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Comparative characterisation of members of the family Francisellaceae

Ph.D. thesis

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Abbreviations

16S rRNA gene	16S ribosomal ribonucleic acid gene
BHI	brain-heart infusion
bp	base pair
canSNP	canonical single nucleotide polymorphism
CFU	colony forming unit
CLSI	Clinical and Laboratory Standard Institute
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
fH	factor H
FLE	*Francisella*-like endosymbiont
HRPO	horseradish peroxidase
IHC	immunohistochemistry
ip	intraperitoneal
kDa	kilodalton
LD_{50}	lethal dose 50
LVS	live vaccine strain (NCTC 10857)
MAMA	mismatch amplification mutation assay
MIC	minimum inhibitory concentration
MLVA	multi-locus variable number of tandem repeats analysis
NMRI	Naval Medical Research Institute
pi	post infection
PCR	polymerase chain reaction
RD	region of genomic difference
RIPA	radioimmunoprecipitation assay
sdhA gene	putative succinate dehydrogenase gene
SMTTBS	skim milk in Tween-20 Tris-Buffered Saline
sp	species (singular)
spp	species (plural)
ssp	subspecies
Tm	melting temperature
tul4 gene	17 kDa lipoprotein precursor gene
VNTR	variable number of tandem repeats
WG	whole genome

1. Summary

The family Francisellaceae is rapidly expanding with several new members described in the last few decades. *Francisella tularensis* is a facultative intracellular, zoonotic bacterium, the causative agent of tularaemia and a potential biological weapon. The moderately pathogenic *F. tularensis* ssp. *holarctica* is endemic in Europe. Phylogenetic analyses revealed that two major genetic clades (B.FTNF002-00 and B.12) of the bacterium are dominant in the continent, which occur in distinct geographic regions. The B.12 genotype of *F. tularensis* ssp. *holarctica* is endemic in Hungary. Tularaemia was first diagnosed in humans in 1951 in the country and in the past 20 years 20-148 cases were reported each year. In Hungary besides the potential threat to public health tularaemia is also important economically. As many as 40,000 brown hares are exported from Hungary each year, which should be free of tularaemia.

In the past few years several new variants of *Francisella*-like endosymbionts (FLEs) were described in ticks. Description of new variants is generally based on the analysis of the sequences of specific genes. A collection of 5806 ticks of 16 species from Hungary and Ethiopia was examined for the presence of members of the family Francisellaceae. *F. tularensis* ssp. *holarctica* was detected in *Haemaphysalis concinna* and *Dermacentor reticulatus* collected in Hungary. FLEs were detected in Hungary in questing *D. reticulatus* ticks and a new variant in a new host species, *Ixodes ricinus*. In Ethiopia a FLE was described in *Hyalomma rufipes*. Phylogenetic analysis revealed close relatedness among endosymbionts from Europe and Africa. The identical sequences of FLE variants harboured by *D. reticulatus* detected in distinct countries in Europe assume host adaptation and a host species–linked evolution of this FLE species.

Phylogenetic analyses of the live vaccine strain (LVS) and 69 *F. tularensis* ssp. *holarctica* strains isolated in Hungary were performed by canonical single nucleotide polymorphism (canSNP) typing and multi-locus variable number of tandem repeats analysis (MLVA). The whole genome (WG) sequencing of nine selected isolates was also carried out. The results revealed relatively high genetic diversity of the Hungarian strains. Long-term survival of the strains was detected in the environment, during which the strains showed no genetic mutations. Epidemiologic analysis of the genotypes in the country reflects the probability of emergence of multiple clones in outbreaks triggered by environmental factors.

In the background of the different susceptibility to tularaemia in animal species the interactions between bacterial membrane proteins and the elements of the host's complement system may play a significant role. Complement sensitivity of different genotypes of wild *F. tularensis* ssp. *holarctica* strains and the attenuated LVS was compared using sera of selected animal species with different susceptibility to the infection. Regardless to their genotypes, all

wild strains survived in the sera of the highly susceptible house mouse (*Mus musculus*), moderately susceptible European brown hare (*Lepus europaeus*) and in the relatively resistant cattle (*Bos taurus*). In contrast, the attenuated LVS cells were lysed in hare serum and killed in cattle serum as well. *F. tularensis* can evade the complement system in humans by binding factor H (fH), a regulator protein of the complement system. Western blot and pull-down assays of wild and attenuated strains of *F. tularensis* ssp. *holarctica* strains showed no specific interactions with fH in the selected animal sera, supposedly for the lack of an intermediate component or because of interspecies differences.

The two genotypes of *F. tularensis* ssp. *holarctica* strains dominant in Europe differ in their geographical distribution as well. For the comparison of the virulence of the two genotypes experimental infection of Fischer 344 rats was performed. The results revealed moderate difference in the pathogenic potential of the two genotypes and suggest that the Western European genotype is more virulent than the Eastern European genotype.

F. tularensis can induce six clinical forms of infection in humans. In the treatment of tularaemia cases aminoglycosides, quinolones and tetracyclines are the drugs of choices. The *in vitro* examinations of antibiotic susceptibility of 29 *F. tularensis* ssp. *holarctica* strains originating from Hungary to 11 antibiotics were carried out. The examinations revealed high effectiveness of antibiotics recommended in clinical use against tularaemia, especially of levofloxacin, ciprofloxacin and doxycycline. The results also showed effectiveness of tigecycline against the pathogen promoting this antibiotic for the therapy of the infection. Application of linezolid or erythromycin is not recommended against this agent in Hungary because of the *in vitro* resistance to these antibiotics detected in the strains.

Összefoglalás

A Francisellaceae családba tartozó baktériumok köre gyors ütemben bővült az utóbbi évtizedekben. A *Francisella tularensis* egy fakultatív intracelluláris, zoonótikus baktérium, a tularaemia kórokozója és potenciális biológiai fegyver. Európában a mérsékelt megbetegítő képességgel rendelkező *F. tularensis* ssp. *holarctica* alfaj endémiás. Filogenetikai vizsgálatok alapján két fő genotípus jelenlétét állapították meg Európában (a B.FTNF002-00 és a B.12), melyek földrajzi elterjedtségükben jól elkülönülnek. Hazánkban a *F. tularensis* ssp. *holarctica* B.12-es genotípusa endémiás. Magyarországon 1951-ben diagnosztizálták az első tularaemiás emberi megbetegést, és az utóbbi 20 évben 20-148 esetet jelentenek minden évben. Országunkban a közegészségügyi jelentősége mellett a tularaemiának gazdasági szempontból is fontos szerepe van. Magyarországról évente 40.000 élő mezei nyulat exportálnak, melyeknek mentesnek kell lenniük többek között tularaemiától is.

Az utóbbi években számos új változatát írták le *Francisella*-szerű endoszimbiontáknak kullancsokban. Az új endoszimbionta változatok meghatározása általában specifikus gének szekvenciaelemzésén alapul. A Francisellaceae családba tartozó baktériumok jelenlétét vizsgáltuk 16 kullancsfaj összesen 5806 egyedében, melyek Magyarországról és Etiópiából származtak. *F. tularensis* ssp. *holarctica* baktériumot hazánkban gyűjtött *Haemaphysalis concinna* és *Dermacentor reticulatus* kullancsokból mutattunk ki. Endoszimbiontákat Magyarországon kimutattunk a környezetből gyűjtött *D. reticulatus* kullancsokban és egy új változatot egy új kullancsgazdában, az *Ixodes ricinus*-ban. Etiópiából származó kullancsok közül *Hyalomma rufipes*-ben írtunk le endoszimbiontát. Az endoszimbionták filogenetikai vizsgálata alapján közeli rokonságot állapítottunk meg az európai és afrikai változatok között. Az Európában *D. reticulatus*-ban leírt endoszimbionták azonos szekvenciája alapján az endoszimbionta kullancsgazdájához való adaptációját és azzal közös törzsfejlődését feltételezzük.

A gyengített vakcina törzs (live vaccine strain, LVS) és 69 hazai *F. tularensis* ssp. *holarctica* törzs genetikai vizsgálatát végeztük el a genotípusokra specifikus pontmutációk meghatározására alkalmas canSNP (canonical single nucleotide polymorphism) analízis és a tandem ismétlődő szakaszok vizsgálatán alapuló MLVA (multi-locus variable number of tandem repeats analysis) módszer segítségével. Kilenc válogatott törzs esetében teljes genom szekvenálást is végeztünk. Az eredmények alapján viszonylag nagy genetikai változatosságot találtunk a magyar törzsek között. Megállapítottuk, hogy a baktérium képes a természetben mutálódás nélkül hosszú ideig fennmaradni. Járványtani elemzéseink azt mutatják, hogy valamely környezeti hatásra ezek a természetben jelenlévő genotípusok együttesen vehetnek részt az újabb járványok kitörésében.

Az egyes állatfajok tularaemiával szembeni fogékonyságának hátterében a gazda komplement rendszere és a baktérium felületi fehérjéi közti kölcsönhatásoknak jelentős szerepe lehet. Különböző genotípusú és virulenciájú *F. tularensis* ssp. *holarctica* törzsek komplement érzékenységét vizsgáltuk a tularaemiára eltérő mértékben fogékony állatfajokban. Genotípustól függetlenül az összes vad, virulens törzs képes volt túlélni a tularaemiára rendkívül fogékony egér (*Mus musculus*), mérsékelten fogékony mezei nyúl (*Lepus europaeus*) és rezisztens szarvasmarha (*Bos taurus*) vérében. Ezzel szemben a gyengített LVS törzs sejtjei szétestek, illetve elpusztultak a mezei nyúl és a szarvasmarha komplement rendszerének hatására. Emberben leírták, hogy a *F. tularensis* képes a komplement szabályozó H-faktor megkötésével kijátszani a komplement rendszer baktériumölő hatását. A vizsgált állatfajokban a *F. tularensis* vad, virulens és gyengített törzsei nem mutattak direkt, specifikus kötődést a H-faktorhoz Western blot és pull-down eljárások során. A kötődéshez vélhetőleg egy köztes komponens szükséges, illetve a kötődés hiányát a fajok közti eltérések is magyarázhatják.

Az Európában jelenlévő két fő *F. tularensis* ssp. *holarctica* genotípus földrajzi elterjedésében különbözik egymástól. A kísérletben Fischer 344 patkányokat mesterségesen fertőztünk a genotípusok virulenciájának összehasonlítására. Az eredmények mérsékelt különbséget mutattak a genotípusok között, és a nyugat-európai genotípus virulensebbnek bizonyult a kelet-európai genotípusnál.

A *F. tularensis* hatféle kórformát képes előidézni emberekben. A tularaemia kezelésére elsősorban aminoglikozidokat, fluorokinolonokat és tetraciklineket javasolnak. A vizsgálatok során 29 hazai *F. tularensis* ssp. *holarctica* törzs antibiotikum érzékenységét határoztuk meg *in vitro* 11 antibiotikummal szemben. A terápiában használatban lévő antibiotikumok megfelelő hatékonyságot mutattak a baktériummal szemben, különösen a levofloxacin, ciprofloxacin és a doxicklin. A tigeciklin is hatékonyan gátolta a baktérium növekedését a vizsgálatok során, ami alapján a későbbiekben ez az antibiotikum is hasznos lehet a betegség kezelésére. A hazai törzsek rezisztenciát mutattak eritromicinnel és linezoliddal szemben, ezért ezeknek a szereknek az alkalmazása nem javasolt a tularaemia kezelésére a térségben.

2. Introduction

2.1. History and taxonomy

In 1911 a plague-like disease was described in ground squirrels in Tulare County, California by McCoy (1911). He and his co-worker managed to isolate the causative agent of the infection a year later and named it *Bacterium tularense* (McCoy and Chapin, 1912). In the following years Dr. Edward Francis (1872-1957) had prominent role in the research of this disease, which he named tularaemia. Dr. Francis discovered that humans get infected by the bites of blood-sucking arthropods and by handling or dissecting rabbits and rodents and he characterized the symptoms of tularaemia in humans (Francis, 1921, Francis *et al*., 1922). He summarized the knowledge on the ecology and clinical signs of tularaemia and determined that similar syndromes from North America, Europe and Japan were all caused by this same disease (Francis, 1928). In honour of Edward Francis Dorofeev (1947) proposed to name the pathogen *Francisella tularensis*.

In the early 60's Olsufyev and co-workers described two variants of the pathogen, the Old World and the New World variants, which differed in their virulence besides their geographical distribution (Olsufyev *et al*., 1959, 1963). Jellison and co-workers refined the classification of *F. tularensis* and termed Type A variant the bacterium population occurring only in North America and Type B variant the subpopulation prevalent in North America and Eurasia as well (Jellison *et al*., 1961).

The pathogen *F. tularensis*, originally *Bacterium tularense*, used to belong to the genus *Pasteurella* and was proposed to be included in the genus *Brucella* as well (Philip and Owen, 1961). Currently, about 100 years after its first isolation, *F. tularensis* is divided into four subspecies (ssp. *tularensis*, *holarctica*, *mediasiatica* and ssp*. novicida*), belongs to the family Francisellaceae with five other *Francisella* species (*F. philomiragia*, *noatunensis* or *piscicida*, *halioticida*, *hispaniensis*, *guangzhouensis*) and several *Francisella* variants originating from humans, ticks and small mammals (*Francisella*-like endosymbionts, FLE) and the environment (de Carvalho *et al*., 2015, DSMZ, 2015, Keim *et al*., 2007, Kugeler *et al*., 2008, Ottem *et al*., 2009, Sjöstedt, 2005). Subpopulations of *F. tularensis* ssp. *tularensis* (Type A.I and A.II) differing in their geographic and genetic characteristics, virulence and host preferences were described in North America, while *F. tularensis* ssp. *holarctica* was suggested to be classified into three biovars (erythromycin sensitive bv. I, erythromycin resistant bv. II and bv. *japonica*) (Olsufyev and Meshcheryakova, 1983, Staples *et al*., 2006).

In Hungary *F. tularensis* ssp. *holarctica* is endemic. Tularaemia was first diagnosed in humans in 1951 in the country and in the past 20 years 20-148 cases were reported each year (Epinfo). In Hungary besides the potential threat to public health tularaemia is also important economically. As many as 40,000 brown hares are exported from Hungary each year, which should be free of tularaemia (Somogyi, 2006).

2.2. Characteristics and ecology of Francisellaceae

Francisella species are fastidious, obligate aerobe, facultative intracellular, small (0.7-1.5 µm), pleomorphic, non-motile, Gram-negative bacteria. Cysteine is essential for most *Francisella* species and it enhances the growth of all species on blood or chocolate agar. *Francisella* species have worldwide distribution; have broad host spectrum and generally long-term survival in the environment probably in association with protozoans (Ellis *et al*., 2002, Friend, 2006, Keim *et al*., 2007). Although genetically *Francisella* is a highly clonal bacterium without any evidence of horizontal gene transfer, the host preference, geographic distribution and virulence of the species and subspecies differ in a wide range within this genus (Keim *et al*., 2007) (Table 1).

Virulence of the strains is categorized based on the number of colony forming units (CFU) in the lethal dose 50 (LD_{50}) of mice, guinea pigs and rabbits. The two main, human pathogen representatives of the genus are the highly virulent (LD_{50} is as low as 10 CFU) *F. tularensis* ssp. *tularensis* and the moderately infectious (LD_{50} in rabbits $>10^6$ CFU) ssp. *holarctica*. These subspecies have two life-cycles, a terrestrial and an aquatic cycle, and they can infect a wide variety of hosts from different taxonomic classes and orders (Fig. 1.).

More than 300 animal species, including mammals, birds, amphibians, reptiles and invertebrates are susceptible to *F. tularensis* and the bacterium can infect a multitude of cell types, especially macrophages, but fibroblasts, epithelial cells, hepatocytes, muscle cells and neutrophils can be affected as well (Cowley and Elkins, 2011, Keim *et al*., 2007). Lagomorphs (*Sylvilagus*, *Lepus* and *Oryctolagus* spp.) and rodents (*Sciuridae*, *Castoridae*, *Hystricidae*, *Myocastoridae*, *Gliridae*, *Spalacidae*, *Cricetidae* and *Muridae* spp.) are considered to be the main reservoirs and amplification hosts for *F. tularensis* and important sources of human infections. Blood-sucking arthropods (ticks, mites, tabanid flies, mosquitos) have important role in the transmission of the pathogens and may serve as reservoirs for *F. tularensis* as well, although only transstadial transmission of the bacteria was proven in ticks and mosquitos (Bäckman *et al*., 2015, Keim *et al*., 2007, Maurin and Gyuranecz, 2016, Mörner and Addison, 2001, Thelaus *et al*., 2014, Vyrosteková, 1994).

Table 1. Selected characteristics of Francisellaceae

species	distribution	host preference	human pathogen	virulence	cultivation
***F. tularensis* ssp. *tularensis* type A.I**	central and eastern parts of USA, sporadically western USA	broad host spectrum	+	high	cysteine, 37°C
***F. tularensis* ssp. *tularensis* type A.II**	western USA	broad host spectrum	+	mild	cysteine, 37°C
F. tularensis* ssp. *holarctica	Northern Hemisphere	broad host spectrum	+	moderate	cysteine, 37°C
F. tularensis* ssp. *mediasiatica	Central Asia (Kazakhstan)	Lagomorphs, Rodents	-	moderate	cysteine, 37°C
F. tularensis* ssp. *novicida	global	environment	+	low	37°C
F. philomiragia	global	fish	+	low	37°C
F. halioticida	Japan	fish	-	n.d.	sea water and cysteine, 20°C
***F. noatunensis* (= *F. piscicida*)**	global	fish, shellfish, molluscs	-	n.d.	cysteine, 25°C
F. hispaniensis	Spain	humans	+	n.d.*	37°C
F. guangzhouensis	China	environment	-	n.d.	37°C
***Francisella*-like endosymbionts (FLE)**	global	soft ticks (Argasidae), hard ticks (Ixodidae)	-	n.d.	no growth on cell-free media; egg yolk sac, tick cell culture

n.d.: no data

* The type strain was isolated from severe septicaemia secondary to acute obstructive pyelonephritis.

