagnostic titers of 1/40 (ambiguous) and 1:80 recommended for this kit (Bioveta) were reached only in two and a single case respectively. Similarly, this kit also demonstrated low antibody titers (1/10 and 1/20) in 42 % (29/69) of *F. tularensis* seropositive hares in a previous study (Treml et al., 2007). Since hares with lower titers showed typical pathological lesions associated with *F. tularensis* specific immunolabeling, as well as clear-cut positivity by slide agglutination and were even culture positive for *F. tularensis*, we would recommend considering the modification of the diagnostic titer of this diagnostic kit to 1/10 for screening European brown hares before translocation. The slide agglutination test was found to be very useful and sensitive tool for the screening of tularemia in both live and dead hares as demonstrated by the present study and by our earlier long term monitoring data. Nevertheless, since *F. tularensis* shares common antigens with *Brucella* spp., *Yersinia* spp. and *Legionella* spp. these pathogens may sometimes be responsible for false positive results with this test (Mörner and Addison, 2001; OIE, 2008). The frequent presence of lesions in the kidneys and lungs correlates with the results of the pathology study (see below) and lends further support to the hypothesis, that shedding of bacteria by urine contributes to the acquisition of *F. tularensis* by the airborne infection route in European brown hares.

According to previous studies rodents play a key role in maintaining enzootic foci of tularemia in Eurasia (Friend, 2006; Keim et al., 2007). The common vole and the water vole are the species most frequently involved in tularemia epizootics. In addition to being highly susceptible to *F. tularensis*, these species may also become chronically infected, thereby serve as disease reservoirs during periods between epizootics and they are also hosts for the immature stages of several important tick vectors (Bell and Stewart, 1975; Friend, 2006). The prevalence of infection observed during tularemia outbreaks was 4.5% (4/88), 5.2% (4/79) and 8.0% (2/25) by bacterial culture and real-time PCR among common voles in Slovakia, Austria (Gurycová et al., 2001) and Germany (Kaysser et al., 2008) respectively. In contrast to these results we did not find any infected voles with either PCR or serology during the inter-epizootic period. Although the population of voles in our study region was low during the year and we managed to trap and sample only 38 individuals, the present data did not confirm the suggestion that common voles become chronically infected and thus serve as a reservoir species between outbreaks. Furthermore, previous results demonstrating a complete lack of seroconversion among voles in spite of PCR positivity (Kaysser et al., 2008) indicate towards the dominance of fatal outcome in *F. tularensis* infections of this species. Mouse species are highly susceptible and sensitive to *F. tularensis*, they die shortly after infection (Bandouchova et al., 2009) and are not known to be important reservoir hosts (Friend, 2006). The population of *Apodemus* spp. at our study site was high with a normal population curve and we did not detect any infected individuals, which is in congruence with the above finding.

As true reservoir hosts, ticks can carry *F. tularensis* for several years through multiple life-stages which may help perpetuate specific enzootic foci of tularemia during inter-epizootic periods (Friend, 2006; Keim et al., 2007). The seasonal activity of *H. concinna* and *I. ricinus* was similar to that observed earlier in Hungary (Széll et al., 2006; Hornok, 2009). *D. reticulatus* is known to be the most frequent carrier of *F. tularensis* in central European flood-plain forests and meadow ecosystems with infection rates of 0.5-2.6% in Slovakia, Czech Republic (Gurycová et al., 1995; Gurycová et al., 2001; Hubálek et al., 1998) and 0.7-5.8% in Austria (Gurycová et al., 2001), whereas the rates of infection in *I. ricinus* were 0.1-0.2% (Gurycová et al., 1995) in forest habitats near the river Danube. *D. reticulatus* was not present at the site of our investigation, which could be explained by the arid habitat dominated by grasslands. Nevertheless, the expansion of this tick species and its preference for xerophylic vegetation was also observed in Hungary (Hornok and Farkas, 2009; Sréter at al., 2005). The 0.59% infection rate found in *H. concinna* collected at the grassland habitat is lower than the prevalence registered in previous studies at forest habitats near the river Danube (2.8%, 1/35) (Gurycová et al.; 1995). However, the sample size of the referred study was much smaller. *H. concinna* probably serves as the primary arthropod vector in the ecology of *F. tularensis* in our study region and with its developmental cycle of three or more years it may also play an important role in the long term persistence of enzootic foci of tularemia as previously described by Gurycová et al. (1995). Although the number of examined fleas was low, our results support earlier findings establishing that fleas should be considered of minor importance in the transmission and maintenance of *F. tularensis* (Keim et al., 2007; Parker and Johnson, 1957).

There are several reports about water-borne *F. tularensis* infections in humans and the detection of *F. tularensis* in natural water bodies during outbreaks (Friend, 2006; Kaysser et al., 2008). Although limited, the number of water samples examined in our study is representative of the water sources at our study site. The negative results could be explained by the lack of carcasses which would usually contaminate the water during epizootics, the lack of aquatic rodent species which may harbour and shed live bacteria into their environment and the arid summer climate of the region allowing only temporary water bodies which regularly dry out every year. Alternatively, the negative PCR results could have also been obtained because the number of *F. tularensis* organisms in water samples was below the limit of detection (100 CFU/250 ml water) or possible inhibitiory material of the water samples as well.

Epizootiologic surveys of domestic animals, i.e. relatively resistant species which survive the infection and develop antibodies, such as sheep or cattle, may indicate the epidemiological status of a given area (OIE, 2008). Since they collect large numbers of ticks while grazing, *F. tularensis* infections and seropositive cases could be expected to occur regularly in endemic

regions. Apart from the low prevalence of the disease in the area, another potential explanation for the absence of seropositive cases in our study could be the low infective dose transmitted by ticks which may be insufficient to initiate seroconversion.

In our study we explored which species take part in the ecological cycle of tularemia in an enzootic area, serving as reservoirs and vectors of the disease between epizootics. According to our results we hypothesize that only a European brown hare – *H. concinna* cycle, confined to the grassland habitat, persists during inter-epizootic periods in our study region. It is potentially complemented by a hare – hare cycle as suggested by pathological findings. This means that hares may acquire infection by the air-borne route, become chronically infected and shed live bacteria by urine into the environment. *H. concinna* does not only serve as an arthropod vector but it can also carry the bacteria for several years through multiple life-stages (Gurycová et al., 1995) and may therefore be an important reservoir of *F. tularensis*.

Various rodent species are probably not true reservoir hosts as they are all highly susceptible and sensitive to *F. tularensis*, dying shortly after infection, so positive cases can only be found during epizootics. The knowledge about the reservoirs of *F. tularensis* is crucial in order to assess potential human infection sources and to establish a systematic, long-term monitoring of tularemia activity in enzootic areas.

7.3. Susceptibility of the common hamster to *F. tularensis* ssp. *holarctica* and its effect on the epizootiology of tularemia

F. tularensis infection is chronic in the European brown hare, a reservoir species which serves as a good indicator for the occurrence of the disease in Central Europe (Mörner, 1994). The study area was considered a tularemia-endemic region during the past decade and the study period, based on the 1-1.2% infection rate in the local European brown hare population (estimated from 3930 hares by slide agglutination test and isolation). Additionally, 14 human clinical cases including hamster trappers occurred in the surrounding villages during the same time period. We suspect that our study was conducted in an interepizootic period as there were no human cases during the study period; the negative results of the tick and organ pools further supports this hypothesis.

Because of the high sensitivity of common hamsters, preliminary diagnosis of tularemia cannot be based on gross pathologic lesions in contrary to European brown hare. Foci are not always found in the spleen and apart from septicemia these same lesions may also be induced by shock. Bacterial isolation and the IHC assay were effective in diagnosing *F. tularensis* infection in hamsters. The intramuscular bacterial challenge in case 1 showed, that a shorter incubation period was associated with more severe gross pathologic lesions, and

larger amounts of bacterial antigen. Since the number of the animals in the trial was limited, this result may require confirmation and infection studies with different inoculation doses and routes may also be needed.

F. tularensis may cause large epizootics among rodents and infection is considered to be a factor in population regulation, preventing overpopulation of these species in nature (Friend, 2006). Our results confirmed previous data (Olsufjev and Dunayeva, 1970; WHO, 2007) that common hamsters are highly sensitive to *F. tularensis* infection and die after a short incubation period. The negative results of the serologic survey confirm the high sensitivity of hamsters to *F. tularensis*. Since they do not survive the infection, there are no antibody-positive individuals in the population after the outbreak.

We conclude that although septicemic hamsters may pose substantial risk to humans during tularemia outbreaks, hamsters in interepizootic periods are not an important constituent of the natural cycle of the disease in that they do not act as a main reservoir of *F. tularensis*.

7.4. Pathology of tularemia in European brown hares

Our study is considered to be the first one describing the gross and histopathological lesions, the results of the IHC examination and bacterial culture performed on a large number of European brown hares naturally infected with *F. tularensis* ssp. *holarctica*. This essential information can facilitate both the suspecting of tularemia in brown hares by hunters and the recognition and identification of this dangerous zoonotic disease by diagnosticians. Beside two conference presentations (Kemenes, 1976; Sterba and Krul, 1986) only a single case report of a European brown hare acutely infected with *F. tularensis* is available in the literature, but no histological or IHC examinations were applied in these studies (Müller et al., 2007). Gross pathological lesions suggestive of the infection were observed in 44 (88%) cases of the 50 seropositive hares in the present study. These results indicate the excellent applicability of gross pathological examination as an initial screening method for the diagnosis of *F. tularensis* in European brown hares. Macroscopically visible, numerous, grayish-white foci and nodules with a diameter of 0.1 to 1 cm in single or multiple organs should warn hunters and diagnosticians to handle the particular carcass with care, and thus help prevent subsequent human infections. The most often affected organs were lungs, pericardia and kidneys, which is in contrast to reports in rodents and other lagomorphs, where liver and spleen were found to be the primary targets (Friend, 2006; Mörner and Addison, 2001) Overall, histological lesions resembled the subacute tularemia reported in the experimentally infected domestic rabbit (Little and Francis, 1937).

Since there are several infections that can produce similar lesions (e.g. *Brucella suis* or *Y. pseudotuberculosis* infections) additional laboratory tests are necessary for final diagnosis of tularemia (Mörner and Addison, 2001). Bacterial culture, serology and IHC methods revealed that IHC was the most sensitive and most specific method according to the present study, although this result may require confirmation on larger number of animals. The advantage of IHC over serology and bacterial culture is that the infectivity of tissue samples is eliminated by the time of laboratory examination due to formalin fixation (McCaffey and Allen, 2006). The IHC assay was shown to be a very useful and sensitive method for the detection of *F. tularensis* in domestic and wild living animals by several authors (Twenhafel et al., 2009; Valentine et al., 2004; Zeidner et al., 2004). The *F. tularensis* LPS-specific reagent used in the present study was a blend of two clones of mouse monoclonal antibodies, which ensures the detection of all subspecies of *F. tularensis*. The reagent cross-reacted neither with *B. suis* nor *Y. pseudotuberculosis* (data not shown), which can both cause infection in hares. In the European brown hare *F. tularensis* antigen was always associated with tissue lesions, especially with necrotic foci.

In areas of granulomatous inflammation, bacterial antigen was present in low quantities or it was totally absent. This may be explained by the successful elimination of bacteria by the host's immune system. Two of the three natural mortality cases presenting weak body condition showed severe, disseminated lesions. Bacterial antigen was identified in affected tissues within lesions and within blood vessels in unaffected tissues. Similar findings were reported in experimentally infected African green monkeys, but no explanation of this phenomenon was presented (Twenhafel et al., 2009). We hypothesize that these two hares died of *F. tularensis* septicemia resulting replication of bacteria within the blood vessels of unaffected tissues.

Serological methods are used for the diagnosis of tularemia in humans and they can also be applied in animals. Serology has limited use in highly susceptible species of animals, which usually die before specific antibodies develop (Mörner and Addison, 2001). The slide agglutination test was found to be very useful in the present study. This test is simple and fast with 100% sensitivity and high specificity, although this result may require confirmation in further studies. *F. tularensis* shares common antigens with *Brucella* spp., *Yersinia* spp. and *Legionella* spp. and subclinical, clinical, or previous infection with any of these organisms may be responsible for the false positive results (OIE, 2008) This may explain the occurrence of 4 seropositive cases with IHC-negative test results. On the other hand, *F. tularensis*-induced tissue lesions may occur in very small numbers and in only a few organs, so this may have also prevented the recognition of these cases by IHC. Bacterial culture had low sensitivity compared to the IHC test. However, this method is necessary for the determination of species and type of the particular *F. tularensis* strain.

Previous experimental studies suggested that the European brown hare is moderately susceptible to *F. tularensis* ssp. *holarctica* compared to the mountain hare and that it serves as a potential reservoir host of *F. tularensis* ssp. *holarctica* in Europe (Mörner et al., 1988). This hypothesis was supported in the present study by finding moderate to good body condition in 48% of IHC-positive animals and the presence of subacute histological lesions indicating a protracted disease in all cases. The frequent lesions in thoracic organs (44/50 cases, 88%), lung, mediastinal lymph nodes and pericardia, suggest that the airborne route is the most common way of acquiring the infection in European brown hares.

In conclusion, the European brown hare is considered to be an important reservoir of *F. tularensis* ssp. *holarctica*. Macroscopically visible lesions can be found in almost all infected hare cases. The IHC assay for the diagnosis of *F. tularensis* infection is more sensitive and has a similar specificity as bacterial culture. Since the combination of slide agglutination and detection of gross pathological lesions has proven to be a very sensitive tool for preliminary diagnosis of tularemia, we would recommend the use of this method for the screening of *F. tularensis* infection in brown hares in the field.

7.5. Generalized tularemia in a vervet monkey and a patas monkey

The presence of tularemia in a vervet monkey and a patas monkey in a zoo was an unusual finding. Although tularemia is not an uncommon disease in Hungary, reservoirs of infection are found in the free-ranging wildlife population. During the last decade (1999-2009), the numbers of reported human cases of tularemia have ranged between 2 and 5 per year in Csongrád County (~425,000 inhabitants; Epinfo).

Although lesions were present in multiple organ systems, the most severe lesions were involved the lungs and regional lymph nodes. This pathological pattern suggests that the most likely route of infection may have been through inhalation. Zoo primates regularly hunt and consume small prey species such as rodents and birds that enter their enclosures. This could be a potential source for contracting *F. tularensis* from free-ranging wildlife. Furthermore, the heavily forested territory of Szeged Zoo may increase the risk of harboring infected wildlife. The narrow time interval between the 2 deaths suggests that there could have been a common source of infection; however, no further evidence of tularemia has been seen at the zoo since this outbreak. Finally, the current report indicates that infected nonhuman primates may be potential sources of zoonotic disease for animal keepers and visitors and appropriate hygienic measures should be taken.

7.6. Establishing of a *F. tularensis* strain collection

The established Hungarian *F. tularensis* strain collection provided the opportunity to the bacteriological, molecular biological examinations.

7.7. Carbon source utilization of *F. tularensis* ssp. *holarctica* strains

The results show that carbon source utilization is a reliable method of characterization and identification of *F. tularensis* strains and the Biolog system can be used for the comparative examination and identification of this bacterium species. The results can be read already after 4 hours of incubation not only after the standard 24 hours.
Comparing our results with the Biolog standard it is evident that some of the fermentation types of *F. tularensis* included in the Biolog database have not been represented in our study, since certain potentially metabolisable carbon sources were not used at all by our isolates. There is no information on the number and the subspecies distribution of *F. tularensis* strains used to build this database. The Biolog software/database does not distinguish between *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica*, though differentiation based on glycerol utilisation (Petersen and Schriefer, 2005) is absolutely necessary because of their different virulence. However, the results for the use of glycerol as carbon source (well H9 of the Biolog microplate) can be manually read and thus use the Biolog system to distinguish between *F. tularensis* ssp. *tularensis* (glycerol positive) and *F. tularensis* ssp. *holarctica* (glycerol negative). Extending the database using our date is recommended.
F. tularensis strains recovered from hares and monkeys were found to be very similar based on carbon source utilization. The cladogramm, derived from the metabolic profile of the strains, supported the notion of the conservative genetic character of *F. tularensis* ssp. *holarctica*.

7.8. Phylogenetic population structure of *F. tularensis* ssp. *holarctica* strains from Hungary

F. tularensis isolates from Hungary belong to the B.Br.013 lineage (Vogler et al., 2009a). These isolates did not descend from a single lineage unique to Hungary, but rather they descended from a diverse set of minor subclades comprised of isolates found throughout Central Europe, Scandinavia and Russia. Unknown previously, *F. tularensis* isolates native to Italy belong to the B.Br.FTNF002-00 subclade, a distinct genetic group comprised of isolates

found in France, Spain, Switzerland, parts of Germany (Pilo et al., 2009; Svensson et al., 2009; Vogler et al., 2009a). Our analyses enabled us to contradict the hypothesis that Central Europe is the direct source of Western European tularemia, through hare importation, on the basis of their distinct genetic differences. The fact the genotype of *F. tularensis* isolated from an imported Central European hare matched a genotype found in Central and Eastern Europe instead of the genotype of the other native Italian samples favors the argument that diseased hares are successfully imported to Italy, despite strict pre-export screening. We have not found supporting evidence for the successful establishment of Central European strains of tularemia in the environments of Italy or in Western Europe. Similar phylogenetic studies should be undertaken in other Central and Eastern European countries, where the phylogenetic structure of *F. tularensis* is not well characterized, to generate a more comprehensive understanding of the evolution and dissemination of *F. tularensis* ssp. *holarctica*.

8. Overview of the new scientific results

Ad 1. Retrospective data collection showed that the number of human tularemia cases ranged between 20 and 148 a year in Hungary in the past two decades (1984-2009) and the percentage of *F. tularensis* seropositive European brown hares, captured for live animal export (2.8-40 thousand exported hares/year) ranged between 0.31% and 20.2%. We established a Hungarian *F. tularensis* strain collection during our study; sixty-three strains were isolated from European brown hares originating from different parts of Hungary and two strains from Austria and two further strains were isolated from a patas monkey and a vervet monkey from Szeged Zoo, Hungary.

Ad 2. According to our data the European brown hare – *H. concinna* cycle is the most probable way of persistance of *F. tularensis* ssp. *holarctica* during interepizootic periods in an enzootic area. *H. concinna* serves as an arthropod vector and can harbor bacteria for three to four years through multiple life stages and act as an important reservoir of *F. tularensis*. Since chronically infected hares shed live bacteria by urine, an additional airborne hare – hare cycle, may complement the main vector borne cycle. Rodent species probably do not act as true reservoir hosts of *F. tularensis*.

Ad 3. Although septicemic common hamsters may pose substantial risk to humans during tularemia outbreaks, hamsters in interepizootic periods do not serve as a main reservoir of *F. tularensis*.

Ad 4. The modification of the diagnostic 1/80 tube agglutination titer to 1/10 for screening European brown hares for *F. tularensis* infection before translocation is recommended based on our results.

Ad 5. Generalized tularemia following natural infection was described the first time in a vervet monkey and a patas monkey.