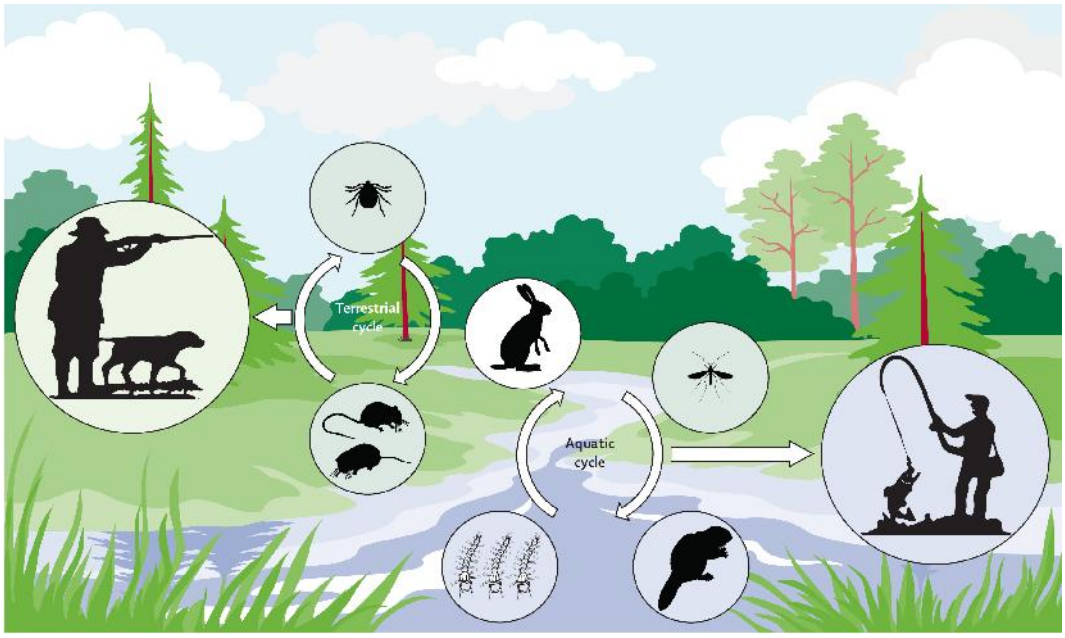

Figure 1. The two main lifecycles of *F. tularensis* in Europe.
The terrestrial cycle involves ticks, mammals and humans (especially hunters, veterinarians, small animal trappers and skinners). The aquatic cycle involves mosquitos (larvae and adults), hares, beavers and muskrats and humans (fishermen, hikers or by drinking from contaminated water sources) (Maurin and Gyuranecz, 2016)

In the past decades, the Francisellaceae family was expanding rapidly. Besides the recently described human pathogen *Francisella* species, many fish pathogens and free-living or symbiont agents from environmental matrices have been reported (Barns *et al*., 2005, Birkbeck *et al*., 2007, Escudero *et al*., 2010, Kamaishi *et al*., 2005, Kugeler *et al*., 2008, Mauel *et al*., 2007, Niebylski *et al*., 1997; Nylund *et al*., 2006, Olsen *et al*., 2006, Ostland *et al*., 2006, Ottem *et al*., 2009, Qu *et al*., 2013). FLEs are small (0.6-3.4 µm), pleomorphic microorganisms without cell wall, and they are harboured both by soft ticks (*Argasidae*) and hard ticks (*Ixodidae*), similarly to *F. tularensis* (Burgdorfer *et al*., 1973, Noda *et al*., 1997). In contrast to *F. tularensis*, FLEs are transmitted transstadially and transovarially in ticks, do not grow on artificial media and information about their virulence is scarce (Barns *et al*., 2005, Noda *et al*., 1997). The first FLE was identified in 1961 in Egypt from the soft tick *Argas arboreus* (previously known as *A. persicus*), and named *Wolbachia persica* according to its phenotypic characteristics (Suitor and Weiss, 1961). In 1973 an endosymbiont from the hard tick *Dermacentor andersoni* was isolated on chicken egg yolk sac, and its pathogenicity against guinea pigs and golden hamsters was described in artificial infection experiments (Burgdorfer *et al*., 1973). Later genetic analyses classified both *W. persica* and *D. andersoni* symbionts into the *Francisella* genus and recent whole genome sequencing of *W. persica* further confirmed this classification (Forsman *et al*., 1994, Niebylski *et al*., 1997, Sjödin *et al*., 2012). It is of question whether these endosymbionts and the virulent *Francisella* species had common ancestor in ticks, which divided into the host specialist symbionts and generalist pathogens. Given the close genetic relatedness among FLEs of soft and hard ticks, it is also hypothesized that FLEs used to spread by an infectious route (e.g. feeding on infected host or co-feeding) and adapted to symbiotic lifestyle secondarily (Noda *et al*., 1997, Scoles, 2004).

2.3. Phylogeography of *Francisella tularensis*

Deeper phylogeographic analyses provide insight into the evolutionary history of *F. tularensis*, especially in the case of the two most concerned subspecies: *tularensis* and *holarctica*. A variety of molecular methods have been developed for the genetic analysis of this highly clonal bacterium, including multi-locus variable number of tandem repeats analysis (MLVA), multi-locus sequence typing, analysis of canonical insertion-deletion markers, canonical single nucleotide polymorphism (canSNP) based typing and whole genome (WG) sequencing (Keim *et al*., 2007, Larsson *et al*., 2007). WG sequencing provides data about all (from the family to the isolate) taxonomic levels. WG SNP analysis is an effective method for the description of the accurate population structure of highly clonal bacteria (Pearson *et al*., 2004, Van Ert *et al*., 2007). Based on this population structure canSNPs can be selected which define the branches specific for species, major lineages or even for individual strains,

thus offering an appropriate method for high resolution genotyping by average laboratory equipment (Vogler *et al*., 2009a). MLVA possesses the highest discriminatory power among closely related isolates (e.g. originating from the same outbreak) (Keim *et al*., 2007).

Autochthon infections by *F. tularensis* ssp. *tularensis* (also known as type A) have been reported solely from North America. The subspecies has been divided into two subpopulations, A.I and A.II according to genetic, pathogenic and geographic characteristics. The highly virulent type A.I subpopulation is prevalent mostly in the central and eastern regions of the U.S.A., with sporadic appearance in western parts as well (Ellis *et al*., 2002). Further three main subtypes (A.I3, A.I8 and A.I12) were distinguished within A.I group based on WG phylogeny, and difference in virulence was also described among these subtypes (Birdsell *et al*., 2014, Molins *et al*., 2010). Subpopulation A.II has milder virulence than the moderately virulent ssp. *holarctica*, and its geographic distribution is restricted to the western parts of the U.S.A., especially the Rocky Mountain region. Distribution of the subpopulations is correlated with vectors and hosts, as prevalence of A.I strains matches with *D. variabilis* and *Amblyomma* (*Am*.) *americanum* ticks and the eastern cottontail rabbit (*Sylvilagus floridans*), while A.II group distribution is associated with *D. andersoni* ticks, *Chrysops discalis* tabanid flies and the mountain cottontail rabbit (*S. nuttallii*). The cause of the detected genetic distance between the two subpopulations is dubious. Separate glacial refugia of the groups during the last ice age may represent one explanation. On the other hand, geographic distribution and vector and host preference support the hypothesis that the subpopulations have distinct ecological niches (Keim *et al*., 2007).

Despite of the fact that *F. tularensis* ssp. *holarctica* is widespread throughout the Northern Hemisphere, the genetic diversity of the strains is low. The homogeny of the strains' genetic characteristics within this subspecies assumes its recent geographic expanding, deriving from a common ancestor (Johansson *et al*., 2004). Those regions where basal clades and higher diversity of the strains are prevalent are assumed to be the sources of emergence of the main *F. tularensis* ssp. *holarctica* branch (Özsürekci *et al*., 2015, Svensson *et al*., 2009a, Vogler *et al*. 2009a, Wang *et al*., 2014). However, retrograde genetic examinations revealed homology between strains isolated from the same region nowadays and decades before, a finding which leads to the hypothesis that *F. tularensis* ssp. *holarctica* has long periods of dormancy in the environment with low replication rate (Johansson *et al*., 2014, Karlsson *et al*., 2013, Petersen *et al*., 2008, Svensson *et al*., 2009b). Four main clades of *F. tularensis* ssp. *holarctica* have been identified by canSNP typing: the B.16 (biovar japonica), B.4 (which was also called clade OSU18 after a strain isolated from a dead beaver in Oklahoma in 1978), B.6 and B.12 clades (Fig. 2.).

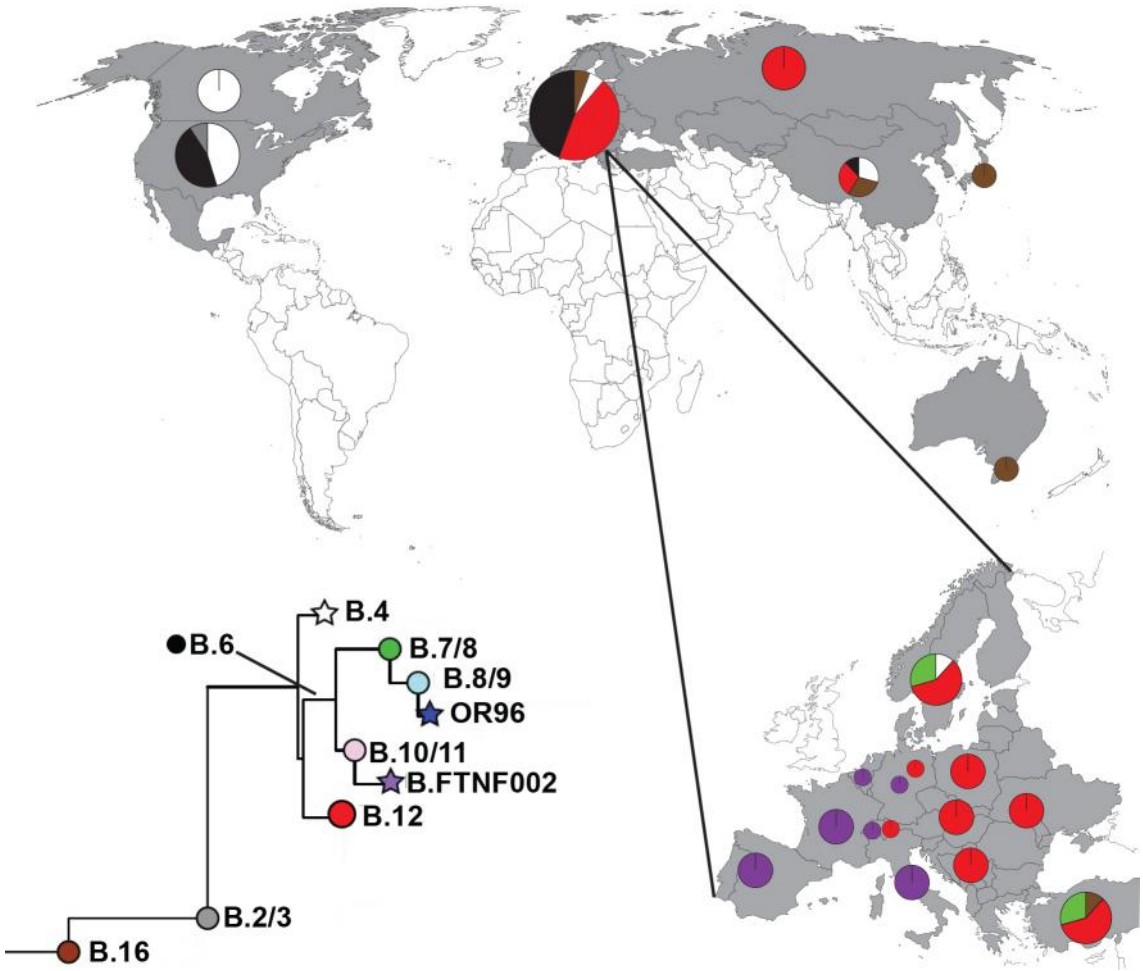

Figure 2. Geographic distribution of the main *F. tularensis* ssp. *holarctica* genotypes. Genotypes dominant in Europe are further detailed. Grey coloured regions represent occurrence of *F. tularensis*. Colour codes of diagrams are consistent with colours on the dendrogram. (Dendrogram adapted from Vogler *et al*. 2009a)

In North America two main clades (B.4 and B.6) and a unique basal clade (B.2/3) of the ssp. *holarctica* are present. Clade B.4 is widespread throughout North America. Strains belonging to the basal clade B.2/3 have been isolated exclusively from California, and based on phylogenetic analyses this clade had diverged from the main *F. tularensis* ssp. *holarctica* branch before the divergence of most European clades (Vogler *et al*., 2009a).

The first detected *F. tularensis* ssp. *holarctica* in the southern hemisphere, a strain from Tasmania had close relatedness to biovar japonica (B.16) strains based on its sequence of the region of genomic difference 1 (RD1) (Jackson *et al*., 2012).

Clades B.16 (biovar japonica), B.4 (OSU18), B.6 and B.12 were all isolated in China, indicating a relatively high diversity of subspecies *holarctica* in this region (Wang *et al*., 2014).

The three main clades B.4, B.6 and B.12 are prevalent in Eurasia, B.12 being the most widespread in the continent (Chanturia *et al*., 2011, Gyuranecz *et al*., 2012a, Vogler *et al*., 2009a) (Fig. 2.). Furthermore, in Turkey a strain belonging to biovar japonica (clade B.16) was

described based on its capability of glycerol fermentation, susceptibility to erythromycin and its genetic region RD1 sequence (Kilic *et al*., 2013). Recent phylogenetic examinations in Turkey revealed the presence of subclades of the main groups B.12 and B.6 (subclade B.7/8). The subclade B.7/8 has been previously described only in Scandinavia (Özsürekci *et al*., 2015). Information about the phylogeny of *holarctica* strains in Russia is scarce; subclades of B.12 have been described so far in this region (Svensson *et al*., 2009a, Vogler *et al*., 2009a). Detailed phylogeographic analyses were conducted in Georgia in 2011 which revealed the presence of clade B.12 in the country, with relatively high diversity of strains on the level of subclades (Chanturia *et al*., 2011).

Strains belonging to the main clades B.4, B.6 and B.12 were described in Scandinavia, representing the highest genetic diversity of subspecies *holarctica* in Europe (Svensson *et al*., 2009a, Vogler *et al*., 2009a). In the continental regions of Europe the two main clades B.12 and B.6 are separated geographically also. In Western European countries (France, Germany, Italy, the Netherlands, Spain and Switzerland) the B.FTNF002-00 subclade of B.6 clade is dominant, while the B.12 clade is most common in Central and Eastern Europe (Austria, Czech Republic, Germany, Hungary, Romania, Slovakia, Switzerland and Ukraine) (Antwerpen *et al*., 2013, Ariza-Miguel *et al*., 2014, Gyuranecz *et al*., 2012a, Maraha *et al*., 2013, Origgi *et al*., 2014, Vogler *et al*., 2009a). WG sequencing based comparison of a B.FTNF002-00 strain and other *holarctica* strains (live vaccine strain /LVS/ from B.12 group, OSU18 of B.4 group) revealed such genetic differences which might correlate with the enhanced pathogenicity and fitness of strain B.FTNF002-00. The described genetic differences included the smaller overall genome size, amino acid changes in virulence associated protein genes and polymorphisms in genes coding essential cellular functions or which are associated with virulence (Barabote *et al*., 2009).

The subspecies *mediasiatica* has been rarely isolated and only in the Central Asian area, but the isolates showed great genetic diversity, similarly to the globally occurring *F. tularensis* ssp. *novicida* (Vogler *et al*., 2009a).

For the lack of WG sequences of FLEs their genetic analyses are based on various genes. FLEs were reported from several continents (America, Europe and Africa) representing global distribution of these microorganisms (Brevik *et al*., 2011, Ivanov *et al*., 2011, Michelet *et al*., 2013, Scoles, 2004). Comparison of the phylogeny of FLEs and their tick hosts revealed no evidence of co-specification (Scoles, 2004).

2.4. Pathogenesis and host responses to *Francisella tularensis*

F. tularensis is a successful pathogen with broad host range, having the ability to infect and replicate in various mammalian and protozoan cell types and also adapted to the extracellular environment for its transmissive phase (Abd *et al*., 2003, Forestal *et al*., 2007, Keim *et al*., 2007, Thelaus *et al*., 2009, Yu *et al*., 2007). The main routes of infection in humans are through the bites of blood-sucking arthropods, skin lesions and consumption of contaminated water or food, and less frequently by inhalation or via the conjunctiva (Ellis *et al*., 2002). In the host the bacteria first replicate in macrophages without triggering exacerbated immune responses (3 to 5 days in humans) (Sjöstedt, 2007). Later ulceration and necrosis at the site of infection occur with invasion of blood and lymph vessels and spreading of the bacteria to the lymph nodes and other organs (Mörner and Addison, 2001). Thus *F. tularensis* is able to adapt many distinct environments and possesses a multitude of mechanisms for evasion, modulation and suppression of the immune system in both extracellular and intracellular compartments (Bosio, 2011).

After transmission of *F. tularensis* to the host, the bacterium is exposed to a variety of anti-microbial factors such as the complement system, antibodies, cationic antimicrobial peptides and phagocytes (Ben Nasr *et al*., 2006, Ben Nasr and Klimpel, 2008, Clay *et al*., 2008, Zarrella *et al*., 2011). The bacterium is able to evade the binding of these factors and to block their subsequent killing effect by using distinct surface structures (e.g. lipopolysaccharide O antigen and capsule) and outer membrane modifications (e.g. capability of changing the surface charge) (Jones *et al*., 2012). During evasion of extracellular defence mechanisms the bacteria prevent the release of pro-inflammatory signals and enhance opsonisation and phagocytosis by host cells (Jones *et al*., 2012).

The complement system is part of the innate immune system, and it is activated by three pathways (classical, mannan-binding lectin and alternative pathways). All pathways lead to a cascade of signalling proteins resulting in lysis or opsonophagocytosis of the pathogen and the triggering of inflammatory responses. The three activation routes join in one key step, where the complement factor C3 is degraded by C3 convertase to its C3b and C3a fragments, initiating the formation of the membrane attack complex and inflammatory activities, respectively (Janeway *et al*., 2001). The glycoprotein factor H (fH) is a member of the regulators of complement activity, expressed by a variety of cell types. Factor H controls C3 convertase and serves as co-factor for factor I in the cleavage and inactivation of C3b (Ferreira *et al*., 2010, Pangburn *et al*., 2008).

As part of the subversion of the host's immunity many pathogens (e.g. *Borrelia hermsii*, *Neisseria meningitidis*, group A *streptococci*, *Yersinia enterocolitica*, *Candida albicans*) developed the ability to bind fH (Biedzka-Sarek *et al*., 2008, Meri *et al*., 2013). Interactions between *F. tularensis* and fH from human serum have also been described (Ben Nasr and Klimpel, 2008).

The binding of the host's plasmin and plasminogen to increase bacterial virulence was described before in the case of *Francisella* and other pathogens (Bosio, 2011, Clinton *et al*., 2010, Lahteenmaki *et al*., 2001). Plasminogen is converted to plasmin that can bind fibrinogen, which was hypothesized to bind fH on the surface of *Francisella* (Jones *et al*., 2012) (Fig. 3.). As a serine protease, plasmin bound to the cell surface can directly cleave C3 and induce proinflammatory response (Amara *et al*., 2010). On the other hand, plasmin can also degrade the opsonising antibodies, preventing antibody-mediated complement activation (Crane *et al*., 2009).

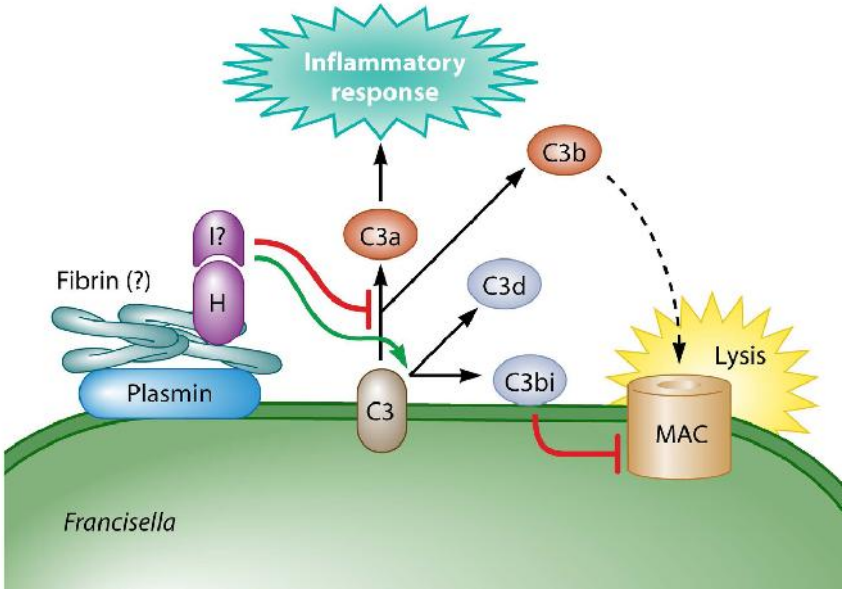

Figure 3. Complement evasion by *Francisella*.
Factor H (H) binding on the surface of *Francisella* by an intermediate component (e.g. plasmin or fibrin) inhibits directly or as a co-factor of factor I the degradation of C3 to its C3a and C3b fragments, thus inhibiting inflammatory activities and cytolysis by the membrane attack complex (MAC). C3bi and C3d fragments generated by the cleavage of C3 are inhibiting MAC formation and promoting opsonophagocytosis (Jones *et al*., 2012).