Ad 6. Tularemia of European brown hare was characterised the first time by a simultaneous pathological, histopathological and immunohistochemical study. The priority of airborne infection route was verified on the basis of tissue lesions in thoracic organs. The results emphasize the importance of the European brown hare as a reservoir of *F. tularensis*.

Ad 7. The Biolog software wasn't able to differentiate the highly virulent *F. tularensis* ssp. *tularensis* and the moderately virulent *F. tularensis* ssp. *holarctica* but the Biolog microplates can be manually read to distinguish the two subspecies based on glycerol source utilisation. The cladogram based on the metabolic relationship of the examined 15 *F. tularensis* ssp. *holarctica* strains showed that the isolates are very similar to each other. Revision of the Biolog software is highly recommended.

Ad 8. The whole genome of a Hungarian *F. tularensis* ssp. *holarctica* isolate was sequenced and compared to 5 other complete genomes showing that the Hungarian strain is grouped closely to the FSC 200 strain found in Sweden but is differred from it by ~60 putative SNPs.

Ad 9. Twenty high-throughput SNP assays were designed from which six identified new subclades and thus presented useful additions to the investigation of *F. tularensis* dispersal throughout Europe.

Ad 10. *F. tularensis* ssp. *holarctica* isolates native to Hungary belong to the B.Br.013 lineage and share identical genotypes with strains found throughout Central Europe, Scandinavia and Russia. *F. tularensis* ssp. *holarctica* isolates from Italy belong to the B.Br.FTNF002-00 subclade, a distinct genetic group comprised of isolates found in France, Spain, Switzerland, parts of Germany. These results enabled us to contradict the hypothesis that Central Europe is the direct source of Western European (e.g. France, Italy) *F. tularensis* ssp. *holarctica* strains through European brown hare importation.

9. References

Abd, H., Johansson, T., Golovliov, I., Sandström, G., Forsman M.: Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*, Appl. Environ. Microbiol., 69. 600-606, 2003.

Achtman, M., Morelli, G., Zhu, P., Wirth, T., Diehl, I., Kusecek, B., Vogler, A.J., Wagner, D.M., Allender, C.J., Easterday, W.R., Chenal-Francisque, V., Worsham, P., Thomson, N.R., Parkhill, J., Lindler, L.E., Carniel, E., Keim, P.: Microevolution and history of the plague bacillus, *Yersinia pestis*, Proc. Natl. Acad. Sci. U. S. A., 101. 17837-17842. 2004.

Amoss, H.L., Sprunt, D.M.: Tularemia: review of literature of cases contracted by ingestion of rabbit and the report of additional cases with necropsy, J. Am. Med. Assoc., 106. 1078-1080, 1936.

Auerbach, R.K.: sDACS: a novel in silico SNP discovery and classification method for bacterial pathogens, Northern Arizona University, Flagstaff, Arizona, 2006.

Avashia, S.B., Petersen, J.M., Lindley, C.M., Schriefer, M.E., Gage, K.L., Cetron, M., DeMarcus, T.A., Kim, D.K., Buck, J., Montenieri, J.A., Lowell, J.L., Antolin, M.F., Kosoy, M.Y., Carter, L.G., Chu, M.C., Hendricks, K.A., Dennis, D.T., Kool, J.L.: First reported prairie dog-to-human tularemia transmission, Texas, 2002, Emerg. Infect. Dis., 10. 483-486, 2004.

Bandouchova, H., Sedlackova, J., Hubálek, M., Pohanka, M., Peckova, L., Treml, F., Vitula, F., Pikula, J.: Susceptibility of selected murine and microtine species to infection by a wild strain of *Francisella tularensis* subsp. *holoarctica*, Vet. Med. (Praha), 54. 64-74, 2009.

Barrow, G.I., Feltham R.K.A.: Cowan and Steel's Manual for the Identification of Medical Bacteria 3rd ed., Cambridge University Press, Cambridge, New York, 1993.

Barry, E.M., Cole, L.E., Santiago, A.E.: Vaccines against tularemia, Hum. Vaccin., 5. 832-838, 2009.

Bányai T., Martyin T.: Tularaemia - ami a tankönyvből kimaradt, Infek. Klin. Mikrob., 13. 17-20, 2006.

Bell, J.F.: Tularemia. In: Steele, J.H.: CRC handbook of zoonoses, Section A, Bacterial, rickettsial and mycotic diseases, 1st ed., CRC Press Inc., Boca Raton, Florida, 161-193, 1980.

Bell, J.F., Stewart, S.J.: Chronic shedding tularemia nephritis in rodents: possible relation to occurance of *Francisella tularensis* in lotic waters, J. Wildl. Dis., 11. 421-430, 1975.

Bell, J.F., Stewart, S.J.: Quantum differences in oral susceptibility of voles, *Microtus pennsylvanicus*, to virulent *Francisella tularensis* type B, in drinking water: implications to epidemiology, Ecol. Dis., 2. 151-155, 1983.

Bihari Z.: Regression in distribution of hamster (*Cricetus cricetus*) in Hungary during the past fifty years, Proceedings of the 11th Meeting of the International Hamster workgroup, Budapest, Hungary, 27-30. 2003.

Bihari Z., Arany I.: Metapopulation structure of common hamster (*Cricetus cricetus*) in agricultural landscape, Jahrbücher des Nassauischen Vereins für Naturkunde, 122. 217-221, 2001.

Brown, S.D., Krisher, K., Traczewski, M.M.: Broth microdilution susceptibility testing of *Francisella tularensis*: quality control limits for nine antimicrobial agents and three standard quality control strains, J. Clin. Microbiol., 42. 5877-5880, 2004.

Capellan, J., Fong, I.W.: Tularemia from a cat bite: case report and review of feline-associated tularemia, Clin. Infect. Dis., 16. 472-475, 1993.

Carlsson, H.E., Lindberg, A., Lindberg, G., Hederstedt, B., Karlsson, K., Agell, B.O.: Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia, J. Clin. Microbiol., 10. 615-621, 1979.

Cha, R.S., Zarbl, H., Keohavong, P., Thilly, W.G.: Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene, PCR Methods. Appl., 2. 14-20, 1992.

Craig, D.W., Pearson, J.V., Szelinger, S., Sekar, A., Redman, M., Corneveaux, J.J., Pawlowski, T.L., Laub, T., Nunn, G., Stephan, D.A., Homer, N., Huentelman, M.J.: Identification of genetic variants using bar-coded multiplexed sequencing, Nat. Methods., 5. 887-893, 2008.

Dennis, D.T., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K.: Tularemia as a biological weapon: medical and public health management, J. Am. Med. Assoc., 285. 2763-2773, 2001.

Dittrich K., Decsi T.: Tularemia gyermekkorban, Gyermekgyógyászat, 50. 386-391, 1999.

DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) Bacterial Nomenclature Up-To-Date (Approved list, Validation list), http://www.dsmz.de/download/bactnom/bactname.pdf. Accessed December 2010.

Ellis, J., Oyston, P.C., Green, M., Titball, R.W.: Tularemia, Clin. Microbiol. Rev., 15. 631-646, 2002.

Epinfo: http://www.oek.hu/

Escudero, R., Toledo, A. Gil, H., Kovácsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B., Bhide, M., Anda, P.: Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts, J. Clin. Microbiol., 46. 3139-3143, 2008.

Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A., Merrick, J.M.: Wholegenome random sequencing and assembly of *Haemophilus influenzae* Rd, Science, 269. 496-512, 1995.

Forsman, M., Sandstrom, G., Sjöstedt A.: Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR, Int. J. Syst. Bacteriol., 44. 38-46, 1994.

Foster, J.T., Beckstrom-Sternberg, S.M., Pearson, T., Beckstrom-Sternberg, J.S., Chain, P.S.G., Roberto, F.F., Hnath, J., Brettin, T., Keim, P.: Whole-genome-based phylogeny and divergence of the Genus *Brucella*, J. Bacteriol., 191. 2864–2870, 2009.

Francis, E.: The occurrence of tularemia in nature as a disease of man. Publ. Health Rep., 36. 1731-1753, 1921.

Francis, E., Mayne, B., Lake, G.C.: Tularemia Francis 1921: A new disease of man, Hyg. Lab. Bull., 130. 1-87, 1922.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, R.D., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F., Hu, P.C.,Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison 3rd, C.A., Venter, J.C.: The minimal gene complement of *Mycoplasma genitalium*, Science, 270. 397-403, 1995.

Friend, M.: Tularemia, 1st ed. U.S. Geological Survey, Circular 1297, Reston, Virginia, 2006.

Fujita, O., Tatsumi, M., Tanabayashi, K., Yamada, A.: Development of a real-time PCR assay for detection and quantification of *Francisella tularensis*, Jpn. J. Infect. Dis., 59. 46-51, 2006.

Füzi M., Kemenes F.: A magyarországi *Francisella tularensis* izolátumok antibiotikum érzékenysége, Orv. Hetil., 113. 2103-2105, 1972.

Gill, V., Cunha, B.A.: Tularemia pneumonia, Semin. Respir. Infect., 12. 61-67, 1997.

Greco, D., Allegrini, G., Tizzi, T., Ninu, E., Lamanna, A., Luzi, S.: A waterborne tularemia outbreak, Eur. J. Epidemiol., 3. 35-38, 1987.

Gurycová, D.: First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. Eur. J. Epidemiol., 14. 797-802, 1998.

Gurycová, D., Kocianová, E., Výrosteková, V., Rehácek, J.: Prevalence of ticks infected with *Francisella tularensis* in natural foci of tularemia in western Slovakia, Eur. J. Epidemiol., 11. 469-474, 1995.