During host-adaptation *F. tularensis* increases the production of several cell surface structures including an O-antigen capsule, lipopolysaccharide O-antigen and other high molecular weight carbohydrates (Zarrella *et al*., 2011). The capsule may limit the access of antibodies to *Francisella* antigens, while lipopolysaccharide O-antigen may regulate the binding of complement factors, and also subvert the production of pro-inflammatory cytokines by bound components (Gunn and Ernst, 2007, Jones *et al*., 2012).

Besides the complement system, other cationic antimicrobial peptides are present in the extracellular compartment, which are able to disrupt the bacterial membrane due to the difference in the surface charge (Cederlund *et al*., 2011). While the capsule and lipopolysaccharide O-antigen is presumed to contribute to the evasion of these peptides, capability of *Francisella* to alter the charge of its surface and to use certain efflux systems to resist the cationic antimicrobials has already been described (Jones *et al*., 2012).

As an intracellular bacterium, following survival of the host's extracellular defence system *Francisella* has to contact with and enter the host cells (Fig. 4.). Host cells possess certain pathogen recognition receptors (e.g. scavenger receptors, mannose receptors, C-type lectins and toll-like receptors) by which they are able to detect conserved pathogen-associated molecular patterns (Janeway and Medzhitov, 2002). Attachment with these receptors triggers phagocytosis and inflammatory signalling contributing to the activation of the innate and adaptive immune cells (Kawai and Akira, 2010). With its modified cell surface structures (e.g. lipopolysaccharide and Tul4 lipoprotein) *Francisella* is capable to evade or suppress toll-like receptors, which are present both on the surface and in the phagosome of the host cells (Bosio, 2011, Jones *et al*., 2012). Also, upon phagocytosis the bacterium attaches to host receptors which do not release pro-inflammatory cytokines (Bosio, 2011).

Opsonized or unopsonized *Francisella* is entering the host cells (preferably macrophages) via pseudopod loops, which are asymmetrical protrusions of the cell wall (Clemens *et al*., 2005). After phagocytosis *Francisella* stays within the phagosome called *Francisella*-containing phagosome, which produces a variety of toxic antibacterials for the disruption of bacterium cells. *Francisella* has a myriad of defence mechanisms (e.g. blockage of NADPH oxidase, production of enzymes for the neutralization of oxidative burst) to prevent killing in the *Francisella*-containing phagosome and release of inflammatory signals by the host cells (Bosio, 2011, McCaffrey *et al*., 2010). The *Francisella*-containing phagosome is maturing in the cytosol by interactions with early and late endosomal markers but it never reaches the phagolysosomal stage (Chong and Celli, 2010). Instead, the bacteria are able to escape from the phagosome to reach cytosol where they can replicate (except in amoebae, where *Francisella* resides and replicates in vesicles) (Jones *et al*., 2012, Abd *et al*., 2003). In the cytosol *Francisella* is able to replicate without activating an effective immune response, and it can also acquire sufficient nutrients from the host cell for its growth (Jones *et al*., 2012).

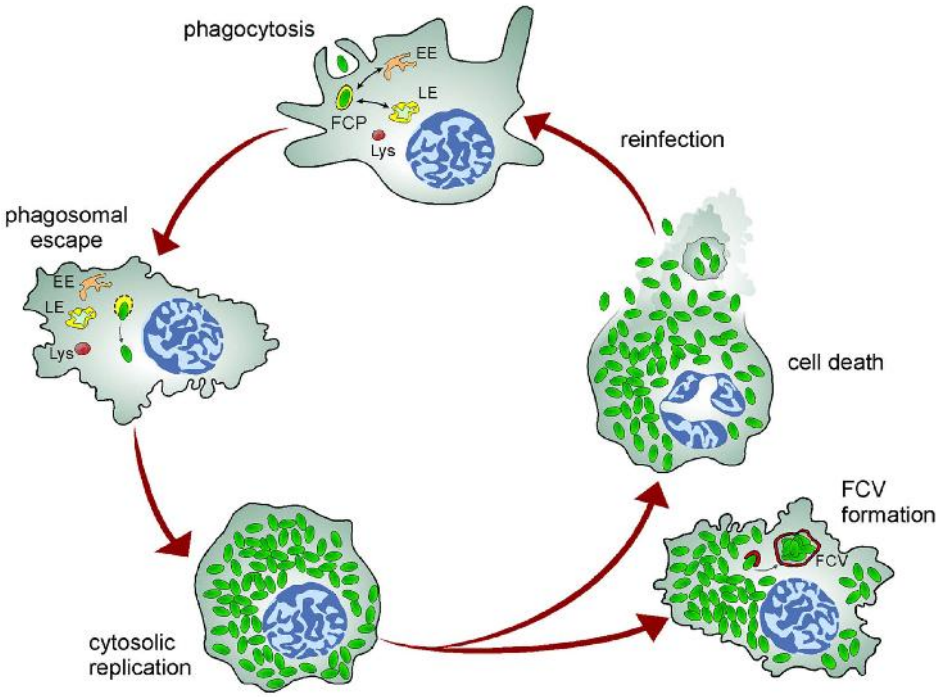

Figure 4. Intracellular phase of *Francisella* in macrophages.
After phagocytosis the *Francisella*-containing phagosome (FCP) is interacting with early (EE) and late (LE) endocytic compartments, but not with lysosomes (Lys). *Francisella* extensively replicates in the cytosol after disruption of the membrane of FCP, which is followed by cell death and the release of the bacteria. In certain cases cytosolic *Francisella* are encapsulated in *Francisella*-containing vacuoles (FCV) via autophagy (Chong and Celli, 2010).

Furthermore, while escaping from the host cell the pathogen can also modulate the expression of genes (e.g. induction of major histocompatibility complex II degradation and production of anti-inflammatory cytokines by antigen-presenting cells) to suppress adaptive immunity also (Chong *et al*., 2008, Jones *et al*., 2012, Wehrly *et al*., 2009, Zarrella *et al*., 2011).

Overall, *Francisella* is able to adapt to a multitude of extracellular and intracellular compartments, thus the bacteria efficiently subvert, modulate and evade the immunity of different hosts (Bosio, 2011, Jones *et al*., 2012, Zarrella *et al*., 2011).

2.5. Clinical signs and pathology of tularaemia

Clinical signs of tularaemia in humans depend on the route of infection and manifest in six main forms: glandular, ulceroglandular, oropharyngeal, oculoglandular, pneumonic and typhoid or tularaemia septicaemia (Sjöstedt, 2007). The most common forms are the glandular and ulceroglandular diseases as results of arthropod bites or through wounds while handling infected animals. After an incubation period of usually 3-5 days flu-like symptoms occur (chills, fever, headache and generalized aches), with the enlargement of regional lymph nodes. An ulcer can form at the site of infection which may persist for several months (Ellis *et al*., 2002, Evans *et al*., 1985, Ohara *et al*., 1991). Inhalation of the bacteria by contaminated aerosols or dust, or complication of less severe forms of tularaemia can cause pneumonia (Gill and Cunha, 1997). The most acute form is typhoidal tularaemia which is characterized by septicaemia without lymphadenopathy or ulcers. Acute pneumonic or typhoidal forms reach mortality rates of 30-60% (Ellis *et al*., 2002, Sjöstedt, 2007). In certain regions (e.g. Scandinavia and Turkey) where drinking wells are commonly used, the oropharyngeal form of tularaemia appears also. Drinking water can be contaminated by carcasses of infected rodents, and these water sources might represent reservoir niche for the bacteria (Afset *et al*., 2015, Karadenizli *et al*., 2015). Painful sore throat, enlargement of the tonsils and formation of yellow-white pseudomembrane accompanied by swollen cervical lymph nodes occur in this case (Ellis *et al*., 2002, Reintjes *et al*., 2002). The ingestion of the bacteria by contaminated food or water may lead to gastrointestinal disease with persistent diarrhoea. In case of heavily contaminated food consumption the extensive ulceration of the bowel may lead to acute fatal disease (Ellis *et al*., 2002). In rare cases, when the conjunctiva is the initial site of infection (e.g. transmission of the bacteria on the surface of the fingertips), oculoglandular tularaemia develops and ulcers or nodules can appear on the conjunctiva, and regional lymph nodes can also be affected (Steinemann *et al*., 1999).

In naturally infected animals clinical manifestations of the disease is rarely recognized, tularaemic wild animals are easy to catch, or found moribund or dead (Friend, 2006, Mörner and Addison 2001). Non-specific clinical signs such as depression, fever, local inflammation or ulceration at the site of infection and swollen regional lymph nodes may be observed in tularaemic animals (Mörner and Addison 2001). Tularaemia septicaemia manifests in highly susceptible animals (e.g. small rodents) with sudden death (Gyuranecz *et al*., 2010a, 2012c). The house mouse (*Mus musculus*) is extremely sensitive to tularaemia; even the attenuated *F. tularensis* ssp. *holarctica* LVS can produce lethal infection in this host (Chen *et al*., 2004, Elkins *et al*., 2003, Jones *et al*., 2012). In domestic animals, tularaemia was described to cause late-term abortions in ewes and death of lambs, and the ulceroglandular form was reported in cat (O'Toole *et al*., 2008, Valentine 2004, Woods 1998). Cattle (*Bos taurus*) are

relatively resistant to the infection; they probably get infected by blood-sucking arthropod bites and seroconvert but do not develop symptoms (Mörner and Sandstedt, 1983; Feldman, 2003). In experimental infections of rats with *Francisella* the main clinical signs were weight loss, ptosis of the eyelids, ruffled fur, ataxia and laboured breathing (Wu *et al*., 2009).

Pathological findings of tularaemia depend on the affected animal species and sometimes on the geographic origin (Maurin and Gyuranecz, 2016). Acute course of the infection results septicaemia, congestion and haemorrhagic lesions and enlargement of the spleen and liver with multifocal coagulation necrosis in multiple organs (Decors *et al*., 2011, Gyuranecz *et al*., 2010a, Kemenes, 1976, Mörner, 1994, Rijks *et al*., 2013) (Fig. 5.). In the case of subacute infection in moderately susceptible species granulomatous lesions in the affected organs (lung, pericardium, kidney, etc.) are observed (Gyuranecz *et al*., 2010b) (Fig. 6.). Pathological findings in tularaemic European brown hare (*L. europaeus*), reservoir species for the pathogen in Central Europe, differ according to the origin of hares. Acute pathological changes and septicaemia were usually described in hares died of tularaemia in France, The Netherlands and in Italy, while lesions of subacute disease were described in this species in Hungary (Decors *et al*., 2011, Gyuranecz *et al*., 2010b, Rijks *et al*., 2013).

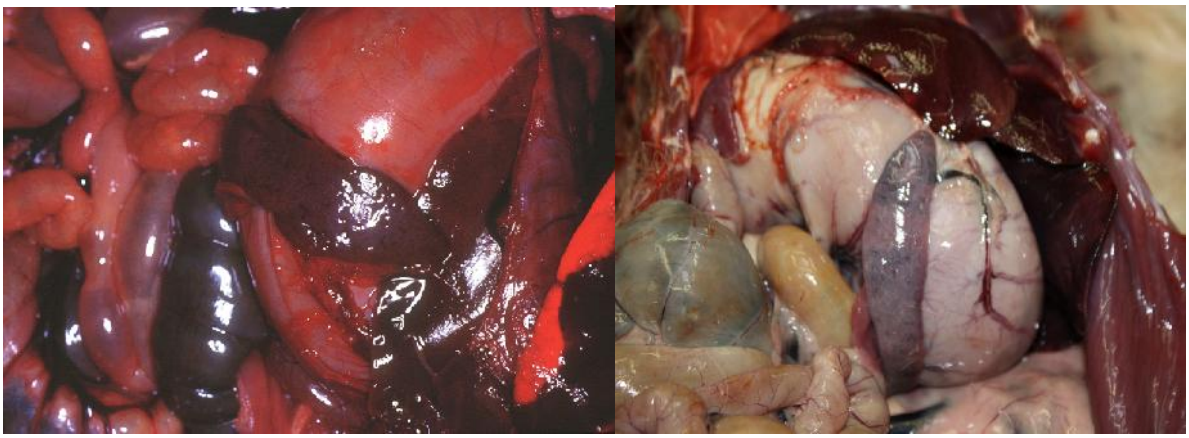

Figure 5. Splenomegaly and congestion in European brown hare with acute tularaemia, infected with B.FTNF002-00 genotype strain (Photos kindly provided by Massimo Fabbi)

Figure 6. Yellowish-white foci in the lung (black arrow), pericardium and kidneys of European brown hare with sub-acute tularaemia, infected with B.12 genotype strain (Gyuranecz *et al*., 2010b)

2.6. Diagnosis, management and control of disease

For the diagnosis of tularaemia in humans compatible epidemiologic or clinical data and positive serological test are required (Hepburn and Simpson, 2008, WHO, 2007, Tärnvik and Chu, 2007). Events in the history of the patients of close contact with wild animals, especially with hares or small rodents (e.g. hunters, veterinarians, hikers or small mammal trappers and skinners), arthropod bites, drinking from natural water sources, inhalation of contaminated dust or aerosol (e.g. dust from hay contaminated by the urine of small rodents) are suspicious for tularaemia infection. The most frequently used serological tests are the tube or microagglutination test, slide agglutination test and the indirect immunfluorescent assay, but enzyme-linked immunosorbent assays and Western blot assays have also been developed (Hepburn and Simpson, 2008, WHO, 2007, Tärnvik and Chu, 2007). Cross reactions with *Brucella abortus*, *B. melitensis*, *B. suis*, *Legionella* spp. and *Yersinia* spp. could occur in serological examinations (WHO, 2007). As antibodies against *Francisella* are usually detectable after 1-2 weeks of the first clinical signs, serological tests in the early phase of the disease often give negative results (Maurin *et al*., 2011).

Animal carcasses with suspected tularaemia infection should be handled with care and in biosafety level 2 or 3 conditions, as the bacteria are highly contagious (OIE, 2008, Sewell, 2003). Diagnosis from the carcasses is usually based on pathological findings and the detection of *F. tularensis* from the tissue samples. The routine diagnostic tests such as direct and indirect fluorescent antibody tests and immunohistochemical (IHC) assays are useful tools for the detection of *F. tularensis* (Karlsson *et al*., 1970; Zeidner *et al*., 2004, OIE, 2008).

The criteria for definition of a confirmed tularaemia case is paired serum samples with significant difference (by enzyme-linked immunosorbent assay or tube or microagglutination test) in titer and at least one positive serum. The isolation and identification of *F. tularensis* in culture by antigen or DNA detection also confirms the infection, according to the World Health Organisation (2007).

F. tularensis is highly fastidious; it requires amino-acid enriched media for its growth and primary isolation might be difficult due to overgrowth by other bacteria. In suspected cases penicillin, polymixin B and cycloheximide can be added to the medium, or the inoculation of mice with the homogenate of the sample as a first passage is recommended (WHO, 2007). Francis medium (peptone agar with cysteine, glucose and rabbit, horse or human blood), McCoy and Chapin medium (egg yolk and normal saline solution, heated to 75ºC), modified Thayer-Martin agar (glucose cysteine agar with haemoglobin and Iso VitaleX /Becton, Dickinson and Company, Franklin Lakes, NJ/), cysteine enriched chocolate agar and cysteine heart agar with chocolate blood are recommended for culturing *Francisella* (WHO, 2007). Colonies of the bacteria are small, greyish-white and round and appear after 24-48 hours of

incubation at 37ºC (OIE, 2008). Some species and subspecies within the family Francisellaceae could be differentiated based on their biochemical characteristics, e.g. *F. tularensis* does not show oxidase activity, while *F. philomiragia* gives positivity, or *F. tularensis* ssp. *tularensis* is able to ferment glycerol while the *holarctica* subspecies is not (WHO, 2007).

Several molecular techniques have been designed for the detection, classification and typing of members of the Francisellaceae family with distinct levels of resolution (Keim *et al*., 2007). Conventional polymerase chain reactions (PCR) and real-time PCRs targeting specific regions or genes of *Francisella* (including the 16S rRNA, the insertion sequence *ISFTu2*, 17 kDa surface lipoprotein coding *tul4* and *lpnA* genes, a putative succinate dehydrogenase locus *sdhA*, a *23kDa* protein coding gene and an outer membrane protein coding *fopA* gene) were designed for the detection of the bacteria (Barns *et al*., 2005). Although initial attempts for the detection of tularaemia based on conventional PCR amplification have led to the misidentification of FLEs and *F. tularensis*, the comparison of the sequences of the target genes or the use of more specific real-time PCR based methods can resolve this problem (Escudero *et al*., 2008, Kugeler *et al*., 2005, Versage *et al*., 2003).

Differential diagnosis of tularaemia involves bacterial infections (*Y. pestis*, *Y. pseudotuberculosis*, *B. anthracis*, mycobacteriosis, staphylococcosis, streptococcosis, pasteurellosis and brucellosis), viral infections (HIV, *Hantavirus*), parasites (toxoplasmosis, *Capillaria hepatica*, ascarid nematodes, larval cestodes) and lymphoma (Mörner and Addison 2001, WHO, 2007).

F. tularensis is a category A priority pathogen, a potential bioweapon, and the disease is to be reported to the World Animal Health Information Database (http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home) (WHO, 2007). The identification of environmental sources of the pathogen is essential in the control of tularaemia (Svensson *et al*, 2009b). In endemic areas the monitoring of wild animals (e.g. small rodents, wild boars), blood-sucking arthropods and water sources for the bacteria provides information for local public authorities and serves as basis for certain precautions in affected regions (Friend, 2006, WHO, 2007, Otto *et al*., 2014). The spreading of the bacteria is difficult to control, as *Francisella* has wide host range and complex ecology (Friend, 2006). At present, there is no licensed vaccine against the pathogen, although LVS has been used as investigational vaccine in humans worldwide (Sandström, 1994). The prevention of human tularaemia cases consists of limitation of contact with vectors and reservoirs of the bacteria such as avoiding direct contact with lagomorphs, rodents and other potentially infected animals or the use of repellents against blood-sucking arthropods (Maurin and Gyuranecz, 2016).

2.7. Treatment

The treatment of human tularaemia cases generally consists of aminoglycosides (streptomycin and gentamicin), quinolones (e.g. ciprofloxacin) and tetracyclines (e.g. doxycycline) (Bossi *et al*., 2004, Hepburn and Simpson, 2008, WHO, 2007). In Hungary, the first-line antibiotics in the treatment of tularaemia are aminoglycosides (streptomycin and gentamicin), while ciprofloxacin and chloramphenicol are recommended in post-exposure prophylaxis according to the National Centre of Epidemiology, Budapest (Herpay *et al*., 2011).

The aminoglycosides streptomycin and gentamicin have bactericidal effect by the inhibition of protein synthesis on the 30S ribosomal subunit, but these antimicrobials are ototoxic and nephrotoxic in humans, thus their use is recommended in the severe forms of tularaemia only (Johansson *et al*., 2002, Maurin and Gyuranecz, 2016). In the therapy of tularaemic patients streptomycin was proved to be highly effective with very low relapse rates (Enderlin *et al*., 1994). Gentamicin is generally used in patients with systemic tularaemia, in pregnant women and in children via intravenous administration for 10 days, although relapses occur more often with its use than with the administration of streptomycin (Kaya *et al*., 2012, Risi *et al*., 1995).

In mild to moderate cases of tularaemia the first choices for antibiotic therapy are quinolones and tetracyclines. Quinolones have bactericidal effect by the inhibition of a DNA-girase enzyme and they reach high concentrations in macrophages, but they may have fetotoxic side effects in pregnant women and they may induce musculoskeletal damage in young children (Hooper, 1999, Johansson *et al*., 2000, Memish and Mah, 2003). Tetracyclines have bacteriostatic effect by the inhibition of protein synthesis on the 30S ribosomal subunit, and they may induce severe side effects in children younger than 8 years old (permanent staining of developing teeth) and in pregnant women (affecting the development of teeth and bones in the fetus) (Ahmad *et al*., 2010, Maurin and Gyuranecz, 2016, Urich and Petersen, 2008). Administration of quinolones (preferably ciprofloxacin) and tetracyclines (generally doxycycline) may require 2-3 weeks for the treatment of tularaemia, but in advantage of aminoglycosides these antibiotics are taken orally (Bossi *et al*., 2004, WHO, 2007). Delayed diagnosis and treatment or suppurated lymphadenopathies may promote treatment failure and relapses with the use of quinolones and tetracyclines (Hepburn and Simpson, 2008, Maurin *et al*., 2011, Maurin and Gyuranecz, 2016, Pérez-Castrillón *et al*., 2001).

In tularaemia meningitis the administration of chloramphenicol (in combination with streptomycin) is recommended (Hofinger *et al*., 2009). Chloramphenicol has bacteriostatic effect by inhibition of protein synthesis on the 50S ribosomal subunit, and due to its severe side effects on the bone marrow, it is used only in exceptional cases (Enderlin *et al*., 1994, Griffin *et al*., 2010).

Considering the side effects of several antibiotics used in the therapy of tularaemia, especially in young children and pregnant women, the benefit of finding alternative drugs for the treatment with less severe side effects is evident. Moreover, therapy of patients with acute severe or chronic suppurative forms needs improvement (Boisset *et al*., 2014). Although naturally acquired resistance in *F. tularensis* to the antibiotics used in the common therapy have not been reported, the bacteria's efflux systems – which effectively protect the agent from the host's antimicrobial peptides – could potentially adapt to antibiotics developing resistance in the pathogen (Bina *et al*., 2008, Gil *et al*., 2006). According to the Clinical and Laboratory Standard Institute (CLSI), antibiotic susceptibility examinations should be performed by broth microdilution tests, determining minimum inhibitory concentrations (MIC) in supplemented Mueller-Hinton broth (CLSI, 2009). MIC value is the lowest concentration of the antibiotics that could still inhibit the growth of the bacteria. In brief, bacteria suspension of 0.5 MacFarland turibidity in physiological saline solution is diluted with Mueller-Hinton broth, containing distinct concentrations of the examined antibiotics. MIC values are determined after incubation for 48 hours at 35±2˚C (CLSI, 2009). Alternatively, the use of MIC test strip on solid medium has been proposed, as a reliable, easy to perform and repeatable assay (Ikäheimo *et al*., 2000, Tomaso *et al*., 2005, Valade *et al*., 2008). Antibiotic susceptibility examinations in eukaryotic cell models were evaluated also, in order to detect the intracellular activity of antimicrobials against *F. tularensis* (Maurin *et al*., 2000, Sutera *et al*., 2014).

F. tularensis produces class A beta-lactamase, which makes the bacteria resistant to most beta-lactam antibiotics (Antunes *et al*., 2012). The pathogen is also resistant to cefalosporins (with few exceptions), and the use of macrolides should be considered upon the epidemiology of the *Francisella* strains, as biovar II *F. tularensis* ssp. *holarctica* strains predominant in Northern, Central and Eastern Europe are resistant to erythromycin (García del Blanco *et al*., 2004, Georgi *et al*., 2012, Hepburn and Simpson, 2008, Ikäheimo *et al*., 2000, Tärnvik and Chu, 2007, Tomaso *et al*., 2005, Yesilyurt *et al*., 2011). There are also differences in the effectiveness of macrolides against type A *F. tularensis* ssp. *tularensis* and biovar I *F. tularensis* ssp. *holarctica* strains, as *in vitro* examinations showed higher effectiveness of azithromycin (azalides) and telithromycin (ketolides) against the pathogen than erythromycin (Ahmad *et al*., 2010, Gestin *et al*., 2010, Maurin *et al*., 2000). Moreover, azythromycin was recommended for alternative therapeutic use in pregnant women with mild tularaemia in regions where erythromycin sensitive strains are dominant (e.g. Western Europe and North America) (Dentan *et al*., 2013, Boisset *et al*., 2014). Although rifampicin *in vitro* is generally effective against *Francisella*, its use is recommended in combination with other drugs because of the possible resistance acquired by the pathogen during monotherapy (Ikäheimo *et al*., 2000, Tomaso *et al*., 2005, Yesilyurt *et al*., 2011). The effectiveness of linezolid (an antibiotic of good activity against Gram-positive pathogens including *Mycobacterium* species, with

potential of intracellular penetration) against *Francisella* was described *in vitro* on solid medium and in cell cultures. In cell cultures lower antibiotic concentrations (~1 mg/L) were sufficient for the inhibition of bacterial growth than on solid media (0.5-8 mg/L) (Sutera *et al*., 2014, Yesilyurt *et al*., 2011). The efficacy of a glycylcycline antibiotic, tigecycline was also examined, as its ability to reach high intracellular concentrations in macrophages and neutrophils made it an interesting alternative drug against intracellular bacteria (George, 2005). The low MIC values of tigecycline against *Francisella* determined in a study in Turkey indicate that this antibiotic might have potential in the therapy of tularaemia (Yesilyurt *et al*., 2011).

Antibiotic susceptibility examinations of *F. tularensis* in Hungary were carried out in 1972 by disc diffusion method. The examined 22 *Francisella* strains showed susceptibility to the aminoglycosides streptomycin, gentamicin, neomycin, kanamycin and paromomycin, to chloramphenicol, tetracycline and novobiocin and most strains were also susceptible to pristinamycin. The resistance of the strains was determined in the case of penicillines (penicillin, meticillin, oxacillin, ampicillin and carbenicillin), polypeptide antibiotics (polymyxin B, colistin and nystatin), macrolides (erythromycin, oleandomycin and spiramycin) and vancomycin (Kemenes and Füzi, 1972).

3. Aims of the study

The aims of the study were:

Ad 1. to investigate the occurrence and prevalence of F. *tularensis* and FLEs in ticks in Hungary and Ethiopia, and to reveal the genetic variability of the described FLEs;

Ad 2. to determine the genetic characteristics of *F. tularensis* ssp. *holarctica* strains originating from Hungary with high resolution molecular methods, including canSNP typing, MLVA and WG sequencing;

Ad 3. to compare the complement sensitivity of *F. tularensis* ssp. *holarctica* strains with different genetic background in the sera of the highly sensitive house mouse, moderately sensitive European brown hare and the resistant cattle, and to discover host-pathogen interactions for immune evasion, especially the binding of fH by *F. tularensis* ssp. *holarctica* in these animal hosts;

Ad 4. to compare the pathogenicity of *F. tularensis* ssp. *holarctica* strains from the two dominant genetic clade (B.FTNF002-00 and B.12) endemic in Europe in artificial infection experiments of rats;

Ad 5. to characterize the *in vitro* antimicrobial susceptibility profile of the Hungarian *F. tularensis* ssp. *holarctica* strains to antibiotics that could potentially be used in clinical therapy.

4. Materials and methods

4.1. *Francisella tularensis* ssp. *holarctica* strains

Sixty six *F. tularensis* ssp. *holarctica* strains were isolated from European brown hares from six counties (Bács-Kiskun, Békés, Csongrád, Győr-Moson-Sopron, Hajdú-Bihar and Jász-Nagykun-Szolnok) of Hungary between 2009 and 2010 and were kindly provided by Miklós Gyuranecz. Further three strains originated from zoo monkeys died in tularaemia outbreaks in Szeged zoo. In 2003 a patas monkey (*Erythrocebus patas*) and a vervet monkey (*Chlorocebus aethiops*) died of tularaemia and in 2014 a red-handed tamarin (*Saguinus midas*) succumbed to the infection (Fig. 7.). Two western European strains were kindly provided by Pedro Anda from Spain and Massimo Fabbi from Italy. The live vaccine strain (LVS, NCTC 10857) was also included in the examinations (Table S1).

Isolation of the strains was performed according to Gyuranecz *et al*. (2010c). Lung and kidney samples of the animals were homogenized with physiological saline solution and injected subcutaneously to NMRI (Naval Medical Research Institute) mice (Charles Rivers Laboratories International, Inc., Research Models and Services, UK). Artificial infection of the animals were in accordance with all national and institutional regulations (permit number: 22.1/2703/003/2009), approved by the ethics committees of the Institute for Veterinary Medical Research. After 7-10 days of the injection the mice died of the infection without showing exacerbated clinical signs. Heart blood and bone marrow samples of the mice were inoculated on modified Francis agar (sheep blood chocolate agar with 1% D-glucose and 0.1% cysteine /Sigma-Aldrich Co. LLC, St. Louis, MO/). Plates were incubated at 37°C with 5% CO_2 atmosphere for 2-4 days and checked daily.

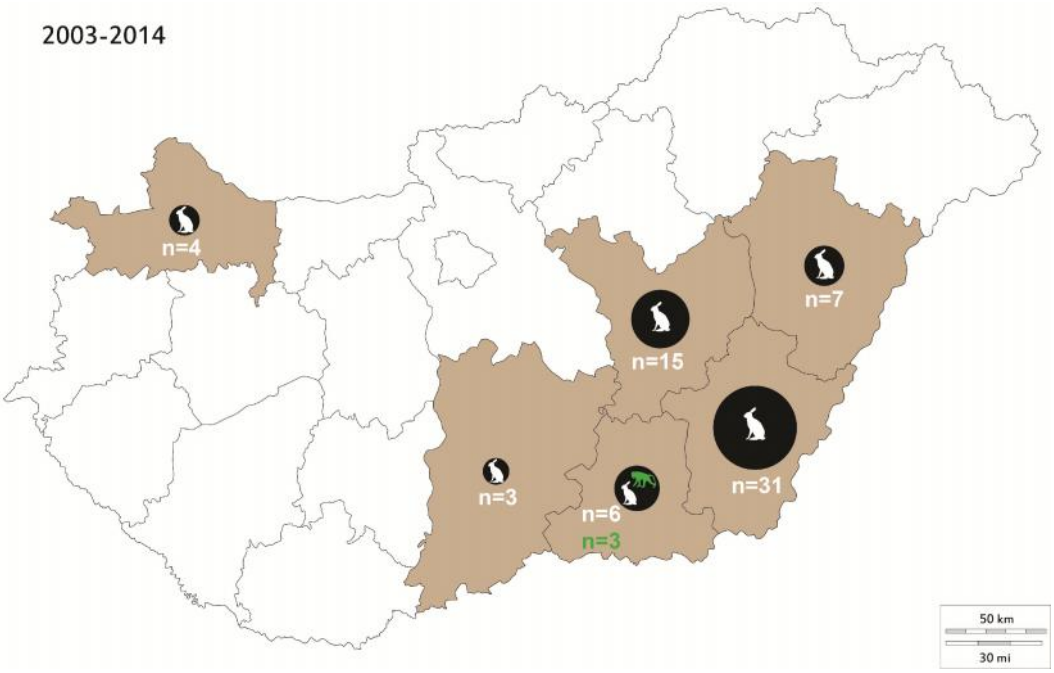

Figure 7. Geographic origin and hosts of 69 *Francisella tularensis* ssp. *holarctica* strains included in the examinations.
The size of circles is in correlation with the number of strains (n) originating from the same county. Animal icons representing host species (brown hares and zoo monkeys).

4.2. Sample collection

Ticks were collected from the environment and from animal hosts in three periods and their DNA were kindly provided by Sándor Hornok and Miklós Gyuranecz. Questing ticks were collected by the dragging-flagging method from 39 different sites of 15 counties (in fringes of pastures on bushy hillsides, fringes of meadows and wide paths in mountain forests and lowland areas) in Hungary between 2007 and 2009 from March until October each year. Ticks removed from common hamsters (*Cricetus cricetus*) and dogs in the same time period were also included in the examinations. In spring of 2011 migratory birds (*n*=1786) were mist-netted at the Ócsa Ringing Station (Duna-Ipoly National Park, Hungary) and were checked for the presence of hard ticks. In 2012 ticks were collected from cattle grazing on moist highland or savannah lowland in Didessa valley, south-western Ethiopia. Identification of the ticks were carried out by microscopy on the basis of their morphology and by species specific PCRs (Babos, 1964, Caporale *et al*., 1995, Hoogstraal, 1956, Rees *et al*., 2003, Rumer *et al*., 2011).

4.3. Molecular methods

4.3.1. DNA extraction from bacteria and ticks

From each *F. tularensis* ssp. *holarctica* strain cultured on modified Francis agar one colony was submitted for DNA extraction using the manufacturer's protocol for Gram-negative bacteria of the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA).

In the case of the ticks collected in Hungary between 2007 and 2009, pools of 10 or fewer ticks (when less than 10 individuals remained) were formed according to their collection date, species, sampling location, developmental stage and gender. The DNA of the ticks originating from migratory birds was extracted either individually or in pools (of 2-7 specimens) separated according to the ticks' hosts, species and developmental stages (Table S2). Ethiopian ticks were submitted for DNA extraction individually. DNA was extracted from the ticks with the QIAmp DNA Mini Kit (Qiagen) (Table S2) in the year of collection and was stored at -20°C. Prevalence rates were calculated from PCR results of individual samples. For pooled ticks, the minimum prevalence was determined from the number of positive pools, expressed as the percentage of all evaluated tick individuals of the same species (provided that there must have been at least one PCR-positive specimen in each PCR-positive pool).

4.3.2. Polymerase chain reactions for the detection of Francisellaceae species

Francisellaceae specific conventional PCRs were performed for the detection of *F. tularensis* and FLEs in ticks targeting the 16S rRNA gene, the *tul4* gene (coding a 17 kDa membrane lipoprotein) and the putative succinate dehydrogenase (*sdhA*) locus (Barns *et al*., 2005, Long *et al*., 1993, Sjöstedt *et al*., 1997).

For the discrimination of *F. tularensis* ssp. *holarctica* genotypes B.FTNF002-00 and B.12 the RD23 region was amplified. B.FTNF002-00 genotype contains the RD23 deletion thus the size of the amplicon is 1380 bp in this case, while from other *Francisella* species a 2970 bp amplicon is produced in this assay (Dempsey *et al*., 2007).

All conventional PCRs were performed in a Biometra–T Personal thermal cycler (Biometra, Analytik Jena AG, Germany). After amplification, 5 µl of each sample was loaded in 1% agarose gel containing GR Safe Nucleic Acid stain (Lab Supply Mall, InnoVita Inc., Gaithersburg, MD) for electrophoresis and visualized in UV light.

For the specific detection of *F. tularensis* fragment of the *tul4* gene was amplified using a real-time TaqMan PCR system (Versage *et al*., 2003). PCR amplifications were performed on a StepOnePLus real-time instrument (Applied Biosystems, Foster City, CA). According to the original description the detection limit of this assay is 1 CFU (Versage *et al*., 2003).

Primer pairs used in the reactions and predicted size of the amplicons are listed in Table 2. Reaction mixtures and programs are presented in Table 3.

Table 2. Primers used in the PCRs and predicted size of the amplicons

target gene	primer ID	primer sequence (5' → 3')	size
[a]**16S rRNA**	Fr153F Fr1281R	GCC CAT TTG AGG GGG ATA CC GGA CTA AGA GTA CCT TTT TGA GT	1020 bp
[b]**tul4**	FT-393 FT-642	ATG GCG AGT GAT ACT GCT TG GCA TCA TCA GAG CCA CCT AA	250 bp
[c]**tul4**	TUL4-435 TUL4-863	GCT GTA TCA TCA TTT AAT AAA CTG CTG TTG GGA AGC TTG TAT CAT GGC ACT	400 bp
[a]**sdhA**	SdhF SdhR	AAG ATA TAT CAA CGA GCK TTT AAA GCA AGA CCC ATA CCA TC	344 bp
[d]**RD23**	RD23F RD23R	GTC TTG TTG AGC AAA TGC CC CGG AGC AGG CTT AAA TAG TGA	1380 bp or 2970 bp
[e]**tul4**	Tul4F Tul4R Tul4P	ATT ACA ATG GCA GGC TCC AGA TGC CCA AGT TTT ATC GTT CTT CT FAM-TTC TAA GTG CCA TGA TAC AAG CTT CCC AAT TAC TAA G-BHQ	100 bp

[a]Barns *et al*., 2005
[b]Long *et al*., 1993
[c]Sjöstedt *et al*., 1997
[d]Dempsey *et al*., 2007
[e]Versage *et al*., 2003

Table 3. Reaction mixtures and PCR programs used in the study.

Reagents		Francisellaceae specific PCR (Volumes /µl/ for 1 sample)			Francisella specific PCR (Volumes /µl/ for 1 sample)	
		16S rRNA[a]	tul4[b,c]	sdhA[a]	RD23[d]	tul4[e]
MilliQ water (EMD Millipore, Merck Millipore, Billerica, MA)		10.45	11.55	12.55	12.05	
5x Green GoTaq Flexi Buffer (ThermoFisher Scientific, Waltham, MA)		5	5	5	5	
25mM $MgCl_2$ (ThermoFisher Scientific)		2.5	2.5	2.5	2	
10mM dNTP (ThermoFisher Scientific)		0.75	0.75	0.75	0.75	
forward primer (10pmol/ µl)		2	2	1	2	
reverse primer (10pmol/ µl)		2	2	1	2	
GoTaq Polymerase (5 unit/µl) (ThermoFisher Scientific)		0.3	0.2	0.2	0.2	
Sample DNA		2	1	2	1	
Total volume		25	25	25	25	
MilliQ water (EMD Millipore)						6.9
AmpliTaq Gold Buffer (Applied Biosystems)						1.25
25mM AmpliTaq Gold $MgCl_2$ (Applied Biosystems)						1.25
10mM dNTP (ThermoFisher Scientific)						0.5
forward primer (10pmol/ µl)						0.5
reverse primer (10pmol/ µl)						0.5
probe (10pmol/ µl)						0.5
AmpliTaq Gold Polymerase (5 unit/µl) (Applied Biosystems)						0.1
Sample DNA						1
Total volume						11
PCR program						
denaturation		95°C - 5’	95°C - 5’	94°C - 5’	95°C - 2.5’	94°C - 10’
number of cycles		45	40	40	45	45
	denaturation	95°C - 1’	95°C - 30”	94°C - 30”	95°C - 30”	95°C - 15”
	primer annealing	60°C - 1’	56°C - 1’	56°C - 45”	64°C - 1’	60°C* - 30”
	extension	72°C - 1’	72°C - 1’	72°C - 1’	72°C - 1’	72°C - 20”
final extension		72°C - 5’	72°C - 5’	72°C - 5’	72°C - 5’	

[a]Barns *et al*., 2005; [b]Long *et al*., 1993; [c]Sjöstedt *et al*., 1997; [d]Dempsey *et al*., 2007; [e]Versage *et al*., 2003

4.3.3. Sanger sequencing and phylogenetic analyses of target genes

Amplicons of 16S rRNA, *tul4* gene and *sdhA* gene based PCRs were extracted from agarose gel and direct cycle sequencing was performed with the primers used for amplification on ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Nucleic acid databases were searched using the BLASTN program in GenBank. The reading errors of the chromatograms were corrected and alignments (16S rRNA, 1025 bp; tul4, 188 bp; sdhA, 270 bp) of the obtained DNA sequences were performed with programs of the Lasergene package

(DNASTAR Inc., Madison, WI). JModeltest was used to identify nucleotide substitution models best fitting for all groups of sequences (Posada, 2008). Based on Akaike information criterion the Tamura–Nei 1993 model was chosen for further analysis from a range of models that possessed a 100% confidence interval, built on the models' cumulative weight gained during the calculations (Posada, 2008). Phylogenetic analysis was conducted with the neighbor-joining method using the maximum composite likelihood model (equivalent with Tamura–Nei 1993 model) and 1000 bootstraps in MEGA5 software (Tamura *et al*., 2011).