Gurycová, D., Výrosteková, V., Khanakah, G., Kocianová, E., Stanek G.: Importance of surveillance of tularemia natural foci in the known endemic area of Central Europe, 1991-1997, Wien Klin. Wochenschr., 113. 433-438, 2001.

Hauri, A.M., Hofstetter, I., Seibold, E., Kaysser, P., Eckert, J., Neubauer, H., Splettstoesser, W.D.: Investigating an airborne tularemia outbreak, Germany, Emerg. Infect. Dis., 16. 238-243, 2010.

Hoelzle, L.E., Corboz, L., Ossent, P., Wittenbrink, M.M.: Tularemia in a captive golden-headed lion tamarin (*Leontopithecus chrysomelas*) in Switzerland, Vet. Rec., 155. 60–61, 2004.

Hollis, D.G., Weaver, R.E., Steigerwalt, A.G., Wenger, J.D., Moss, C.W., Brenner, D.J.: *Francisella philomiragia* comb. Nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease, J. Clin. Microbiol., 27. 1601-1608, 1989.

Holt, K.E., Parkhill, J., Mazzoni, C.J., Roumagnac, P., Weill, F.X., Goodhead, I., Rance, R., Baker, S., Maskell, D.J., Wain, J., Dolecek, C., Achtman, M., Dougan, G.: High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi, Nat. Genet., 40. 987–993, 2008.

Hopla, C.E.: The ecology of tularemia, Adv. Vet. Sci. Comp. Med., 18. 25-53, 1974.

Hopla, C.E., Hopla, A.K.: Tularemia. In: Beran, G.W.: CRC handbook of zoonoses, Section A, Bacterial, rickettsial and mycotic diseases, 2nd ed., CRC Press Inc., Boca Raton, Florida, 113-125, 1994.

Hornok S.: Allochronic seasonal peak activities of *Dermacentor* and *Haemaphysalis* spp. under continental climate in Hungary, Vet. Parasitol., 163. 366-369, 2009.

Hornok S., Farkas R.: Influence of biotope on the distribution and peak activity of questing ixodid ticks in Hungary, Med. Vet. Entomol. 23. 41-46, 2009.

Hubalek, Z., Sixl, W., Halouzka, J.: *Francisella tularensis* in *Dermacentor reticulatus* ticks from the Czech Republic and Austria, Wien Klin. Wochenschr., 110. 909-910, 1998.

Karlsson, K.A., Dahlstrand, S., Hanko, E., Soderlind, O.: Demonstration of *Francisella tularensis* in sylvan animals with the aid of fluourescent antibodies, Acta Pathol. Microbiol. Immunol. Scand., 78. 647-651, 1970.

Kaysser, P., Seibold, E., Matz-Rensing, K., Pfeffer, M., Essbauer, S., Splettstoesser, W.D.: Re-emergence of tularemia in Germany: Presence of *Francisella tularensis* in different rodent species in endemic areas, BMC Infect. Dis., 8. 157, 2008.

Keim, P.S., Johansson, A., Wagner, D.M.: Molecular epidemiology, evolution and ecology of *Francisella*. Ann. N Y. Acad. Sci., 1105. 30-66, 2007.

Keim, P.S., Van Ert, M.N., Pearson, T., Vogler, A.J., Huynh, L.Y., Wagner, D.M.: Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales, Infect. Genet. Evol., 4. 205-213, 2004.

Keim, P.S., Wagner, D.M.: Humans and evolutionary and ecological forces shaped the phylogeography of recently emerged diseases, Nat. Rev. Microbiol., 7. 813-821, 2009.

Kemenes F.: Die Epizootologie der Hausen-tularemie in Ungarn. Proceedings 2. Internationales Arbeitskolloquium über “Naturherde von Infektionskrankheiten in Zentraleuropa”, Graz, Austria, 285-289, 1976.

Kemenes F., Szécsényi I., Dózsa I.: A fácán (*Phasianus colchicus torquatus*) *Pasteurella tularensis* és *Past. multi-* (*avi-*) *septica* iránti fogékonyságának kísérletes vizsgálata, Magy. Állatorvosok., 20. 270-272, 1965.

Kirimanjeswara, G.S., Olmos, S., Bakshi, C.S., Metzger, D.W.: Humoral and cell-mediated immunity to the intracellular pathogen *Francisella tularensis*, Immunol. Rev., 225. 244-255, 2008.

Kocsis Z.: Négy tagú család tagjain előforduló tularemia fertőzés, Orv. Hetil., 105. 414-416. 1964.

Kugeler, K.J., Gurfield, N.: Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR, Appl. Environ. Microbiol., 71. 7594-7597, 2005.

Kugeler, K.J., Pappert, R., Zhou, Y., Petersen, J.M.: Real-time PCR for *Francisella tularensis* type A and type B, Emerg. Infect. Dis., 12. 1799-1801, 2006.

Kurtz, S., Phillippy, A., Delcher, A.L, Smoot, M., Shumway, M., Antonescu, C., Salzberg, S.L.: Versatile and open software for comparing large genomes, Genome Biol., 5. R12, 2004.

Little, R.D., Francis, E.: Pathology of tularaemia in the Belgian hare (*Oryctolagus cuniculus*), NIH. Bull., 167. 83-114, 1937.

Mätz-Rensing, K., Floto, A., Schrod, A., Becker, T., Finke, E.J., Seibold, E., Splettstoesser, W.D., Kaup, F.J.: Epizootic tularemia in an outdoor housed group of cynomolgus monkeys (*Macaca fascicularis*), Vet. Pathol., 44. 327-334, 2007.

McCaffey, R.L., Allen, L.A.H.: Pivotal Advance: *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape, J. Leukoc. Biol., 80. 1224-1230, 2006.

McCoy, G.W., Chapin, C.W.: Further observations on a plague-like disease of rodents with a preliminary note on the causative agent *Bacterium tularense*, J. Infect. Dis., 10. 61-72, 1912.

Moore, F.M., Petrak, M.L., McMillan, M.C.: Diagnosis of clamydial infection in pet birds: comparison of cloacal-swab culture and peroxidase-antiperoxidase methods, Avian Dis., 32. 157-162, 1988.

Mörner, T.: The use of FA technique of detecting *Francisella tularensis* in formalin fixed material, Acta Vet. Scand., 22. 296-306, 1981.

Mörner, T.: The ecology of tularemia, Rev. Sci. Tech., 11. 1123-1130, 1992.

Mörner, T.: Tularemia in hares in Sweden, MS Thesis, Swedish University of Agricultural Science, Uppsala, Sweden, 1994.

Mörner, T., Addison, E.: Tularemia. In: Williams, E.S., Barker, I.K.: Infectious diseases of wild mammals, 3rd ed. Iowa State University Press, Ames, Iowa, 303-312, 2001.

Mörner, T., Gunnar, S., Mattsson, R., Nilsson, P. O.: Infection with *Francisella tularensis* biovar *palaearctica* in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden, J. Wildl. Dis., 24. 422-433, 1988.

Mörner, T., Sandstrom, G., Mattson, R.: Comparison of sera and lung extracts for surveys of wild animals for antibodies against *Francisella tularensis* biovar *palaearctica*, J. Wildl. Dis., 24. 10-14, 1988.

Müller, W., Bocklisch, H., Schüler, G., Hotzel, H., Neubauer, H., Otto, P.: Detection of *Francisella tularensis* subsp. *holarctica* in a European brown hare (*Lepus europaeus*) in Thuringia, Germany, Vet. Microbiol., 123. 225-229, 2007.

Münnich D., Lakatos M.: Clinical, epidemiological and therapeutical experience with human tularemia: the role of hamster hunters, Infection, 7. 61-63, 1979.

Nakazawa, Y., Williams, R., Peterson, A.T., Mead, P., Staples, E., Gage, K.L.: Climate change effects on plague and tularemia in the United States, Vector Borne Zoonotic Dis., 7. 529-540, 2007.

Nechay, G.: Status of Hamsters *Cricetus cricetus*, *Cricetus migratorius*, *Mesocricetus newtoni* and other hamster species in Europe, Nature and environment, No. 106. Council of Europe Publishing, 2000.

Nano, F.E.: *Francisella*. In: Collier L.H.: Topley and Wilson's Microbiology and Microbial Infections, 9th ed., B.C. Decker Inc., Philadelphia, Pennsylvania, 234-241, 1998.

Nubel, U., Roumagnac, P., Feldkamp, M., Song, J.H., Ko, K.S., Huang, Y.C., Coombs, G., Ip, M.,Westh, H., Skov, R., Struelens, M.J., Goering, R.V., Strommenger, B.,Weller, A., Witte, W., Achtman, M.: Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*, Proc. Natl. Acad. Sci. U.S.A., 105. 14130-14135, 2008.

Ohara, H., Kobayashi, T., Kudo. J.: A Study on Pleomorphism of *Bacterium tularense* ("Yato Byo" bacteria, Ohara) and their Special Flagellum, Tohoku J. Exp. Med., 25. 520-530, 1935.

Office International des Epizooties (OIE): Chapter Tularemia, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.18_TULAREMIA.pdf, 2008.

Olusfjev, N.G., Emelyanova, O.S., Dunayeva, T. N.: Comparative study of strains of *Bacterium tularense* in the old and new world and their taxonomy, J. Hyg. Epidemiol. Mikrobiol. Immunol., 3. 138–49, 1959.

Olsufjev, N.G., Dunayeva, T.N.: Natural focality, epidemiology and prophylaxis of tularemia, Medicina, Moskva, U.S.S.R., 1970.

Olsufjev, N.G., Meshcheryakova, I.S.: Subspecific taxonomy of *Francisella tularensis*, Int. J. Syst. Bacteriol., 33. 872-874, 1983.