4.3.4. Genotyping of the Hungarian *F. tularensis* ssp. *holarctica* strains

The canSNP typing of 70 *F. tularensis* ssp. *holarctica* strains (69 isolates from Hungary and the LVS) was performed using 14 primer sets in melt analysis of mismatch amplification mutation assays (melt-MAMA) (Chanturia *et al*., 2011, Gyuranecz *et al*., 2012a, Vogler *et al*., 2009a) (Table S3). The melt-MAMA is based on competing allele specific primers, which are distinguished by a 15-19 bp GC-clamp at the 5'end. The SNPs are identified by the melting temperature (Tm) of the amplicons on a real-time PCR platform (Applied Biosystems StepOnePlus real-time PCR system, StepOne Software v2.2.2) (Birdsell *et al*., 2012). Primers used in the reactions are listed in Table S3. The reaction mixture and program are presented in Table 4. The Tm of the amplicons was measured in a melt curve by ramping from 60°C to 95ºC with increment of 0.3 ºC/min.

The MLVA of the *F. tularensis* ssp. *holarctica* strains was performed by using 11 primer pairs to further resolve genetic relationships within subclades determined by canSNP typing (Vogler *et al*., 2009b) (Table S4). This MLVA uses genome markers with repeat unit sizes between 5-23 bp, and the genetic analysis is based on the strains' profiles resulted from the number of repeat units on each examined loci. Primers used in the reactions are listed in Table S4. The reaction mixture and program are presented in Table 4. The PCR was performed in a Biometra–T Personal thermal cycler (Biometra). Fragment analysis of the amplicons was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and results were analyzed with Peak Scanner™ Software v.1.0 (Applied Biosystems).

The evolutionary relatedness among the allelic profiles of the strains was examined with neighbour-joining algorithm in MEGA5 software (Tamura *et al*., 2011).

Table 4. Reaction mixtures and PCR programs used for genotyping

Reagents		canSNP (Volumes /µl/ for 1 sample)	MLVA (Volumes /µl/ for 1 sample)
MilliQ water (EMD Millipore)		4.37-4.67	10.8-15
5x Colorless GoTaq Flexi Buffer (ThermoFisher Scientific)		3	5
25mM $MgCl_2$ (ThermoFisher Scientific)		1	2
10mM dNTP (ThermoFisher Scientific)		0.5	0.8
EvaGreen™ dye (Biotium Inc., Hayward, CA)		0.5	
primers (10pmol/ µl)		according to Table S3	according to Table S4
GoTaq Polymerase (5 unit/µl) (ThermoFisher Scientific)		0.08	0.2-0.4
Sample DNA		1	1
Total volume		10	25
PCR program			
denaturation		95°C - 10’	94°C - 5’
number of cycles		40	35
	denaturation	95°C - 15”	94°C - 30”
	primer annealing	60°C* - 1’	58°C - 30”
	extension		72°C - 30”
extension			72°C - 5’
melt curve		60-95°C 0.3°C/min	

4.3.5. Whole genome sequencing

To further resolve phylogenetic structure of Hungarian isolates the sequencing of the WG of nine Hungarian strains was performed. The diverse selection of strains was based on the year of isolation, geographic origin and host (Table S1). WG sequencing was accomplished by sequence-by-synthesis next-generation sequencing technology on MiSeq desktop sequencer (Illumina Inc., San Diego, CA) in the Swedish Defence Research Agency (FOI, Umea, Sweden). The library for the samples was prepared according to the manufacturer’s instructions by Nextera XT DNA Library Prep Kit (Illumina). After the tagmentation of the DNA of the samples, each DNA library was marked with commercial index primers. The DNA libraries were then normalized and pooled for cluster generation and sequencing. Image analysis for base calling and alignments were performed with ABySS sequence assembler and based on previous publications (Craig *et al*., 2008, Simpson *et al*., 2009).

4.4. Complement sensitivity assay

Complement sensitivity of different genotypes of *F. tularensis* ssp. *holarctica* strains (B.FTNF002-00 and B.12) was compared using sera of selected animal hosts. *Francisella* strains originated from Spain and Italy (strain IDs: Ft6 and 21581/2006, respectively; B.FTNF002-00 genotypes) and Hungary (strain ID: FTH24/08; B.12 genotype). The attenuated LVS (B.12 genotype) was included in the examinations also. Sera of NMRI mice (Charles River Laboratories), European brown hares and cattle (Holstein-Friesian breed) were used to represent hosts that are highly or moderately (reservoir) sensitive or resistant to tularaemia, respectively. The sera were collected from healthy individuals (mouse, *n*=30; hare, *n*=10; cattle, *n*=10) in accordance with all national and institutional regulations (permit number: 22.1/2703/003/2009). All sera were negative for antibodies against *F. tularensis* by slide and tube agglutination tests (Bioveta Inc., Ivanovice na Hané, Czech Republic). None of the animals were under antibiotic therapy during sampling, and sera were filtered through a 0.2 µm filter (Minisart NML, Sartorius AG, Göttingen, Germany) before use.

For the complement sensitivity assay *F. tularensis* ssp. *holarctica* strains were cultured in filtered (0.2 µm pore size, Minisart NML), modified brain-heart infusion (BHI) medium, containing 0.1% L-cysteine and 1% D-glucose (Sigma-Aldrich). An amount of 200 µl of four-day-old bacterium culture of adjusted cell numbers (300 bacterial cells in 10 µl BHI) was incubated together in 1:1 dilution with each serum at 37 °C for 4 h. Heat-inactivated sera (30 min at 56 °C) were used as inactive complement control. Each examination included a live cell control from the broth culture of the examined *Francisella* strains and a dead cell control from gentamicin- (100 µg/ml; Sigma-Aldrich) killed bacteria from each strain. After incubation, cells were stained with propidium iodide (adding 1 µl propidium iodide /Sigma-Aldrich/ to 50 µl broth culture and incubating for 8 min at room temperature with constant shaking) and examined by flow cytometry and fluorescent microscopy.

The analyses were run on a Fluorescence Activated Cell Sorting single-laser flow cytometer (Becton, Dickinson and Company). Events were counted in the list mode for one minute, with 10 µl/min sample fluid flow rate. Live and dead cell controls were analysed first to construct the gates. For the discrimination and enumeration of live and dead cells, gates were read on the logarithmically amplified FL-2 vs. FL-3 fluorescence dot plot. Data were analysed using the WinMDI software (Windows Multiple Document Interafce for Flow Cytometry, Version 2.8, The Scripps Research Institute, La Jolla, CA).

Examinations were carried out in triplicates on each sample and the mean values were used in the evaluations.

4.5. Proteomic methods

4.5.1. Gaining whole cell lysates

Whole cell lysates of *F. tularensis* ssp*. holarctica* strains originating from Spain, Italy and Hungary (strain IDs: Ft6, 21581/2006 and FTH24/08, respectively) and of LVS were extracted for proteomic examinations. Bacterial cells in broth cultures were centrifuged (15 min at 5500 rpm at 4 °C), then treated with 1% protease inhibitor cocktail (ProteoBlock Protease Inhibitor Cocktail, Thermo Fisher Scientific), and after sonication whole cell lysates were obtained from the supernatant of the centrifuged (30 min at 13,000 rpm at 4 °C) samples.

4.5.2. Membrane protein extraction

Membrane proteins of *F. tularensis* ssp*. holarctica* strains originating from Spain, Italy and Hungary (strain IDs: Ft6, 21581/2006 and FTH24/08, respectively) and of LVS were extracted for proteomic examinations. Membrane proteins were gained from whole cell lysates using the Proteojet Membrane Protein Extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Membrane proteins of each *F. tularensis* strain were run in polyacrylamide gels (6% spacing gel and 10% resolving gel) and transferred to nitrocellulose membranes (0.45 µm pore size, Thermo Fisher Scientific) by electroblotting (2 h at 200 V, XCell SureLock Electrophoresis Cell, Invitrogen, Carlsbad, CA).

4.5.3. Western blot assay

Western blot assays were performed for the detection of possible interactions between animal host fH and *Francisella*. Interactions between *F*. *tularensis* ssp. *holarctica* strains of different genotypes (B.FTNF002-00 and B.12) and the sera of selected animal hosts of distinct susceptibility to the pathogen (mouse, hare and cattle) were examined. The same sera were used for Western blots as were for the complement sensitivity assays. *Francisella* strains originated from Spain and Italy (strain IDs: Ft6 and 21581/2006, respectively; B.FTNF002-00 genotypes) and from Hungary (strain ID: FTH24/08; B.12 genotype). The attenuated LVS (B.12 genotype) was included in the examinations also.

Bacterial membrane proteins of the *Francisella* strains were blocked in 2% SMTTBS (2% skim milk in 0.05% Tween-20 Tris-Buffered Saline /Sigma-Aldrich/) for 1 h, then incubated with the sera of animal hosts for 2 h at room temperature. For the detection of possible interactions between membrane proteins and the complement regulator fH, nitrocellulose

membrane bound proteins were incubated for 1 h with polyclonal primary antibody of goat (concentration 1:200 in 0.5% SMTTBS; anti-factor H, Abcam PLC, Cambridge, UK) and for 1 h with rabbit anti-goat HRPO (horseradish peroxidase; concentration 1:50,000 in 0.5% SMTTBS; Sigma-Aldrich) secondary antibody. After a final step of 5 min incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), the chemiluminescence of the samples was measured with a blot scanner (LI-COR C-DiGit, LI-COR Biotechnology, Lincoln, NE).

Membrane proteins of *B. hermsii* were incubated together with serum of mouse and the antibodies described above for use as positive control (Bhide *et al*., 2009). *B. hermsii* binds fH with its 20 kDa protein, FhbA.

4.5.4. Pull-down assay

Pull-down assays were performed to show possible interactions between host and pathogen proteins. Interactions between *F. tularensis* ssp. *holarctica* strains of different genotypes (B.FTNF002-00 and B.12) and the sera of selected animal hosts of distinct susceptibility to the pathogen (mouse, hare and cattle) were examined. The same sera and bacterium strains were used for pull-down assays as were for the Western blots.

Protein G binding agarose beads (20 µl/sample; Abcam PLC) were washed with radioimmunoprecipitation assay (RIPA) buffer (Abcam PLC) three times by vortexing the beads in 300 µl buffer and then centrifuged for 1 min at 10,000 rpm, supernatants were discarded. Then goat antibodies (7.4 µl/sample; anti-factor H, Abcam PLC) were conjugated to the surface of the beads by incubation at 4°C for 1 h with continuous shaking in the presence of protease inhibitor (0.5 µl; ThermoFischer Scientific). After incubation, beads were washed three times with RIPA buffer. Sera were prepared for conjugation by centrifugation at 15,000 rpm for 5 min. Supernatants of the sera were then diluted (1:1) in RIPA buffer and added to the beads in 1:5 or 1:10 concentrations. Sera and protein G bound goat anti-factor H antibodies were incubated for 1 h at 4°C with continuous shaking. After incubation the beads were washed three times with RIPA buffer, supernatants were discarded. Preparation of *Francisella* whole cell lysates was performed by centrifugation at 15,000 rpm for 5 min. Supernatants of the whole cell lysates were then diluted (1:1) in RIPA buffer and added to the beads in 1:5 or 1:10 concentrations. After incubation for 1 h at 4°C with continuous shaking, beads were washed three times in RIPA buffer and supernatants were discarded. Finally, glicin HCl (60 µl/sample; Abcam PLC) of pH 2.7 was added to the beads to release protein-complexes and vortexed for 15 min. Acidity was neutralized by adding tris HCl (4.1 µl; Abcam PLC) of pH9. After centrifugation of the samples for 1 min at 10,000 rpm, supernatants were taken and dried completely with Savant SpeedVac Concentrator (Thermo Fisher Scientific)

(53°C at 878 rpm in vacuum). Samples were then diluted in molecular grade water (5 µl in 15 µl) and run in polyacrylamide gel (6% spacing gel and 10% resolving gel) and stained with Coomassie Blue (Abcam PLC).

Conjugated proteins were then extracted from the polyacrylamide gel, destained and identified by protein mass fingerprinting on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Ultraflex, Bruker Corporation, MA) and data were analysed by Mascot software (Matrix Science Ltd., London, UK), a search algorithm for mass spectral proteomics peaklists.

4.6. Artificial infection

4.6.1. Preparation of infectious inoculum

The virulence of different genotypes of *F. tularensis* ssp *holarctica* strains (B.FTNF002-00 and B.12) was compared in rats. Bacterial strains originating from Italy (strain ID: 21851/2006; B.FTNF002-00 genotype) and Hungary (strain ID: FTH24/08; B.12 genotype) were cultured on modified Francis agar for 48 h at 37°C and 5% CO_2. First, colonies were suspended in sterile saline and adjusted to 0.5 McFarland turbidity. Then 100 µl of each dilution from a tenfold dilution series of the suspension was inoculated on modified Francis agar and incubated for 48 h to determine the CFUs ($4*10^7$).

For the infection of the Fischer 344 rats, fresh bacteria colonies were suspended in sterile saline and adjusted to 0.5 McFarland turbidity, then diluted in sterile saline to get 10^0, 10^1 and 10^2 concentrations. Following the artificial infection of the rats, CFUs were re-checked from the used dilutions on modified Francis plates after 48 h of incubation.

4.6.2. Animal model and infection

Age matched (7 weeks) female Fischer 344 rats were purchased from Charles River Laboratories. The animals were kept in accordance with all national and institutional regulations (permit number: PEI/001/1927-4/2015). The rats (6 animals/group) were injected intraperitoneally (ip) with 100 µl of the B.FTNF002-00 and B.12 genotypes of *F. tularensis* ssp. *holarctica* (10^0, 10^1 and 10^2 concentrations). A group of 6 Fischer 344 rats injected ip with 100 µl sterile saline was used as negative control in the experiment.

After infection, the animals were checked and measured daily for 21 days. Rats that did not succumb to the infection were euthanized by CO_2 over exposure at the end of the experiment. Slide agglutination test was performed at necropsy with heart blood, using the commercially available Antigen *Francisella tularensis* (Bioveta). Tissue samples were excised from the lung, thymus, liver, spleen, kidney, small and large intestine, muscle, bone marrow and brain and preserved in 10% formalin for histological and IHC examinations.

4.6.3. Histology and immunohistochemistry

Histopathological changes were detected by light microscope on 10% formalin-fixed, paraffin-embedded tissue samples stained with hematoxylin and eosin.

IHC examinations were performed as described before (Gyuranecz *et al*., 2010b). In brief, formalin-fixed, paraffin-embedded tissue samples were deparaffinised and *F. tularensis* lipopolysaccharide antigen was retrieved by heating the slides in citrate buffer (pH 6.0) for 20 min in microwave oven at 750 W. Rabbit polyclonal antibody in 1:30,000 dilution was used as primary antibody and incubated overnight at 37°C with the samples. Antibody binding was detected by a HRPO-labelled polymer (EnVisionTM+ Kit; Dako Inc., Glostrup, Denmark). A serial section incubated with phosphate buffer solution was used as a negative control.

4.7. Antimicrobial susceptibility test

The susceptibility of 30 *F. tularensis* ssp. *holarctica* strains (29 isolates from Hungary and the LVS) to 11 antibiotics (erythromycin, streptomycin, gentamicin, ciprofloxacin, levofloxacin, tetracycline, doxycycline, tigecycline, rifampicin, linezolid and chloramphenicol; Table S5) was determined. The strains were isolated between 2003 and 2010 from European brown hares shot during hunting and from zoo monkeys, originating from different parts of Hungary.

Antibiotic susceptibility tests were performed by MIC test strips (Liofilchem s.r.l., Roseto degli Abruzzi, Italy; Table S5) on 5 mm thick modified Francis agar plates. The strains were cultured for 48 h on modified Francis agar at 37°C in a 5% CO_2 atmosphere. Three to four colonies were suspended in 3 ml of physiological saline, with the turbidity adjusted to be equivalent to that of a 0.5 McFarland standard. The plates were inoculated using sterile cotton swabs and one MIC test strip was placed on each plate within 15 min. After 48 h of incubation at 37°C in a 5% CO_2 atmosphere, the MIC results were read according to the manufacturer's instructions. The *F. tularensis* ssp. *holarctica* LVS was included as a quality control. The breakpoints were interpreted according to CLSI standards for *F. tularensis*, where available, and to CLSI standards for Enterobacteriaceae, staphylococci or *Streptococcus pneumoniae* where specific standards were unavailable (CLSI, 2009).

4.8. Statistical analysis

Independent *t*-test was performed to evaluate differences in the results of complement sensitivity assays. The absolute values of the differences between mean values of number of events in normal and inactivated sera in the case of wild and attenuated *F. tularensis* spp. *holarctica* strains, and in groups B.FTNF002-00 and B.12 within the wild strains were compared in each host species.

The results of the artificial infection experiments were compared with independent *t*-test. The categories of the severity of clinical signs were converted into numbers and results of all groups infected with B.FTNF002-00 genotype were compared with data of all groups infected with B.12 genotype.

5. Results

5.1. Francisellaceae in ticks from Hungary and Ethiopia

A total of 5024 questing ticks of 6 species (3222 *Ixodes ricinus*, 369 *D. marginatus*, 361 *D. reticulatus*, 315 *Haemaphysalis* /*Ha*./ *inermis*, 735 *Ha. concinna* and 22 *Ha. punctata*) were collected and 378 ticks were removed from animal hosts (374 *I. acuminatus* from common hamsters and 4 *D. reticulatus* from dogs) in a two-year period in Hungary. In addition, 108 (104 *I. ricinus*, 1 *Ha. concinna* and 3 *Hyalomma /Hy./ marginatum*) ticks were collected from 62 migratory birds in an examination in 2011 during which 1786 birds were checked for infesting ticks. In Ethiopia a total of 296 ticks, 118 *A. variegatum*, 100 *A.cohaerens*, 2 *A. lepidum*, 50 *R. decoloratus*, 17 *R. evertsi*, 8 *R. praetextatus* and 1 *Hy. rufipes* were collected from cattle in 2012. Altogether, 352 individual and 568 pooled tick samples were screened for the presence of members of the Francisellaceae family.

The same *F. tularensis* ssp. *holarctica* strain was detected in 1 nymph and 1 female *Ha. concinna* pool collected from a meadow in Békés county in 2009, and in 1 *D. reticulatus* pool of females collected from the environment in Zala county in 2007. The positive samples representing a minimum prevalence (calculating with only 1 infected tick per pool) of 0.27% within the examined tick species (2/735 of *Hy. concinna* and 1/361 of *D. reticulatus*). Both 16S rRNA and *tul4* gene coding regions were sequenced and genetic relationships with other Francisellaceae species was demonstrated by neighbor-joining phylogenetic analysis (GenBank No.: JQ942363, JQ942364, JQ942366, JQ942367) (Fig. 8.). *F. tularensis* specific DNA was not detected in any of the ticks collected from migratory birds in Hungary or from cattle in Ethiopia.