Olsufjev, N.G., Shlygina, K.N., Ananova. E.V.: Persistence of *Francisella tularensis* McCoy et Chapin tularemia agent in the organism of highly sensitive rodents after oral infection, J. Hyg. Epidemiol. Mikrobiol. Immunol., 28. 441–454, 1984.

O'Toole, D., Williams, E.S., Woods, L.W., Mills, K., Boerger-Fields, A., Montgomery, D.L., Jaeger, P., Edwards, W.H., Christensen, D., Marlatt, W.: Tularemia in range sheep: an overlooked syndrome, J. Vet. Diagn. Invest., 20. 508-513, 2008.

Papp, A.C., Pinsonneault, J.K., Cooke, G., Sadee, W.: Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves, BioTechniques, 34. 1068-1072, 2003.

Parker, D.D., Johnson, D.E.: Experimental transmission of *Pasteurella tularensis* by the flea, *Orchopeas leucopus* (Baker), J. Infect. Dis., 101. 69-72, 1957.

Pearson, T., Busch, J.D., Ravel, J., Read, T.D., Rhoton, S.D., U'Ren, J.M., Simonson, T.S., Kachur, S.M., Leadem, R.R., Cardon, M.L., Van Ert, M.N., Huynh, L.Y., Fraser, C.M., Keim, P.: Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphisms from whole-genome sequencing, Proc. Natl. Acad. Sci. U. S. A., 101. 13536-13541, 2004.

Pearson, T., Okinaka, R.T., Foster, J.T., Keim, P.: Phylogenetic understanding of clonal populations in an era of whole genome sequencing, Infect. Genet. Evol. 9. 1010-1019, 2009.

Petersen, J.M., Schriefer, M.E.: Tularemia: emergence/re-emergence, Vet. Res., 36. 455-467, 2005.

Petersen, J.M., Schriefer, M.E., Carter, L.G., Zhou, Y., Sealy, T., Bawiec, D., Yockey, B., Urich, S., Zeidner, N.S., Avashia, S.B., Kool, J.L., Buck, J., Lindley, C., Celeda, L., Monteneiri, J.A., Gage, K.L., Chu, M.C.: Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002, Emerg. Infect. Dis., 10. 419-425, 2004.

Pérez-Castrillón, J.L., Bachiller-Luque, P., Martín-Luquero, M., Mena-Martín, F.J., Herreros, V.: Tularemia epidemic in northwestern Spain: clinical description and therapeutic response, Clin. Infect. Dis., 33. 573-576, 2001.

Pikula, J., Beklova, M., Holesovoska, Z., Treml, F.: Ecology of European brown hare and distribution of natural foci of tularemia in the Czech Republic, Acta Vet. Brno 73. 267-273, 2004.

Pilo, P., Johansson, A., Frey, J.: Identification of *Francisella tularensis* cluster in central and western Europe, Emerg. Infect. Dis., 15. 2049-2051, 2009.

Pintur, K., Popovic, N., Alegro, A., Severin, K., Slavica, A., Kolic, E.: Selected indicators of brown hare (*Lepus europaeus* Pallas, 1778) population dynamics in northwestern Croatia, Vet. Archive, 76. 199-209, 2006.

Porsch-Özcürümez, M., Kischel, N., Priebe, H., Splettstösser, W., Finke, E. J., Grunow, R. Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia, Clin. Diagn. Lab. Immunol., 11. 1008-1015, 2004.

Posthaus, H., Welle, M., Mörner, T., Nicolet, J., Kuhnert, P.: Tularemia in common marmoset (*Callithrix jacchus*) diagnosed by 16S rRNA sequencing, Vet. Microbiol., 61. 145-150, 1998.

Reintjes, R., Dedushaj, I., Gjini, A., Jorgensen, T.R., Cotter, B., Lieftucht, A., D'Ancona, F., Dennis, D.T., Kosoy, M.A., Mulliqi-Osmani, G., Grunow, R., Kalaveshi, A., Gashi, L., Humolli, I.: Tularemia outbreak investigation in Kosovo: case control and environmental studies, Emerg. Infect. Dis., 8. 69-73, 2002.

Relman, D.A., Universal Bacterial 16S rDNA Amplification and Sequencing. In: Persing, D.H., Smith, T.F., Tenover, F.C., White, T.J.: Diagnostic Molecular Microbiology, Principles and Applications, Mayo Foundation, Rochester, U.K., 489-495, 1993.

Rydén, P., Sjöstedt, A., Johansson, A.: Effects of climate change on tularemia disease activity in Sweden. Global Health Action, DOI: 10.3402/gha.v2i0.2063, 2009.

Sandström, G., Sjöstedt, A., Forsman, M., Pavlovich, N.V., Mishankin, B.N.: Characterization and classification of strains of *Francisella tularensis* isolated in the central Asian focus of the Soviet Union and in Japan, J. Clin. Microbiol., 30. 172-175, 1992.

Scoles, G.A.: Phylogenetic analysis of the Francisella-like endosymbionts of *Dermacentor* ticks, J. Med. Entomol., 41. 277-286, 2004.

Sewell, D.L.: Minireview, laboratory safety practices associated with potential agents of biocrime or bioterrorism, J. Clin. Microbiol., 41. 2801-2809, 2003.

Sjöstedt, A.: *Francisella*. In: Garrity, G.: The Proteobacteria, Part B, Bergey's Manual of Systematic Bacteriology, 2nd ed., Springer, New York, New York, 200–210, 2005.

Sjöstedt, A., Eriksson, U., Berglund, L., Tärnvik, A.: Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR, J. Clin. Microbiol., 35. 1045-1048, 1997.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A., Miller, S.I., Ramsey, B.W., Speert, D.P., Moskowitz, S.M., Burns, J.L., Kaul, R., Olson, M.V.: Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients, Proc. Natl. Acad. Sci. U.S.A., 103. 8487-8492, 2006.

Somogyi F.: A mezei nyúl hasznosítás kereskedelmi lehetőségei. In: Nagy E.: European brown hare, 1st ed., Dénes Natúr Műhely Kiadó, Százhalombatta, Hungary, 62-72, 2006.

Sréter T, Széll Z, Varga I.: Spatial distribution of *Dermacentor reticulatus* and *Ixodes ricinus* in Hungary: evidence for change?, Vet. Parasitol., 128. 347-351, 2005.

Sréter-Lancz Zs., Széll Z., Sréter, T., Márialigeti, K.: Detection of a novel Francisella in *Dermacentor reticulatus*: A need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks, Vector Borne Zoonotic Dis., 9. 123-126, 2009.

Sterba, F., Krul, J.: Pathologisch-morphologische untersuchungen zur differentialdiagnose von brucellose, tularame und pseudotuberculose beim hasen (*Lepus europaeus* L.), XVIIth Congress of the international union of Game Biologists, Brussels, Belgium, 763-771, 1985.

Strauss, E., Pohlmeyer, K.: Population density of European hares (*Lepus europaeus* Pallas, 1778) and hunting activity in Lower Saxony, Z. Jagdwiss. 47. 43-62, 2001.

Sun, L.V., Scoles, G.A., Fish, D., O'Neill, S.L.: *Francisella*-like endosymbionts of ticks, J. Invertebr. Pathol., 76. 301-303, 2000.

Svensson, K., Granberg, M., Karlsson, L., Neubauerova, V., Forsman, M., Johansson, A.: A real-time PCR array for hierarchical identification of *Francisella* isolates, PLoS One, 4:e8360, 2009.

Széll Z, Sréter-Lancz Zs, Márialigeti K, Sréter T.: Temporal distribution of *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* in Hungary, Vet. Parasitol. 141. 377-379, 2006.

Sztojkov V.: A hazai mezei nyúl-állomány egészségügyi helyzete. In: Nagy E.: European brown hare, 1st ed., Dénes Natúr Műhely Kiadó, Százhalombatta, Hungary, 16-24. 2006.

Tärnvik, A., Priebe, H.S., Grunow, R.: Tularemia in Europe: An epidemiological overview, Scand. J. Infect. Dis., 36. 350-355, 2004.

Tomaso, H., Al Dahouk, S.: Antimicrobial susceptibilities of Austrian *Francisella tularensis holarctica* biovar II strains, Int. J. Antimicrob. Agents, 26. 279-284, 2005.

Treml, F., Pikula, J., Bandouchova, H., Horakova, J.: European brown hare as a potential source of zoonotic agents, Vet. Med. (Praha), 52. 451-456, 2007.

Twenhafel, N.A., Alves, D.A., Purcell, B.K.: Pathology of inhalational *Francisella tularensis* spp. *tularensis* SCHU S4 infection in African Green Monkeys (*Chlorocebus aethiops*), Vet. Pathol. 46. 698-706, 2009.

Valentine, B.A., DeBey, B.M., Sonn, R.J., Stauffer, L.R., Pielstick, L.G.: Localized cutaneous infection with *Francisella tularensis* resembling ulceroglandular tularemia in a cat, J. Vet. Diagn. Invest., 16. 83-85, 2004.

Van Ert, M.N., Easterday, W.R., Huynh, L.Y., Okinaka, R.T., Hugh-Jones, M.E., Ravel, J., Zanecki, S.R., Pearson, T., Simonson, T.S., U'Ren, J.M., Kachur, S.M., Leadem-Dougherty, R.R., Rhoton, S.D., Zinser, G., Farlow, J., Coker, P.R., Smith, K.L., Wang, B., Kenefic, L.J., Fraser-Liggett, C.M., Wagner, D.M., Keim, P.: Global genetic population structure of *Bacillus anthracis,* PLoS One, 2, e461, 2007.