FLEs were found in 11 pools of *D. reticulatus* questing ticks collected in 2007 (2 pools of nymphs from Nógrád county, 1 pool of males from Borsod-Abaúj-Zemplén county, and 8 pools of females from Bács-Kiskun, Csongrád, Nógrád, Pest /n=2/, Somogy, Vas and Zala counties), showing a minimum prevalence of 3% (11/361). Both 16S rRNA and *tul4* gene coding sequences were identical in all 11 FLEs of *D. reticulatus* (GenBank No.: JQ942365, JQ942368) (Fig. 8.). The comparison of the obtained sequences with those deposited in GenBank revealed that the detected 16S rRNA gene sequence was identical to the FLE of *D. reticulatus* from Bulgaria (GenBank No.: HQ705173) and differed in 2 nucleotides from the endosymbiont found earlier in 3 *D. reticulatus* samples in Hungary (GenBank No.: EU234535) (Fig. 8., 16S rRNA). However, the *tul4* gene coding sequences of the present FLEs proved to be identical to the endosymbiont found earlier in Portugal (GenBank No.: GU113085) and in Hungary (GenBank No.: EU126640) (Fig. 8., tul4).

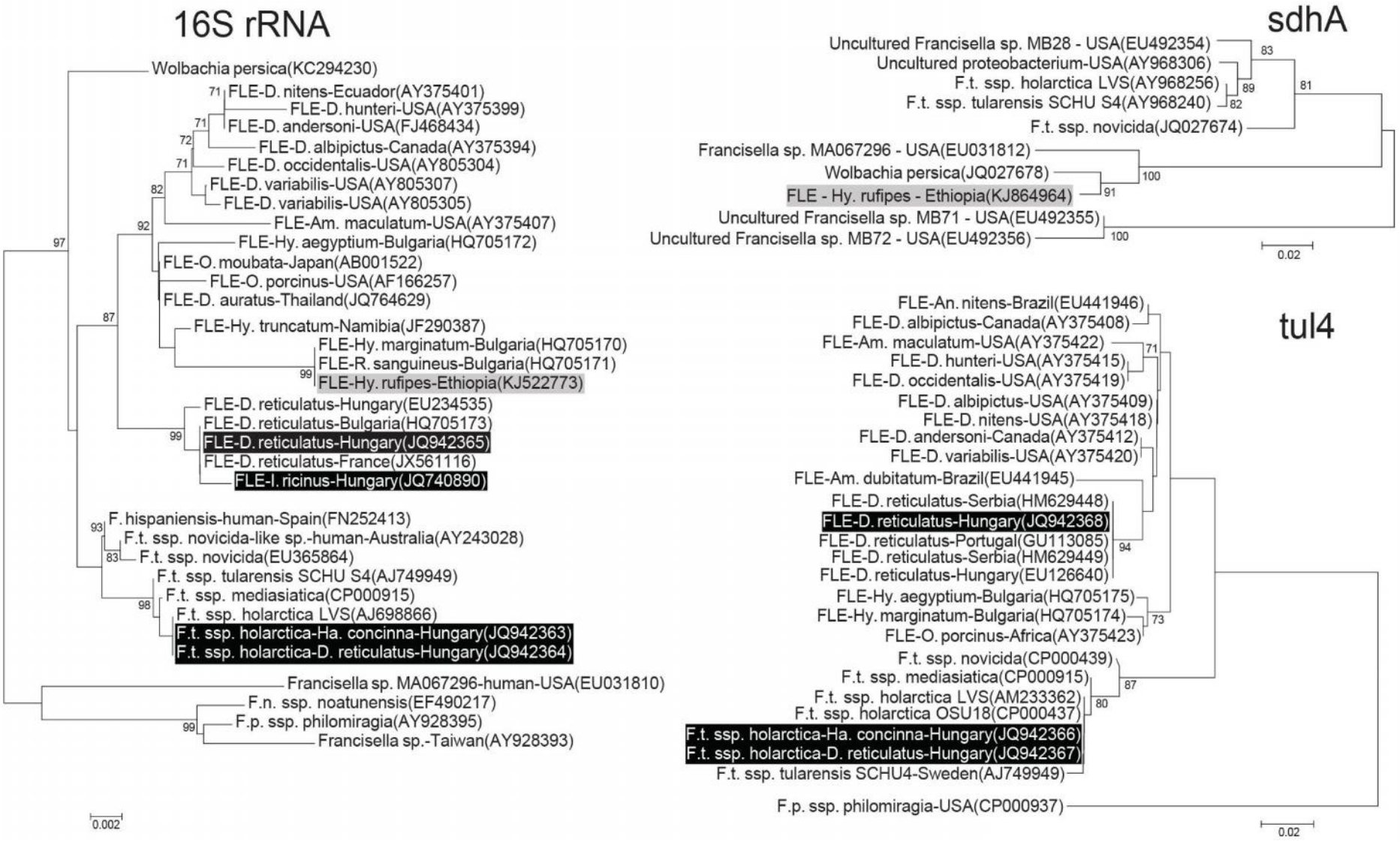

Figure 8. Neighbor-joining phylogenetic analyses of Frnacisellaceae based on 16S rRNA, *sdhA* and *tul4* genes. Bootstrap values of neighbor-joining (1000 replicates) of equal or higher than 70 are shown. Strains originating from Hungary are highlighted in black; the strain originating from Ethiopia is highlighted in grey.

According to its 16S rRNA gene sequence, a novel FLE was obtained from an *I. ricinus* larva removed from a European robin (*Erithacus rubecula*) in 2011. The 16S rRNA sequence of the novel FLE (GenBank No.: JQ740890) showed closest similarity (99%) to endosymbionts previously described in *D. reticulatus* in Hungary and Bulgaria (Fig. 8., 16S rRNA).

The 16S rRNA and *sdhA* genes of a FLE were found in the sole *Hy. rufipes* specimen originating from Ethiopia. The sequence of the 16S rRNA gene fragment of this endosymbiont (GenBank No.: KJ522773) resulted to be identical with that of the endosymbionts described in *R. sanguineus* and *Hy. marginatum* collected in Bulgaria (Fig. 8., 16S rRNA). The sequence of the *sdhA* gene fragment of the present Ethiopian endosymbiont (GenBank No.: KJ864964) showed 99% identity with *W. persica* (GenBank No.: JQ027678) detected in Egypt (Fig. 8., sdhA). The amplification of the *tul4* gene fragments failed with both primer pairs used (Long *et al*., 1993, Sjöstedt *et al*.,1997).

GenBank accession numbers of the detected genes of Francisellaceae species are summerized in Table S2.

5.2. Genotyping of *F. tularensis* ssp. *holarctica* strains by high resolution molecular methods

The comprehensive study of the genotyping of 69 *F. tularensis* ssp. *holarctica* strains and the LVS was performed by canSNP and MLVA methods, and 9 strains were submitted for WG sequencing as well. The strains originated from six counties from regions where the European brown hare, the reservoir of the bacterium is prevalent in the country (Table S1, Fig. 7.).

Phylogenetic analyses showed that all Hungarian *F. tularensis* ssp. *holarctica* strains belong to subclades of the main genetic clade B.12. CanSNP typing classified the Hungarian strains and LVS into 9 subclades (B.LVS, B. 23/14/25, B.20/21/33, B.33/34, B.34/35, B.35/36, B.36/37, B.37/38 and B.Tul07/2007), out of which B.33/34 subclade was the most dominant as 68% (47/69) of the strains showed this genotype and 89.85% (62/69) belonged to the B.33/34 subclade or derivated subclades (Fig. 9.).

The MLVA showed variability on two loci out of the examined 11 loci among the 69 *F. tularensis* ssp. *holarctica* strains. The three subclades: B.20/21/33, B.33/34 and B.34/35 determined by canSNP typing were further resolved with MLVA into 4, 6 and 4 subgroups, respectively (Fig. 10.).

Strains originating from zoo monkeys from the 2003 and 2014 tularaemia outbreaks in Szeged Zoo showed identical MLVA profiles (Fig. 10.). The canSNP and MLVA profiles of the strains are presented in Tables S6 and S7.

Analysis of the WG sequences of 9 selected strains and a previously sequenced Hungarian strain (FTH7 a.k.a. Tul7/2007, Gyuranecz *et al*., 2012a) confirmed results of canSNP and MLVA typing (Fig. 10.). No association was found between genotypes and geographical origins, year of isolation or host species of the samples.

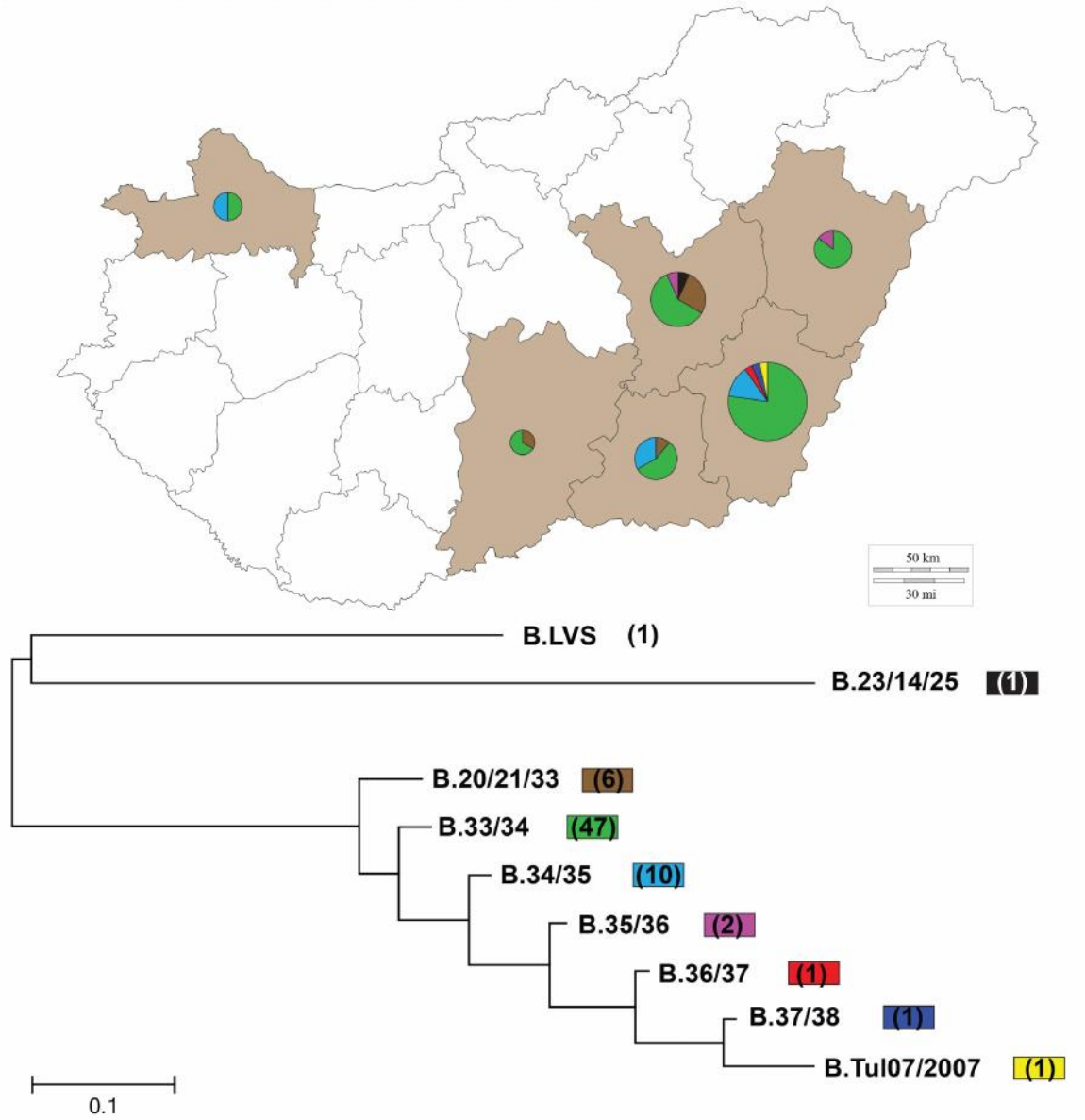

Figure 9. CanSNP typing based genotypes and geographic origin of 69 *F. tularensis* ssp. *holarctica* strains from Hungary.
Colour codes representing genotypes, number of isolates of the same genotype is in brackets, and circles are representing number of strains according to Fig. 7.

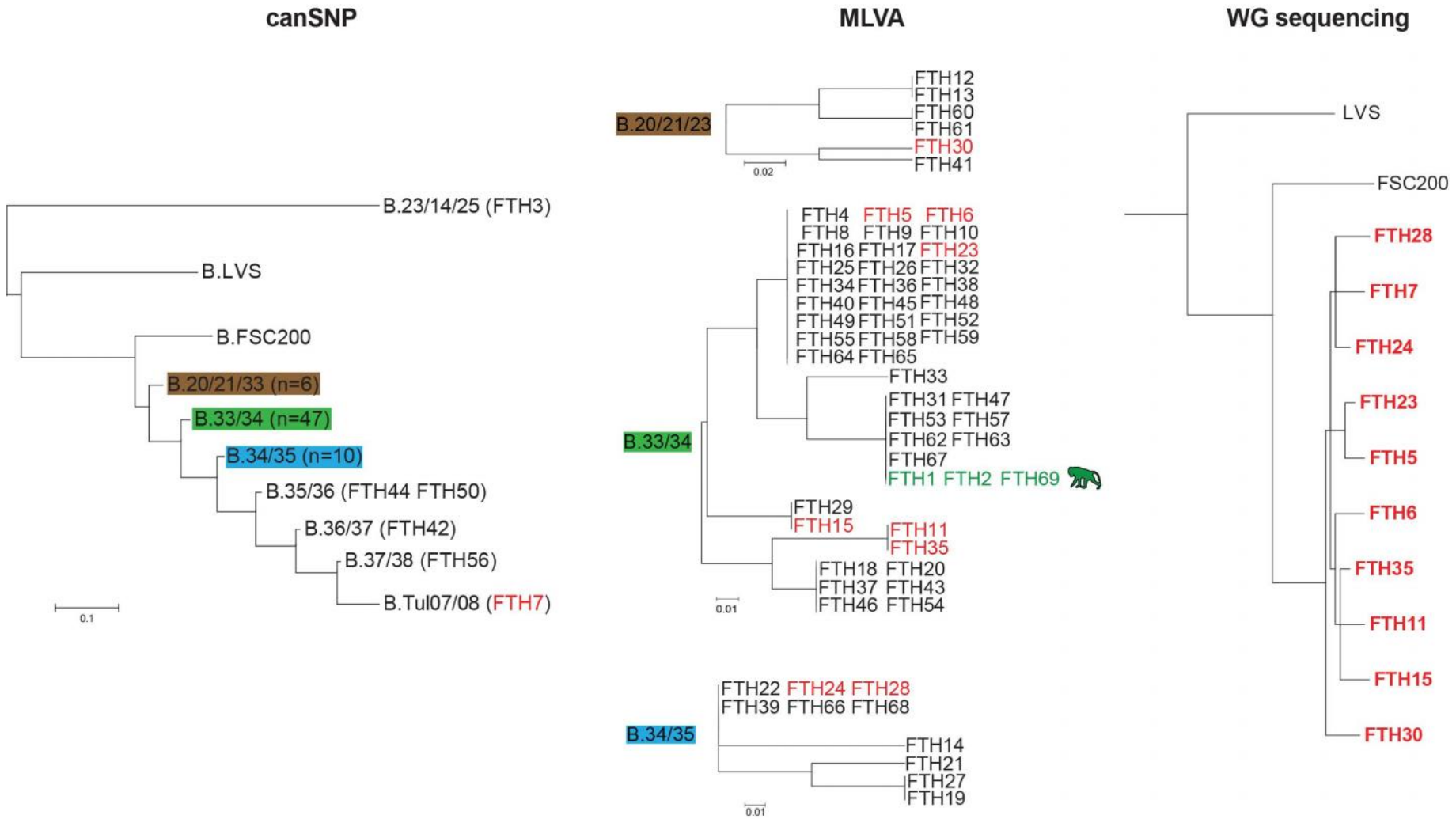

Figure 10. Neighbor-joining trees of the Hungarian *F. tularensis* ssp. *holarctica* strains based on canSNP, MLVA and WG sequencing. Number of strains (n) or strain IDs (FTH number) are shown in brackets on the canSNP based dendrogram. Strain IDs of WG sequenced Hungarian strains are coloured red. Strain IDs originating from zoo monkeys are coloured green.

5.3. Host-pathogen interactions between *Francisella* strains and selected animal species

The complement sensitivity assays were performed on one attenuated (LVS) and three wild *F. tularensis* ssp. *holarctica* strains, originating from Italy and Spain (clade B.FTNF002-00), and from Hungary (clade B.12) using sera of the highly susceptible house mouse, moderately susceptible European brown hare and relatively resistant cattle. Differences were observed in the resistance of the strains to serum killing and among the hosts' susceptibility (Fig. 11.).

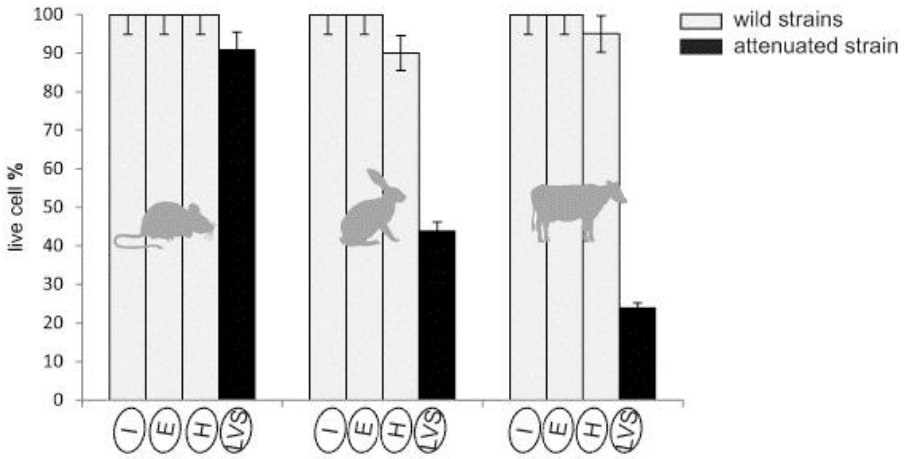

Figure 11. Percentage of live *F. tularensis* ssp. *holarctica* bacterial cells in the sera of house mouse, brown hare and cattle in complement sensitivity assays.
Values represent the percentage of live bacterial cells in normal sera compared to live cell numbers in heat-inactivated sera after incubation at 37 °C for 4 h, determined by flow cytometry. Animal icons (mouse, hare and cattle) represent the origin of the sera examined. Abbreviations represent the origin of the strains: Italy, B.FTNF002-00 group (**I**), Spain, B.FTNF002-00 group (**E**), Hungary, B.12 group (**H**) and live vaccine strain, B.12 group (**LVS**)

The complement sensitivity assays showed that most bacterial cells stayed intact after incubation with mouse serum in every case examined by flow cytometry (Fig. 11.) and fluorescent microscopy as well. In the case of hare serum, flow cytometric analyses showed a decreased number of events in the LVS broth culture after incubation with normal serum compared to cultures incubated with heat-inactivated hare serum (Fig. 11.). Examinations by fluorescent microscope confirmed bacterial cell lysis in the assays with normal hare serum and LVS. Noticeable elevation of fluorescence emission and a significant decrease in the number of live cell events ($p=0.003$) were observed in LVS broth culture after incubation with normal cattle serum compared to incubation with inactivated cattle serum due to mass bacterial cell killing (Figs. 11 and 12.).

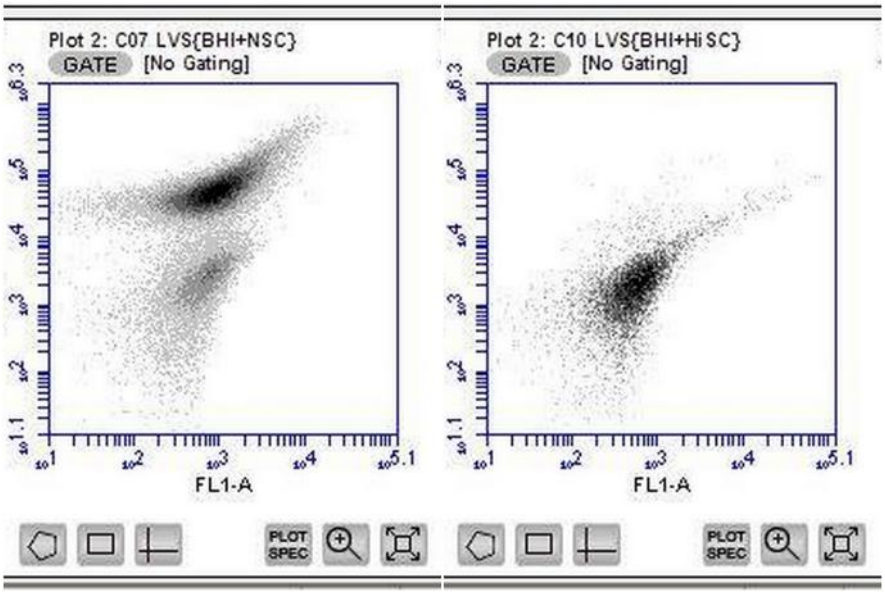

Figure 12. Flow cytometric analyses of complement sensitivity assay of LVS in normal (left side) and heat inactivated (right side) cattle serum.
Elevated fluorescent emission is observed in the case of LVS incubated with normal cattle serum due to mass bacterial cell killing.

In contrast with the attenuated strain, the wild strains (both B.FTNF002-00 and B.12 isolates) stayed intact after incubation with normal hare and cattle sera. No significant differences were observed between live cell rates of distinct genotypes of *Francisella* wild strains in the examined sera (Table 5).

Table 5. Mean values of event counts by flow cytometry after complement sensitivity assays of *Francisella tularensis* ssp. *holarctica* strains in the sera of selected animal species.

Strain ID	Origin	Genotype	Virulence	mouse serum		hare serum		cattle serum	
				inact.	norm.	inact.	norm.	inact.	norm.
21851/2006	Italy	B.FTNF	wild	121	129	290	321	249	254
FT6	Spain	B.FTNF	wild	95	87	168	167	164	184
FTH24/08	Hungary	B.12	wild	114	104	172	144	215	205
LVS	Russia	B.12	attenuated	104	92	256	50	364	88

B.FTNF= B.FTNF002-00; inact.=heat inactivated serum; norm.= normal serum

Despite the observed host–pathogen interactions in complement sensitivity assays, further examinations on fH binding to bacterial cell membrane proteins using Western blot assays and pull-down assays did not reveal specific interactions in any of the animal species examined (Fig. 13.). Protein mass fingerprinting identified the unspecific binding of an undefined 42 kDa membrane protein of *F. tularensis* to serum fH and a 72 kDa competence protein of *F. tularensis* to the primary antibody sheep anti-fH.

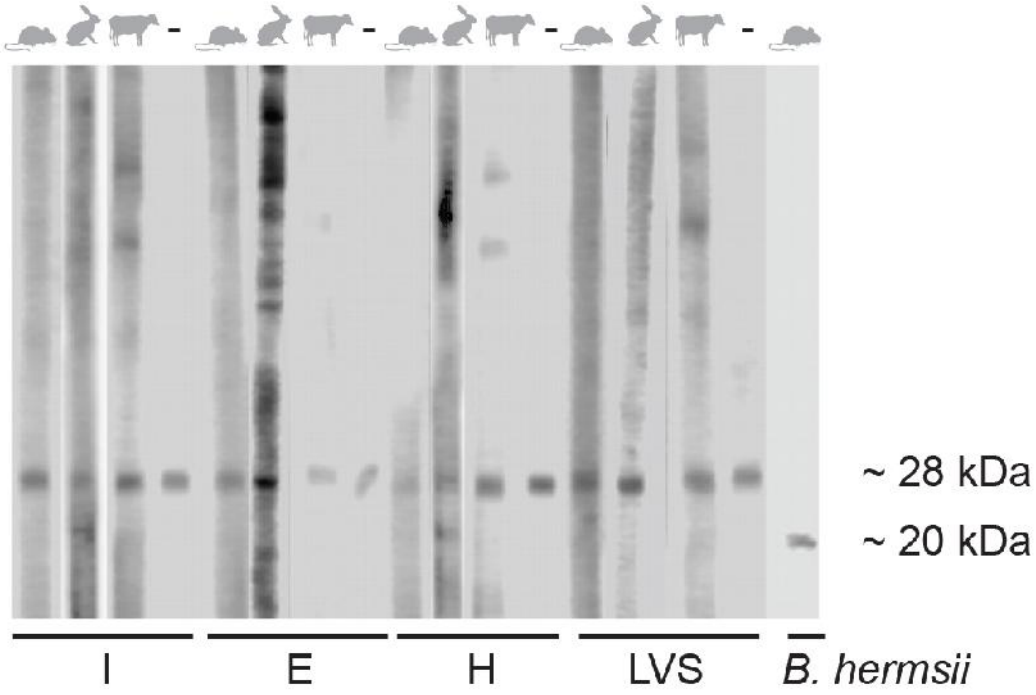

Figure 13. Western blot assays for the detection of fH binding with *F. tularensis* ssp. *holarctica* strains in selected animal species.
Animal icons (mouse, hare and cattle) represent the origin of the sera examined; negative control (–) excluding sera from the reagents are presented in each case. Abbreviations represent the positive control *Borrelia hermsii* (***B. hermsii***) and the origin of *Francisella* strains according to Fig. 11. *B. hermsii* binds fH with its 20 kDa protein, FhbA. Non-specific binding of the primary antibody by ~28 kDa *Francisella* membrane protein can be observed.

5.4. Comparison of pathogenicity of *Francisella* genotypes B.12 and B.FTNF002-00

The virulence of *F. tularensis* ssp. *holarctica* strains of the two *Francisella* genotypes, B.FTNF002-00 and B.12 endemic in Europe was compared in three concentrations infecting Fischer 344 rats intraperitoneally (ip). All rats showed clinical signs after ip inoculation of *F. tularensis* ssp. *holarctica* strains (both genotypes B.12 and B.FTNF002-00), between days 4-12 post infection (pi.). Clinical signs included porphyrin accumulation around the eyes, nasal discharge, weight loss, weakness, ruffled fur, inactivity, diarrhea and laboured breathing. According to the severity of clinical signs three categories (mild, moderate and severe) were distinguished (Table 6), but the categories did not correlate with the challenge dose.

Table 6. Categories of clinical signs shown by *Francisella* infected Fischer 344 rats

Severity of disease	Clinical signs
mild	weight loss, accumulation of porphyrin around the eyes (one or both sides), nasal discharge
moderate	weight loss, definite porphyrin secretion (one or both sides), nasal discharge, ruffled fur, decreased activity, diarrhoea
severe	weight loss, definite porphyrin secretion (both sides), nasal discharge, ruffled fur, inactivity, diarrhoea, laboured breathing, weakness

More than 50% of the rats survived the ip challenge by *F. tularensis* ssp. *holarctica* strains in all, but one group (genotype B.FTNF002-00, 10^0 CFU) and severity of the disease did not correlate with the challenge dose, thus further analysis is based on the comparison of the two main groups differing in the infective agent. More rats showed severe clinical signs infected with the B.FTNF002-00 genotype, although the difference was not significant (p=0.066) (Fig. 14.).

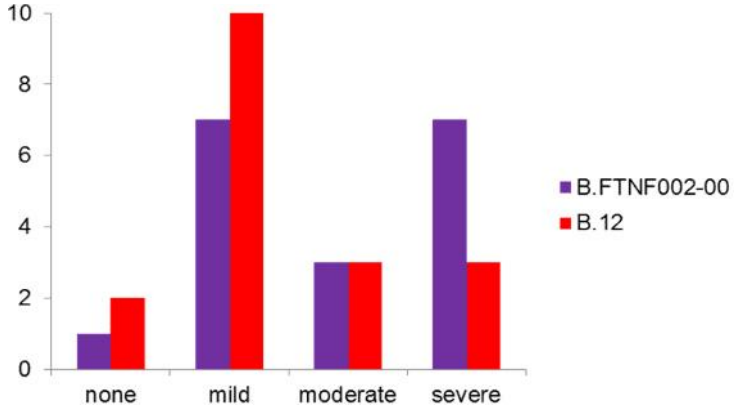

Figure 14. Number of rats showing different severity of clinical signs

In the B.FTNF002-00 genotype infected group 33% (6/18) of the animals succumbed between days 4-12 pi., losing 5.6-35.5% of their body weight. In contrast, only 11% (2/18) of the rats died of the disease caused by B.12 genotype on days 8 and 10 pi., with 18.1% and 24.0% weight loss, respectively. At necropsy, the deceased rats were seronegative while all the survived rats showed positive reaction in slide agglutination test on day 21 pi. Macroscopic pathological findings were scarce, enlarged spleen was occasionally observed in the deceased and euthanized rats as well. Histopathological examinations showed similar pathological changes in the case of both infecting agents. Histological findings in rats that succumbed to the infection consisted of acute multiplex necrotic foci in the liver and spleen and IHC showed high amounts of antigens in these organs (Fig. 15A.). Sub-acute interstitial lymphohistiocytic inflammation was also observed in the lung with high or moderate amounts of antigens in rats that died of the infection (Fig. 15B.). Seropositive rats which were sacrificed on day 21 pi. showed sub-acute serous inflammation in the liver and spleen with no or low amounts of antigens.

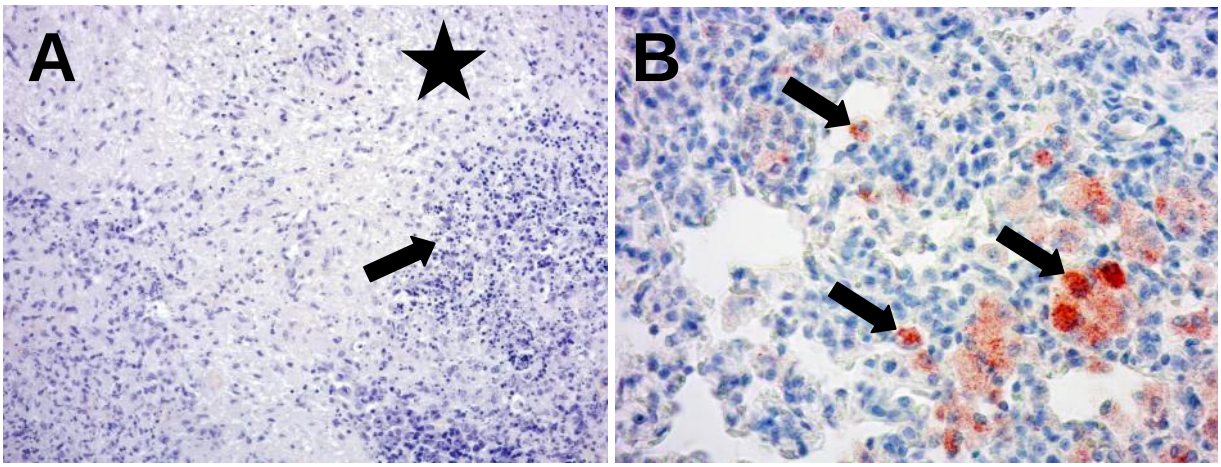

Figure 15. Histopathological and IHC findings in the spleen (**A**) and and lung (**B**) of succumbed rats.
Signs of acute infection are shown in the hematoxylin eosine stained slide of spleen (**A**) with lymphocyte depletion (**asterix**) and necrotic cells (**arrow**), 20x magnification. In the lung (**B**) high amounts of stained *Francisella* antigen accumulated in the macrophages (**arrows**) are observed with IHC, 40x magnification.

5.5. Antibiotic susceptibility examinations of *F. tularensis* ssp. *holarctica* strains from Hungary

From the collection of *F. tularensis* ssp. *holarctica* strains originating from Hungary 29 isolates were systematically chosen for antibiotic susceptibility examination considering their geographical origin, host species and genetic characteristics. The selected strains originated from European brown hares (28 strains) and a patas monkey from different parts of Hungary. Phylogenetic analyses demonstrated that the strains belonged to the subclades B.23/14/25 (*n*=1), B.20/21/33 (*n*=4), B.33/34 (*n*=18), B.35/36 (*n*=1), B.37/38 (*n*=4) and B.Tul07/2007 (*n*=1) of group B.12. According to the MIC values that inhibited the growth of 90% of the strains (MIC_{90}), resistance to erythromycin (>256 mg/L) and linezolid (32 mg/L) and susceptibility to aminoglycosides (gentamicin, 0.75 mg/L; and streptomycin, 6.0 mg/L), quinolones (ciprofloxacin, 0.047 mg/L; and levofloxacin, 0.023 mg/L), tetracyclines (tetracycline, 0.5 mg/L; and doxycycline, 1.0 mg/L), rifampicin (1.0 mg/L), tigecycline (0.19 mg/L) and chloramphenicol (1.5 mg/L) were observed in all 29 strains (Fig. 16., Tables 7 and S8).

Figure 16. *In vitro* susceptibility of *F. tularensis* ssp. *holarctica* strains from Hungary. The strains were susceptible to quinolones recommended in clinical use (e.g. levofloxacin, **A**), and to tigecycline (**B**) which represents the potential of clinical use of this antibiotic against tularaemia. All strains were resistant to erythromycin (**C**).

Table 7. *In vitro* activity of 11 antibiotics against 29 Hungarian *F. tularensis* ssp. *holarctica* clinical strains and *F. tularensis* reference strains (LVS and Schu S4), and the CLSI susceptibility breakpoints.

Antibiotics	MICs (mg/L) for clinical strains			MICs (mg/L) for LVS	MICs (mg/L) for Schu S4[a]	CLSI susceptibility breakpoints
	MIC range	MIC_{50}	MIC_{90}			
streptomycin	3.0-8.0	4	6	0.38	0.0125	<8[b]
gentamicin	0.38-1.0	0.5	0.75	0.094	0.032	<4[b]
ciprofloxacin	0.012-0.047	0.032	0.047	0.008	0.016	<0.5[b]
levofloxacin	0.004-0.023	0.016	0.023	0.006	NA	<0.5[b]
tetracycline	0.19-0.72	0.38	0.5	0.19	NA	<4[b]
doxycycline	0.125-1.5	0.75	1	0.25	0.25	<4[b]
chloramphenicol	0.5-1.5	1	1.5	1	0.5	<8[b]
rifampicin	0.5-2.0	1	1	0.094	0.25	<1[c]
tigecycline	0.094-0.19	0.125	0.19	0.064	NA	<2[d]
erythromycin	>256.0	>256.0	>256.0	>256.0	2	<1[e]
linezolid	12.0-48.0	24	32	6	1	<4[c]

NA: data not available.
[a]Values originating from the study of Johansson *et al*. (2002)
CLSI standard breakpoints for [b]*F. tularensis*, [c]staphylococci, [d]Enterobacteriaceae and for [e]*S. pneumoniae*

6. Discussions

6.1. Francisellaceae in ticks from Hungary and Ethiopia

Tularaemia occurs mainly in the Northern Hemisphere and knowledge on the presence of *F. tularensis* and FLEs in Africa is limited (Brevik *et al*., 2011, Keim *et al*., 2007, Mohamed *et al*., 2012, Scoles, 2004). Previous studies in Central Europe (Austria, Czech Republic and Slovakia) demonstrated a 0.1–2.8% prevalence of *F. tularensis* in Ixodid ticks (Gurycová *et al*., 1995, Hubálek *et al*., 1997). FLEs were detected in several countries throughout Europe by amplifying the sequences of the 16S rRNA, *tul4*, *lpnA* and/or *sdhA* genes. While *D. marginatus*, *D. reticulatus*, and *I. ricinus* ticks have been reported to harbour *F. tularensis* in Europe, *Francisella*-like agents were detected in *Amblyomma* spp., *Dermacentor* spp., *Hyalomma* spp. and in *Rhipicephalus* spp. (de Carvalho *et al*., 2011, Escudero *et al*., 2008, Franke *et al*., 2010, Hubálek *et al*., 1997, Ivanov *et al*., 2011, Milutinovic *et al*., 2008, Sréter-Lancz *et al*. 2009, Stanek, 2009, Toledo *et al*., 2009, Tomanovic *et al*., 2013, Wicki *et al*., 2000).

Tularaemia is known to be endemic in Hungary, and the results of the study confirm the role of ticks in the ecology of the disease and highlights that ticks carrying the pathogen could pose a threat to public health (Gyuranecz *et al*., 2010d). The prevalence of *F. tularensis* (0.27%) in the Hungarian tick population was within the range found in the neighbouring countries (Gurycová *et al*., 1995, Hubálek *et al*., 1997). The prevalence of the pathogen in ticks is in correlation with the moderate activity of the disease in the tick collection period, as seropositivity in the European brown hare population (0.66–1.1%) and the annual number of human cases 20–25 were relatively low at that time (Gyuranecz *et al*., 2010d).

Genetic analysis of the FLE of *D. reticulatus* revealed, that although the *tul4* gene sequence of this FLE was identical to Hungarian (GenBank No.: EU126640) and Portuguese (GenBank No.: GU113085) FLEs found earlier, and the 16S rRNA sequence was also identical to the sequence of the endosymbiont of *D. reticulatus* described in Bulgaria (GenBank No.: HQ705173), these 16S rRNA gene coding sequences differed in 2 nucleotides from the one found earlier in this tick species in Hungary (GenBank No.: EU234535) (Fig. 7., 16S rRNA and tul4). This divergence may appear to be a minor difference between the sequences, but Francisellaceae have a very conservative genetic character and this 2-nucleotide-divergence between the FLEs is equivalent in magnitude to the difference between the type strain (Schu S4, accession number: AJ749949) of the highly virulent *F. tularensis* ssp. *tularensis* and the attenuated *F. tularensis* ssp. *holarctica* LVS (accession number, AJ698866;

Fig. 7., 16S rRNA) (Keim *et al*. 2007). Thus, this is a notable difference that could lead to the hypothesis that there may be 2 distinct FLEs circulating in *D. reticulatus* populations in Hungary and therefore in Europe. However, the FLEs of *D. reticulatus* from Hungary differing in their 16S rRNA genes showed identical *tul4* gene sequences, and the samples were collected from the same geographical region within a relatively short time, for which a technical error during sequencing cannot be ruled out. Based on the identical sequences of the 17 kDa lipoprotein and 16S rRNA genes of the FLE species harboured by *D. reticulatus* in Europe host adaptation and a host species–linked evolution of this FLE species could be assumed.