Versage, J.L., Severin, D.D., Chu, M.C., Petersen, J.M. Development of multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens, J. Clin. Microbiol., 41. 5492-5499, 2003.

Vogler, A.J., Birdsell, D., Price, L.B., Bowers, J.R., Beckstrom-Sternberg, S.M., Auerbach, R.K., Beckstrom-Sternberg, J.S., Johansson, A., Clare, A., Buchhagen, J.L., Petersen, J.M., Pearson, T., Vaissaire, J., Dempsey, M.P., Foxall, P., Engelthaler, D.M., Wagner, D.M., Keim, P.: Phylogeography of *Francisella tularensis*: global expansion of a highly fit clone, J. Bacteriol., 191. 2474-2484, 2009.

Vogler, A.J., Birdsell, D., Wagner, D.M., Keim, P.: An optimized, multiplexed multi-locus variable-number tandem repeat analysis system for genotyping *Francisella tularensis*, Lett. Appl. Microbiol. 48. 140-144, 2009.

World Animal Health Information Database, http://www.oie.int/wahis/public.php?page=home

Wherry, W.B., Lamb, B.H.: Infection of man with *Bacterium tularense*, J. Infect. Dis., 15. 331-340, 1914.

Whipp, M.J., Davis, J.M., Lum, G., de Boer, J., Zhou, Y., Bearden, S.W., Petersen, J.M., Chu, M.C., Hogg, G.: Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia, J. Med. Microbiol., 52. 839-842, 2003.

WHO Guidelines on Tularemia. WHO Press, Geneva, Switzerland, www.cdc.gov/tularemia/resources/whotularemiamanual.pdf, 2007.

Woods, J.P., Panciera, R.J., Morton, R.J., Lehenbauer, T.W.: Feline tularemia, Compendium on Continuing Education for the Practicing Veterinarian, 20. 442, 1998.

Zeidner, N.S., Carter, L.G., Monteneiri, J.A,, Petersen, J.M,, Schriefer, M., Gage, K.L., Hall, G., Chu, M.C.: An outbreak of *Francisella tularensis* in captive prairie dogs: an immunohistochemical analysis, J. Vet. Diagn. Invest., 16. 150-152, 2004.

10. Scientific publications

In peer-reviewed journals

Gyuranecz M., Birdsell, D.N., Beckstrom-Sternberg, S.M., Makrai L., Fodor L., Fabbi, M., Vicari, N., Busch, J.D., Wagner, D.M., Keim, P.S.: Phylogenetic population structure of *Francisella tularensis* subsp. *holarctica* from Hungary and the consequence of tularemia dispersal resulting from transcontinental relocation of the European brown hare (*Lepus europaeus*), *Emerg. Infect. Dis.,* [submitted]

Gyuranecz M., Rigó K., Dán Á., Földvári G., Makrai L., Dénes B., Fodor L., Majoros G., Tirják L., Erdélyi K.: Investigation of the ecology of *Francisella tularensis* during an inter-epizootic period, *Vector Borne Zoonotic Dis.*, [in press]

Gyuranecz M., Dénes B., Dán Á., Rigó K., Földvári G., Szeredi L., Fodor L., Sallós A., Jánosi K., Erdélyi K., Krisztalovics K., Makrai L.: Susceptibility of the common hamster (*Cricetus cricetus*) to *Francisella tularensis* and its effect on the epizootiology of tularemia in an area where both are endemic, *J. Wildl. Dis.*, 46. 1316-1320, 2010.

Gyuranecz M., Szeredi L., Makrai L., Fodor L., Ráczné Mészáros Á., Szépe B., Füleki M., Erdélyi K.: Tularemia of European brown hare (*Lepus europaeus*): a pathological, histopathological and immunhistochemical study, *Vet. Pathol.*, 47. 958-963, 2010.

Gyuranecz M., Erdélyi K., Fodor L., Jánosi K., Szépe B., Füleki M., Szőke I., Dénes B., Makrai L.: Characterisation of *Francisella tularensis* strains, comparing their carbon source utilization, *Zoonoses Public Health*, 57. 417-422, 2010.

Gyuranecz M., Fodor L., Makrai L., Monse L., Krisztalovics K., Dénes B., Szépe B., Füleki M., Erdélyi K.: A tularemia járványtana, különös tekintettel a mezei nyúl (*Lepus europaeus*) fertőzésére, *Magy. Állatorvosok Lapja*, 132. 39-45, 2010.

Gyuranecz M., Fodor L., Makrai L., Szőke I., Jánosi K., Krisztalovics K., Erdélyi K.: Generalized tularemia in a vervet monkey (*Chlorocebus aethiops*) and a patas monkey (*Erythrocebus patas*) in a zoo, *J. Vet. Diagn. Invest.*, 21. 384-387, 2009.

In book

Gyuranecz M.: Tularemia. In: Duff, J.P., Gavier-Wieden, D., Meredith, A.: Infectious diseases of wild birds and mammals in Europe, *Wiley-Blackwell Publishing* [in press]

Conference oral presentations

Gyuranecz M.: Tularemia, Day of Hungarian Science, TIT stúdió, Budapest, Hungary, 2010.

Gyuranecz M.: Tularemia, Conference on Antibiotics, Szent György Hospital, Székesfehérvár, Hungary, 2010.

Gyuranecz M.: Tularemia, Annual conference of the Hungarian Society on Zoonoses, Budapest, Hungary, 2010.

Gyuranecz M., Rigó K., Dán Á., Földvári G., Makrai L., Dénes B., Fodor L., Majoros G., Tirják L., Erdélyi K.: Investigation of the ecology of *Francisella tularensis* during an inter-epizootic period, IX. Conference of the European Wildlife Disease Association, Vlieland, The Netherlands, 2010.

Gyuranecz M., Szeredi L., Makrai L., Fodor L., Ráczné Mészáros Á., Szépe B., Füleki M., Dénes B., Jánosi K., Erdélyi K.: Immunohistochemical findings in tularemia infected brown hares (*Lepus europaeus*), Annual conference of the Hungarian Society on Zoonoses, Tiszafüred, Hungary, 2009.

Gyuranecz M.: Tularemia, Med-Vet-Net WiREDZ (Wildlife Related Emerging Diseases and Zoonoses) Special Interest Group meeting, Budapest, Hungary, 2008.

Gyuranecz M., Erdélyi K., Fodor L., Jánosi K., Szépe B., Füleki M., Szőke I., Dénes B., Makrai L.: Tularemia of the european brown hare (*Lepus europaeus*), Annual conference of the Hungarian Society on Zoonoses, Ráckeve, Hungary, 2008.

Conference poster presentations

Madar, M., **Gyuranecz M.**, Bencurova, E., Bhide, M.: Detection of neuroinvasive *Francisella* in *Lepus europaeus*, VIII. International Symposium on Molecular Diagnostics, Graz, Austria, 2010.

Gyuranecz M., Szeredi L., Makrai L., Fodor L., Ráczné Mészáros Á., Szépe B., Füleki M., Dénes B., Jánosi K., Erdélyi K.: Pathological, histopathological and immunohistochemical findings in tularemia infected brown hares (*Lepus europaeus*), VI. International Conference on Tularemia, Tularemia International Society, Berlin, Germany, 2009.

Gyuranecz M., Szeredi L., Makrai L., Fodor L., Ráczné Mészáros Á., Szépe B., Füleki M., Dénes B., Jánosi K., Erdélyi K.: Pathology of tularemia infected brown hares (*Lepus europaeus*), III. Workshop of the Student Chapter of the European Wildlife Disease Association, Veyrier-du-Lac, France, 2009.

Gyuranecz M., Erdélyi K., Fodor L., Jánosi K., Szépe B., Füleki M., Szőke I., Makrai L.: comparison of *Francisella tularensis* strains on the basis of carbohydrate utilization, Annual conference of the Hungarian Society for Microbiology, Keszthely, Hungary, 2008.

Gyuranecz M., Erdélyi K., Fodor L., Jánosi K., Szépe B., Füleki M., Szőke I., Makrai L.: Carbon source utilization of *Francisella tularensis s*trains isolated in Hungary, VIII. Conference of the European Wildlife Disease Association, Rovinj, Croatia, 2008.

Gyuranecz M., Makrai L., Fodor L., Szőke I., Jánosi K., Erdélyi K.: Generalized tularemia in vervet monkey (*Chlorocebus aethiops*) and patas monkey (*Erythrocebus patas*), Annual conference of the Hungarian Association of Zoo and Wildlife Veterinarians, Budapest, Hungary, 2008.

Other publications in peer-reviewed journals

Gyuranecz M., Erdélyi K., Makrai L., Fodor L., Szépe B., Ráczné Mészáros Á., Dán Á., Dencső L., Fassang E., Szeredi L.: Brucellosis of the European brown hare (*Lepus europaeus*), *J. Comp. Pathol.*, [in press]

Gyuranecz M., Szeredi L., Rónai Zs., Dénes B., Dencső L., Dán Á., Pálmai N., Hauser Zs., Lami E., Makrai L., Erdélyi K., Jánosi Sz.: Detection of *Brucella canis* induced reproductive diseases in a kennel, *J. Vet. Diagn. Invest.*, 23. 143-147, 2011.

Jánosi K., Hajtós I., Makrai L., **Gyuranecz M.**, Varga J., Fodor L.: First isolation of *Histophilus somni* from goats, *Vet. Microbiol.*, 133. 383-386, 2009.

Jánosi K., Stipkovits L., Glávits R., Molnár T., Makrai L., **Gyuranecz M.**, Varga J., Fodor L.: Aerosol infection of calves with *Histophilus somni*, *Acta Vet. Hung.*, 57. 347-356, 2009.

Jánosi K., Stipkovits L., Schreck O., Glávits R., Molnár T., Makrai L., **Gyuranecz M.**, Varga J., Szathmáry Zs., Fodor L.: A tüdő kórbonctani és kórszövettani elváltozásai mesterséges *Histophilus somni* fertőzést követően borjakban, *Magy. Állatorvosok Lapja*, 131. 202-209, 2009.

11. Supplements

Table S1. The genome locations, SNP state, primer sequences and thermal cycler parameters for the previously published canSNP assays (Svensson et al., 2009; Vogler et al., 2009a).

SNP	SCHU S4 position	Genome SNP state (D/A)*	Melt-MAMA primer†	Melt-MAMA primer sequences‡	Primer concentration (µM)	Annealing temperature (°C)	Melting T_m (°C)
A.I.Br.002	387 311	A/G	A D C	ttttttttttttggATCAAATATCGAAAGTTTCAATCAGag cggggcggggcggggATCAAATATCGAAAGTTTCAATCAGca gcccCTTTAATGACCCGGTATCATCATAGAC	0.600 0.200 0.200	60	71.5 76.5
A.II.Br.002	201 094	T/C	A D C	ttttttttttttggTAAAATGATCTCTAAAGAAATATTGATGCAgc cggggcggggcggggATAAAATGATCTCTAAAGAAATATTGATGCAct ccccTTTGCCATCGGTGTAGGCATCC	0.200 0.200 0.200	60	73.5 76.3
A.II.Br.003	688 455	C/T	A D C	ttttttttttttggCATTATTTTTGAGCCGTACTTATCga cggggcggggcggggCATTATTTTTGAGCCGTACTTATCtg gcccCCTATTTATGCAATATCACAAGTTCCAG	0.600 0.200 0.200	60	74.0 78.0
A.II.Br.004	640 668	G/C	A D C	ttttttttttttttCCCATAGTGGTTGATGATGATTCac cggggcggggcggggCCCATAGTGGTTGATGATGATTCtg GACATACAGAAACATATTTGGTATCTATCTCTTTA	0.200 0.200 0.200	60	69.0 74.5
A.II.Br.006	29 478	T/C	A D C	ttttttttttttttCCAGAGCAATATAAAATTTGGTATCAag cggggcggggcggggTCCAGAGCAATATAAAATTTGGTATCAta gcccCATTAGATAGTTGTTACTATCAAGTCCTCTATTATCT	0.200 0.200 0.200	60	70.0 74.5
B.Br.003	470 842	A/G	A D C	ttttttttttttttGGAGGTATAATAATGTGCCATCgc cggggcggggcggggATGGAGGTATAATAATGTGCCATCtt AATGCGTGACTGTGATATTGGATCTTTTA	0.300 0.200 0.200	60	72.5 76.0
B.Br.004	823 673	T/A	A D C	ttttttttttttttGACTTTTGGGGTTTTTGCTGga cggggcggggcggggGACTTTTGGGGTTTTTGCTGgt GCGATCACCAAAACTACCYATAACCACACTG	0.800 0.200 0.200	60	75.5 79.5
B.Br.005	1 853 699	T/C	A D C	ttttttttttttttttttGCTTCAATAGTAGCATCATCAGCTAAtg cggggcggggcggggcggggGCTTCAATAGTAGCATCATCAGCTAAca GGGTTTTAGAAATAGGAGTTTTYCGTGG	0.200 0.200 0.200	60	72.2 79.5
B.Br.006	713 648	A/G	A D C	ttttttttttttttttttTGTTATTGAATGTAAGGAAAATGATTCgg cggggcggggcggggcggggTGTTATTGAATGTAAGGAAAATGATTCca GAAATAGTAAGTCCAAACGCATGAAATA	0.200 0.200 0.200	60	72.0 77.0
B.Br.007	599 475	C/T	A D C	ttttttttttttttttttTGGTTTTGCAGCTAATAATTTCATTGgt cggggcggggcggggcggggGTTTTGCAGCTAATAATTTCATTGgc GTCCTTGTTAGTCAAAGCGCTATAA	0.200 0.300 0.200	60	73.0 78.0
B.Br.008	686 383	T/G	A D C	ttttttttttttTTAAATGATAGTGGCAGTATGAAGACTgg cggggcggggcgggTATTAAATGATAGTGGCAGTATGAAGACTtt ATTAAAAGAATTCCTAAAACATTTTGCC	0.200 0.200 0.200	60	70.5 75.5
B.Br.009	943 136	G/A	A D C	ttttttttttttttttttAGTTGGTACAGCTGCTGCGAca cggggcggggcggggcggggCAGTTGGTACAGCTGCTGCGAag TCCTCTCCACCAGCAACTCTTAATAAA	0.200 0.200 0.200	60	76.0 81.0
B.Br.010	387 538	G/A	A D C	ttttttttttttttttttTGCCATCACTAGTAAATACCACATTAAgt cggggcggggcggggcggggGCCATCACTAGTAAATACCACATTAAac TTGTAATATTAGCTMGAAAAGTAGATGAA	0.200 0.200 0.200	60	70.0 74.5

B.Br.011	1 282 031	A/G	A D C	ttttttttttttttttttttTGTGATCAAGGGAAATGCTCAtg cggggcggggcggggcggggTGTGATCAAGGGAAATGCTCAga TATTCTGTTATATTGTTGCGCATAGGCATAC	0.200 0.200 0.200	60	73.0 79.0
B.Br.012	109 781	T/A	A D C	ttttttttttttttttttttCSGGTTCAGCACGTCAATATTtt cggggcggggcggggcggggSGGTTCAGCACGTCAATATca YARKACAATYGCAAATGCAAATG	0.200 0.200 0.200	60	72.5 77.5
B.Br.013	948 767	G/A	A D C	ttttttttttttttttttttGGTATATTGGGTATGGGCGAATTt cggggcggggcggggcggggGTATATTGGGTATGGGCGAATgc GCAGCAGGTAGTTGTAATAACTCTAGTAATAAA	0.200 0.200 0.200	60	70.0 73.5/80.0#
FtB20M	1396117	G/A	A D C	gcgggcTCTGATGAAGAATATCTTAaAa gcgggcgcgggcagggcggcTCTGATGAAGAATATCTTAgAg ATTATGGCAAAACTATACCTT	0.200 0.200 0.200	60	71.7 74.7
FtB21M	701320	A/G	A D C	gcgggcgcgggcagggcggcACCAAGGTAGATTTGCAGCTcCg gcgggcACCAAGGTAGATTTGCAGCTtCa ATCCCTGTTGGGATATCCTCGACTAA	0.200 0.200 0.200	60	78.1/82.1# 75,9
FtB22M	1113320	A/G	A D C	gcgggcgcgggcagggcggcTGAATACTCTACGCGATAAGgTg TGAATACTCTACGCGATAAGtTa ATCAGACTTAGGTGTTAGATCAGAGTT	0.200 0.200 0.200	60	76.2/80.2# 73,6
FtB23M	253121	T/G	A D C	gcgggcgcgggcagggcggcTTACTACAAATTCGCCTCTgAg TTACTACAAATTCGCCTCTtAt AGCAAAAGAGCTTACTAAACAATTTGA	0.200 0.200 0.200	60	77,3 72,8
FtB24M	1419996	G/T	A D C	gcgggcTATCGCCAGGTTTAATTTGtTt gcgggcgcgggcagggcggcTATCGCCAGGTTTAATTTGgTg TCTGCAGCATCTATCCCATTAGCCTTA	0.400 0.200 0.200	60	75,8 80,6
FtB25M	1534495	T/C	A D C	gcgggcgcgggcagggcggcTGTATCTAAGACAGCAGTGAgGc gcgggcTGTATCTAAGACAGCAGTGAtGt ATGGTAGCATAGTTCTAGGAATAAACT	0.200 0.200 0.200	60	76.6/80.6# 73,5

*SNP states are presented according to their orientation in the SHU S4 reference genome (NC_006570); D: Derived SNP state; A: Ancestral SNP state.

†D: Derived; A: Ancestral; C: Consensus.

‡Primer tails and antepenultimate mismatch bases are in lower case.

Two melting temperatures reflecting differential dissociation of the product.

Table S2. Primers used in *Francisella tularensis* MLVA (Vogler et al., 2009b).

Locus	Primer	PCR	Conc. (µmol l^{-1})	Primer Sequence (5'-3')	Dye
Ft-M22	Ft-M22-2F	1A	0.075	gtggaaatgcaaaaacaatatcgaggaacttta	6FAM
	Ft-M22-2R	1A	0.075	gttttttctcgtccgctgttagtgattttacatc	None
Ft-M23	Ft-M23-2F	1A	0.2	gctggattattaaagcatatgacagacgagtagg	NED
	Ft-M23-2R	1A	0.2	gttccctcaggtttatccaaagttttaatgttttatt	None
Ft-M24	Ft-M24-2F	1A	0.8	gaatcttaatccatacggtcctaataatattcctgtcaat	NED
	Ft-M24-2R	1A	0.8	gttggtacttatgggctatagcggatattatttcagt	None
Ft-M03	Ft-M03-2F	1B	0.2	gcacgcttgtctcctatcatcctctggtagtc	HEX
	Ft-M03-2R	1B	0.2	gaacaacaaaagcaacagcaaaattcacaaaa	None
Ft-M20A	Ft-M20-2AF*	2	0.1	gtatatcttggaataagccggagttagatggttct	6FAM
	Ft-M20-2AFcold*	2	0.1	gtatatcttggaataagccggagttagatggttct	None
	Ft-M20-2AR†	2	0.2	gcaataactttatcacccttattgtagactgcttctgc	None
Ft-M10	Ft-M10-2F	2	0.15	gctaatttttttcatatttatctccatttttacttttttgc	HEX
	Ft-M10-2R‡	2	0.15	gctcagctcgaactccgtcataccttcttc	None
Ft-M05	Ft-M05-2F	2	0.6	gtttgttacgccaataaacaaaaagtgtaaataatg	NED
	Ft-M05-2R‡	2	0.6	gctcagctcgaactccgtcataccttcttc	None
Ft-M04	Ft-M04-2F	3A	0.2	gcgcgctatctaactaatttttatattgaaacaatcaaat	6FAM
	Ft-M04-2R	3A	0.2	gcaaatataccgtaatgccacctatgaaaactc	None
Ft-M20B	Ft-M20-2BF†	3A	0.2	gggtgataaagttattgttaatggtgtgacttatgaa	None
	Ft-M20-2BR	3A	0.2	gtaactacttgaccgccagtatatgcttgacct	HEX
Ft-M06	Ft-M06-2F	3A	0.6	gtttttggtgaactgccaacaccataactt	NED
	Ft-M06-2R	3A	0.6	gcaattcagcgaaaccctatcttagcctc	None
Ft-M02	Ft-M02-2F	3B	0.2	gctgctgtggctgtaaatgttgctatgct	6FAM
	Ft-M02-2R	3B	0.2	gcagggcacaattcttgaccatcagg	None

* Equal amounts of 6FAM-labeled and unlabeled (cold) Ft-M20-2AF were added to PCR Mix 2 to decrease signal strength for multiplexing.

† Ft-M20-2AR and Ft-M20-2BF have overlapping primer sequences and so cannot be run in the same PCR.

‡ Ft-M05-2R and Ft-M10-2R have the same primer sequence.

Table S3. Number of exported European brown hares, number and percentage of *F. tularensis* seropositive hares, and the absolute number of human cases in Hungary between 1984-2009.

Year	European brown hare			Number of human cases
	Number of exported	Number of positive	Percentage of positive	
1984	31376	188	0.60	23
1985	30033	489	1.63	27
1986	17509	167	0,95	106
1987	18933	173	0.91	40
1988	14595	1150	7.88	77
1989	19783	1808	9.14	100
1990	16347	698	4.27	59
1991	5585	85	1.52	32
1992	13814	1558	11.28	49
1993	15049	443	2.94	56
1994	15116	3053	20.20	96
1995	13426	936	6.97	131
1996	2629	82	3.12	107
1997	5115	309	6.04	148
1998	5160	16	0.31	89
1999	8682	785	9.04	131
2000	9923	163	1.64	69
2001	11806	621	5.26	87
2002	6512	228	3.50	69
2003	2864	39	1.36	28
2004	5116	643	12.57	36
2005	7816	410	5.25	87
2006	2824	19	0.67	139
2007	9235	61	0.66	20
2008	4633	51	1.10	25
2009	5224	48	0.92	37

Table S4. Identification number, host, geographic origin and year of isolation of the collected *F. tularensis* ssp. *holarctica* strains.

	ID number	Host	Origin	Year of isolation
1	TUL M1/03	patas monkey	Szeged Zoo	2003
2	TUL M2/03	vervet monkey	Szeged Zoo	2003
3	TUL1/07	European brown hare	Alattyán	2007
4	TUL3/07	European brown hare	Kengyel	2007
5	TUL4/07	European brown hare	Békés	2007
6	TUL6/07	European brown hare	Battonya	2007
7	TUL7/07	European brown hare	Szarvas	2007
8	TUL11/07	European brown hare	Kőrösladány	2007
9	TUL12/07	European brown hare	Kőröstarcsa	2007
10	TUL13/07	European brown hare	Csökmő	2007
11	TUL14/07	European brown hare	Jászberény	2007
12	TUL17/08	European brown hare	Kecel	2008
13	TUL19/08	European brown hare	Jászárokszállás	2008
14	TUL21/08	European brown hare	Hegyeshalom	2008
15	TUL22/08	European brown hare	Mosonszentjános	2008
16	DV52/08	European brown hare	Dévaványa	2008
17	DV57/08	European brown hare	Dévaványa	2008
18	TUL24/08	European brown hare	Szegvár	2008
19	TUL25/08	European brown hare	Szegvár	2008
20	TUL26/08	European brown hare	Mindszent	2008
21	TUL28/08	European brown hare	Bucsa	2008
22	TUL30/08	European brown hare	Szeghalom	2008
23	TUL31/08	European brown hare	Püspökladány	2008
24	TUL32/08	European brown hare	Orosháza	2008
25	TUL33/08	European brown hare	Gerendás	2008
26	TUL34/08	European brown hare	Szeghalom	2008
27	TUL35/08	European brown hare	Orosháza	2008
28	TUL37/08	European brown hare	Csanádpalota	2008
29	TUL40/08	European brown hare	Bucsa	2008
30	TUL41/09	European brown hare	Alattyán	2009
31	TUL42/09	European brown hare	Füzesgyarmat	2009
32	TUL43/09	European brown hare	Füzesgyarmat	2009
33	TUL44/09	European brown hare	Surján	2009
34	TUL45/09	European brown hare	Gyomaendrőd	2009
35	TUL47/09	European brown hare	Jászjákóhalma	2009
36	TUL48/09	European brown hare	Gyomaendrőd	2009
37	TUL49/09	European brown hare	Törökszentmiklós	2009
38	AUS02/09	European brown hare	Zürndorf (Austria)	2009
39	TUL39/09	European brown hare	Zürndorf (Austria)	2009
40	TUL40/09	European brown hare	Kevermes	2009
41	TUL41/09	European brown hare	Ópusztaszer	2009
42	TUL42/09	European brown hare	Gyomaendrőd	2009

43	TUL43/09	European brown hare	Szarvas	2009
44	TUL44/09	European brown hare	Szajol	2009
45	TUL45/09	European brown hare	Szajol	2009
46	TUL46/09	European brown hare	Szajol	2009
47	TUL47/09	European brown hare	Okány	2009
48	TUL48/09	European brown hare	Okány	2009
49	TUL49/09	European brown hare	Kétegyháza	2009
50	TUL50/09	European brown hare	Püspökladány	2009
51	TUL51/09	European brown hare	Dévaványa	2009
52	TUL52/09	European brown hare	Kétegyháza	2009
53	TUL53/09	European brown hare	Püspökladány	2009
54	TUL54/09	European brown hare	Dévaványa	2009
55	TUL55/09	European brown hare	Békés	2009
56	TUL56/09	European brown hare	Békés	2009
57	TUL57/10	European brown hare	Báránd	2010
58	TUL58/10	European brown hare	Püspökladány	2010
59	TUL59/10	European brown hare	Battonya	2010
60	TUL60/10	European brown hare	Jászfelsőszentgyörgy	2010
61	TUL61/10	European brown hare	Jászfelsőszentgyörgy	2010
62	TUL62/10	European brown hare	Borota	2010
63	TUL63/10	European brown hare	Battonya	2010
64	TUL64/10	European brown hare	Szajol	2010
65	TUL65/10	European brown hare	Szajol	2010
66	TUL66/10	European brown hare	Báránd	2010
67	TUL67/10	European brown hare	Borota	2010

12. Acknowledgements

I would like to express my honest gratitude to my revered supervisor Professor **László Fodor** for his support and intensive efforts in supervising my scientific work. I am grateful to my co-supervisor **László Makrai** for his continuous help and encouragement throughout the years.

A special thank has to be granted to **Károly Erdélyi** for his generous help and advices in all parts of my research studies.

I am grateful to **Ádám Dán** for his help during the molecular biology examinations. It is my honor to acknowledge **Béla Dénes** for his contribution in the serology studies. I would like to thank **Levente Szeredi** for his help in the immunohistochemistry works. I am grateful to **Gábor Földvári**, **Krisztina Rigó** and **Gábor Majoros** for their contribution in the parasitology examinations. I would like to thank Professor **Paul S. Keim** and **Dawn N. Birdsell** for the opportunity to spend a three-months-long research externship at Northern Arizona University and their help to perform the genotyping studies with state of art techniquies. I am also grateful to **Steven M. Beckstrom-Sternberg** for his contribution with the whole genome sequencing and analysis.

It is my honor to ackowledge **Katalin Krisztalovics** for providing me the retrospective human tularemia data and **László Monse** the European brown hare data. I would like to thank for **Bálint Szépe** and **Miklós Füleki** for their enormous help in the collection of tularemia seropoitive hares. I am grateful to **István Szőke** for providing the tularemic zoo monkeys. I would like to thank for **Massimo Fabbi** for providing the Italian *F. tularensis* strains and his constructive remarks to various parts of my work. I would like to express my appreciation to **László Tirják** who has provided the physical means to carry out the ecological studies in the Körös-Maros National Park and **Tibor Lengyel** and the other rangers for their help during my field studies. I am grateful to **Zoltán Lévai**, **Miklós Lévai** and **János Pősze** and their collagues for helping me in hamster trapping and sampling. I would like to thank for **Zsófia Müller** and **Zsófia Ozsvár** for their contribution in human serum sample collection.

I would like to express my appreciation to **Katalin Jánosi** for sharing the winding road of PhD students with me and I am grateful to **Ágnes Ráczné Mészáros**, **Teréz Halasi**, **Éva Kolozsvári, Ágnes Juhász** and **Mária Ernőné Ottinger** for their outstanding laboratory assistance. I would like to thank **Edit Oláh** for her help in library search.

Last, but not least I would like to acknowledge **my family** for their endless sacrifices, love and support so that I could stand where I am now.

The financial support was granted by the Hungarian Scientific Research Fund, **OTKA-78139**.