Birds in the epidemiology of tick-borne diseases may act as transporters of ticks, frequently disseminating them to large distances, especially during seasonal migration. Birds can also serve as reservoirs of the pathogens, providing the source of infection for ticks during bacteraemia (Elfving *et al*., 2010, Hubálek, 2004). The significance of birds in these situations, especially in the case of zoonotic pathogens may be particularly high in urban and periurban habitats. The European robin is a synanthropic migratory bird, which arrives to Hungary from the Mediterranean countries (Csörgő *et al*., 2009). These birds can cover few hundreds of kilometres during a single day of migration, and tick larvae and nymphs are known to attach and feed for several days, thus it is assumed that most of the ticks collected from European robin in this study derived from southern Europe (Babos, 1964). The *I. ricinus* tick containing the FLE detected in the study most likely acquired the endosymbiont transovarially, because an engorged nymph from the same bird was PCR negative. However, the horizontal transfer (e.g. by co-feeding) from other ticks cannot be excluded, as suggested in the case of the ancestors of these endosymbionts (Scoles, 2004). The identification of a FLE in *I.ricinus* from a European robin in 2012 was the first molecular evidence of their occurrence in *Ixodes* spp. and it was indicated for the first time that FLEs may associate with bird ticks. The sequence divergence between the *Francisella*-like agent of *I. ricinus* in the present study and those already reported from *D. reticulatus* exceeds in magnitude the difference between *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica* in the same part of their 16S rRNA genes, thus the FLE is considered to be a new *Francisella* variant.

F. tularensis occurs primarily in North-America and Eurasia and the *Francisella*-like agents might be more prevalent in those areas than in Africa. The screening of 296 individual ticks of 6 species collected from cattle in Ethiopia resulted in the detection of the 16S rRNA and *sdhA* gene fragments of a FLE in a *Hy. rufipes*. Unfortunately, *sdhA* gene has not been used for FLE detection and comparison previously, thus the comprehensive analysis of this sequence was not possible. The amplification of the *tul4* gene fragments of this FLE using two different primer pairs failed. Similar results were gained during the examination of ticks from Bulgaria, where only six out of twelve 16S rRNA gene based PCR-positive FLEs resulted positive with the *tul4* gene based PCR assay as well, using the TUL4B-F/TUL4B-R primer pair

(Ivanov *et al*., 2011). These findings suggest that the *tul4* gene of some FLEs may significantly differ from that of *F. tularensis*, whilst others' are similar enough for causing misidentification using PCR assay without sequencing (Escudero *et al*., 2008, Kugeler *et al*., 2005, de Carvalho *et al*., 2011). The sequence of the 16S rRNA gene fragment of the detected endosymbiont of *Hy. rufipes* was identical with that of the endosymbionts described in *R. sanguineus* and *Hy. marginatum* collected in Bulgaria (Ivanov *et al*., 2011). The detection of endosymbionts with identical 16S rRNA gene sequences in a *Rhipicephalus* and two *Hyalomma* species supports the hypotheses, that most FLEs had independent evolution from their tick hosts (Ivanov *et al*., 2011, Scoles, 2004). Since the first detection of a FLE (*W. persica* in a soft tick in Egypt, 1961), FLEs have been detected only twice from Africa: in the hard tick *Hy. truncatum* from Namibia (GenBank No.: JF290387) and in the soft tick *Ornithodoros porcinus* from Southern Africa (Brevik *et al*., 2011, Scoles, 2004). Phylogenetic analysis of the 16S rRNA gene fragments revealed close relatedness among endosymbionts of hard ticks from Europe and Africa (Fig. 7.). Sporadic occurrence of *Hy. rufipes* was reported in Europe, probably transported by migrating birds, but the detection of FLE in this species has not been documented so far, thus this is the first molecular evidence of a FLE in this tick species (Hornok and Horváth, 2012).

More recent examinations on FLEs in France, Germany and Poland confirmed the presence of the same FLE, what we had found in *D. reticulatus* in other parts of Europe, which supports the hypothesis that this FLE has host species–linked evolution (Gehringer *et al*., 2013, Michelet *et al*., 2013, Wójcik-Fatla *et al*., 2015). Currently, FLEs with identical sequences of the 17 kDa lipoprotein gene were reported in *Ixodes* spp. (de Carvalho *et al*., 2015, Wójcik-Fatla *et al*., 2015). The identical sequences of these FLEs of *Ixodes* spp. (Prostriata, *Ixodinae*) and of the FLE of *D. reticulatus* (Metastriata, *Rhipicephalinae*), tick species of another subfamily likely support the hypothesis that FLEs can be transmitted horizontally (e.g. by co-feeding) (de Carvalho *et al*., 2015, Wójcik-Fatla *et al*., 2015). Even more, FLEs have been described lately in wood mouse (*Apodemus sylvaticus*) in Portugal, and one of these FLEs was found in ticks as well (in *D. marginatus*, *R. pusillus* and in *R. sanguineus*), representing the potential of FLEs to occur in small mammals, and based on these results the horizontal transmission of FLEs between small mammals and ticks might be also possible (de Carvalho *et al*., 2015).

6.2. Genotyping of *F. tularensis* ssp. *holarctica* strains by high resolution molecular methods

Phylogenetic analysis of *Francisella* species with high resolution molecular typing methods promotes understanding of epidemiologic characteristics and evolutionary history of the bacteria. The performed canSNP and MLVA typing of 69 Hungarian *F. tularensis* ssp. *holarctica* strains showed close genetic relationships between the isolates, and no correlations were found between genotypes and other characteristics of the strains (e.g. host, year of isolation or geographic origin). All strains belonged to the main clade B.12 showing lower overall genetic diversity of the pathogen in Hungary compared to Scandinavian countries or Turkey (Karadenizli *et al*., 2015, Özsürekci *et al*, 2015, Svensson *et al*., 2009a, Vogler *et al*., 2009a). However, higher resolution of the strains' genetic characteristics revealed the presence of 8 subclades of the B.12 group in the country, which supports the hypothesis that *F. tularensis* ssp. *holarctica* has descended from a diverse set of minor subclades in Hungary shared with isolates from central Europe, Scandinavia and Russia (Gyuranecz, 2012a). The majority of the strains (88.4%) belonged to subclade B.33/34 and to its derivated subclades, which were detected and predominant in all examined counties. The WG sequencing of 9 selected strains further confirmed the relative diversity of the strains in Hungary described by canSNP and MLVA typings, and these sequences will be used in a comprehensive global phylogenetic analysis of *Francisella* species as well.

In previous studies *Francisella* was hypothesized to have landscape-epidemiology, consisting of the presence of phylogenetically distinct clones in restricted regions, which could persist in the environment for decades (Johansson *et al*., 2014, Karlsson *et al*., 2013, Petersen *et al*., 2008, Svensson *et al*., 2009b). Environmental factors (e.g. climate or the density of host populations) are suggested to be involved in the triggering of tularaemia outbreaks, thus genetically distinct clones are usually detected in epidemics instead of the spread of a certain clone with enhanced infectivity and fitness (Gyuranecz *et al*., 2012b, Johansson *et al*., 2014, Karlsson *et al*., 2013). Nevertheless, during longer periods, clones with higher fitness may predominate in certain regions, completing a selective genetic sweep in the area (Svensson *et al*, 2009b). Considering this landscape-epidemiology of the pathogen, the interpretation of genetic similarities of the isolates in epidemiological investigations during tularaemia outbreaks should be handled with caution (Johansson *et al*., 2014).

In the current study, isolates of different genotypes were involved in tularaemia outbreaks in many regions (e.g. strains FTH18 and 19 from Szegvár, FTH50 and 53 from Püspökladány or strains FTH57 and 66 from Báránd, Fig. 10.), which is in accordance with the suggestion that epidemics are triggered by ecological factors rather than the increased infectivity of a specific *F. tularensis* clone. The detection of identical genotypes of *Francisella* strains from zoo monkeys succumbed to tularaemia in 2003 and 2014 present an example for long-term environmental phase of the pathogen, which is to be considered in the prevention of human infections as well.

6.3. Host-pathogen interactions between *Francisella* strains and selected animal species

As an intracellular bacterium *F. tularensis* has to evade a diverse spectrum of extracellular and intracellular defence reactions during its pathogenesis. Moreover, for its rapid dissemination in the host system, the bacterium survives and replicates in the extracellular compartments; thus the subversion of a first-line defence system, the complement system is crucial in the bacterial invasion (Clinton *et al*., 2010, Yu *et al*., 2008).

Complement sensitivity assays were performed on three wild and one attenuated *F. tularensis* ssp. *holarctica* strains of two genotypes (B.FTNF002-00 and B.12) in the sera of selected animal species with different susceptibility to tularaemia. The comparison of host-pathogen interactions in the *in vitro* experiments showed differences in the resistance of the strains to serum killing, in conformity with previous observations (Jones *et al*., 2012).

In tularaemia and intracellular bacteria research, a frequently used experimental infection model is the mouse infected with *F. tularensis* ssp. *holarctica* LVS, as the strain has attenuated virulence in humans, but still can cause lethal disease in mice (Elkins *et al*., 2003). In the experiments most bacterial cells - disregarded of their genotypes or attenuation - stayed intact after incubation with mouse serum. The demonstrated resistance of the attenuated LVS strain to serum killing in mouse is consistent with the known high susceptibility of this animal species to tularaemia (Elkins *et al*., 2003).

The European brown hare, a main source of human infections, is considered to be a reservoir species for *F. tularensis* ssp. *holarctica* B.12 strains in Central and Eastern Europe, developing sub-acute pathological changes during infection (Gyuranecz *et al*., 2010b, 2012ab). In the experiments the attenuated LVS cells were lysed in normal hare serum, which highlights the capability of this host to control tularaemia infection. The wild strains (both B.FTNF002-00 and B.12 isolates) stayed intact after incubation with normal hare serum, which is in accordance with the virulence characteristics of the strains.

The relative resistance of cattle to *F. tularensis* suggests a limited role of this host in the epidemiology of the pathogen, as the animals most probably eliminate the bacteria during seroconversion (Mörner and Sandstedt, 1983). The complement sensitivity assays showed mass bacterial cell killing in the case of the attenuated LVS cells in cattle serum. However, wild bacterial strains (B.FTNF002-00 and B.12 isolates as well) were resistant to serum killing in cattle, which assumes that cattle eliminate the pathogen after seroconversion with the help of the adaptive immune system.

While differences in virulence among *F. tularensis* subspecies or even among genetic clades of subspecies are noticeable (e.g. in the case of *F. tularensis* ssp. *tularensis* clade A1 and clade A2), the phylogeographically distinct *F. tularensis* ssp. *holarctica* wild strains, originating from Italy and Spain (B.FTNF002-00 genetic group) and from Hungary (B.12 genetic group) revealed no relevant differences in their survivability in the serum of the animal species examined (Keim *et al*., 2007; Molins *et al*., 2010).

Previously, the binding of fH complement regulator protein to the surface of both *F. tularensis* ssp. *tularensis* strain Schu S4 and *F. tularensis* ssp. *holarctica* strain LVS has been described in humans (Ben Nasr and Klimpel, 2008). However, the exact mechanism of this binding has not been discovered yet, but fibrinogen and/or plasmin are hypothesised to have a promoter role in fH binding to *F. tularensis* cell surface (Crane *et al*., 2009, Jones *et al*., 2012). Although in the present study host–pathogen interactions were observed by complement sensitivity assays, further examinations on fH binding to bacterial cell membrane proteins using Western blot assays and pull-down assays did not reveal specific interactions in any of the animal species examined. The lack of direct fH binding to *F. tularensis* membrane proteins might result from the absence of a co-factor (e.g. fibrinogen or plasmin) or may represent difference in the individual hosts' immunity and might suggest that the pathogen does not use fH binding during complement evasion in animal hosts. Further examinations are needed for the identification and characterization of the unspecific binding of certain proteins of the pathogen to fH and anti-fH antibodies found by pull-down assays. Future experiments are required also for the description of interactions between the different hosts' C3 component (key member of the complement system) and the pathogen to reveal differential kinetics among *Francisella* and the animal hosts of distinct susceptibility to tularaemia.

6.4. Comparison of pathogenicity of *Francisella* genotypes B.FTNF002-00 and B.12

While clear differences are described among *F. tularensis* ssp. *tularensis* subpopulations, little or no information is available about the subpopulations of the widespread *holarctica* subspecies. The two genotypes of *F. tularensis* ssp. *holarctica* described in Europe (B.12 and B.FTNF002-00) differ in their geographic distribution, and the difference in the pathological signs of tularaemia in European brown hare originating from distinct geographic regions might assume the probability of difference in virulence of these genotypes as well (Decors *et al*., 2011, Gyuranecz *et al*., 2010b, Rijks *et al*., 2013).

The susceptibility of Fischer 344 rats to tularaemia was described in previous examinations (Jemski, 1981, Signarovitz *et al*., 2012, Wu *et al*., 2009). The artificial infection of the rats with ip inoculation of 10^1 CFU of a *F. tularensis* ssp. *holarctica* strain from Sweden (B.12 genotype) manifested fatal disease within 10 days in this species (Raymond and Conlan, 2009). In the current study, the virulence of two *F. tularensis* ssp. *holarctica* strains of genotypes B.12 and B.FTNF002-00 was compared in Fischer 344 rats by ip inoculation of 10^0, 10^1 and 10^2 CFU bacteria. The severity of the disease did not correlate with the challenge dose and mortality rates reached the LD_{50} in only one group of the animals (infected with 10^0 CFU of the B.FTNF002-00 strain), which might be in connection with a possible attenuation process during culturing of the bacteria on artificial media and suggests the need of higher bacterial load for experimental infection. However, clinical signs manifested in most rats, and they were in accordance with previously described symptoms in Fischer 344 rats infected subcutaneously with *F. tularensis* ssp. *tularensis* SCHU S4 strain (Wu *et al*., 2009). The number of rats with severe clinical signs was higher in the B.FTNF002-00 infected group, compared to the B.12 genotype infected group. Most of the rats (n=6/8) succumbed to the infection by day 8 pi., and detectable difference was observed in the mortality rates between the two groups. The results revealed difference in the pathogenic potential of the two strains and supports the hypothesis that B.FTNF002-00 genotype is moderately more virulent than the B.12 genotype. Nevertheless, experimental infections repeated in the brown hare, the host which shows the presumptive pathological changes and involving higher number of strains of the two genotypes probably would enlighten better the possible differences between the genotypes' virulence.

6.5. Antibiotic susceptibility examinations of *F. tularensis* ssp. *holarctica* strains from Hungary

The examination of the susceptibility of selected 29 Hungarian *F. tularensis* strains to 11 antibiotics with potential to be used in clinical therapy was performed in the study. As the resistance of *F. tularensis* ssp. *holarctica* to beta-lactam antibiotics and cephalosporins (with few exceptions) has already been confirmed in several studies, these antibiotics were excluded from the present study (García del Blanco *et al*., 2004, Georgi *et al*., 2012, Ikäheimo *et al*., 2000, Tomaso *et al*., 2005, Yesilyurt *et al*., 2011).

F. tularensis ssp. *holarctica* strains could be categorized into two biovars based on their erythromycin susceptibility, where biovar I is the erythromycin sensitive (present in Western Europe: France, Germany, Spain and Switzerland; genotype B.FTNF002-00) while biovar II is the resistant type (present in Northern and Eastern Europe: Austria, Germany, Sweden and Turkey; all other genotypes of the *holarctica* subspecies) (Georgi *et al*., 2012, Keim *et al*., 2007, Yesilyurt *et al*., 2011). All Hungarian strains proved to be consistently resistant to erythromycin, thus confirming their classification into biovar II (B.12 genotype).

Linezolid is used in the treatment of infections caused by Gram-positive bacteria, and it is especially active against vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*. The *in vitro* susceptibility of *F. tularensis* to linezolid has been also demonstrated recently (Yesilyurt *et al*., 2011, Sutera *et al*., 2014). In the previous studies linezolid showed 0.5-4 mg/L MIC values on solid medium and 1 mg/L MIC values in the extracellular compartment when examined in cell cultures (Yesilyurt *et al*., 2011, Sutera *et al*., 2014). Contrary to these findings, all Hungarian *F. tularensis* strains were resistant to linezolid (MIC range: 12-48 mg/L), similarly to North American *F. tularensis* ssp. *holarctica* strains (Johansson *et al*., 2002).

In the treatment of human tularaemia infections, the aminoglycosides gentamicin and streptomycin are the antibiotics of choice in Hungary (Herpay *et al*., 2011). All strains were susceptible *in vitro* to both antibiotics, but it should be noted that in one case (the strain from the patas monkey) the MIC value for streptomycin reached the limit of intermediate susceptibility (8 mg/L).

In 2011, the National Centre of Epidemiology (Budapest, Hungary) recommended ciprofloxacin and chloramphenicol for post-exposure prophylaxis of tularaemia (Herpay *et al*., 2011). The examined *F. tularensis* strains showed high susceptibility to quinolones (ciprofloxacin and levofloxacin) and chloramphenicol as well, although the latter has serious side effects thus its use in therapy is limited to exceptional cases (e.g. tularaemia with meningitis) (Hofinger *et al*., 2009, Tomaso *et al*., 2005).

The WHO's guidelines on tularaemia also recommend tetracyclines and especially doxycycline for the therapy of tularaemia (WHO, 2007). The examined strains showed good *in vitro* susceptibility to both tetracycline and doxycycline; however, the risk of relapse should be considered during the clinical use of these antibiotics (Ahmad *et al*., 2010, Hepburn and Simpson, 2008, Urich and Petersen, 2008).

F. tularensis susceptibility to tigecycline was detected for the first time in Turkey (Yesilyurt *et al*., 2011). Tigecycline is a member of the glycylcyclines, a new class of antibiotics that achieves high intracellular concentrations; hence, its use in the treatment of tularaemia has also been recommended (Yesilyurt *et al*., 2011). Examining the Hungarian strains' susceptibility to tigecycline, the results were consistent with the susceptibility reported in the publication of Yesilyurt and co-workers (2011). Due to the low *in vitro* MIC values of tigecycline, this antibiotic may have potential in the clinical therapy of tularaemia in Hungary as well.

Rifampicin was also effective *in vitro* against the *F. tularensis* strains, but due to its tendency for emerging resistance in monotherapy, its use is only recommended in combination with other antibiotics (e.g. tetracyclines) (Ikäheimo *et al*., 2000, Tomaso *et al*., 2005, Yesilyurt *et al*., 2011).

In conclusion, on the basis of *in vitro* examinations, quinolones are recommended as first choice in the therapy of tularaemia in Hungary. Oral application of ciprofloxacin (2x500 mg for adults and 2x10-15 mg/kg for children) or levofloxacin (500 mg for adults) for tularaemia treatment takes 2 weeks of daily administration (Bossi *et al*., 2004). The use of aminoglycosides, tetracyclines and chloramphenicol is also appropriate against *F. tularensis* in Hungary. In the case of moderate clinical signs, the daily administration of doxycycline for 3 weeks (2x100 mg for adults and 2x2.2 mg/kg for children) is recommended, while in severe forms the intravenous application of gentamicin for 10 days (5 mg/kg for adults and 2.5 mg/kg for children) is suggested (Bossi *et al*., 2004). The *in vitro* effectiveness of tigecycline against *F. tularensis* ssp. *holarctica* suggests the applicability of this antibiotic in tularaemia treatment as well, but further *in vivo* examinations are required for confirmation. The use of macrolides (e.g. erythromycin) and linezolid in the treatment of tularaemia should be avoided in Hungary.

7. Overview of the new scientific results

Ad 1. Ticks possess epidemiologic importance in the case of tularaemia in Hungary. Host adaptation of the FLE of *D. reticulatus* is hypothesised, while most FLEs had independent evolution from their tick hosts. A novel FLE variant was detected in *I. ricinus*, a new tick host of the agent. FLEs from Europe and Africa are closely related.

Ad 2. Relatively high genetic diversity was described of *F. tularensis* ssp. *holarctica* in Hungary. The population structure of the strains suggests the parallel emergence of multiple clones from the environment during outbreaks. The pathogen has long-term dormancy with low replication rates in the environment.

Ad 3. The wild, virulent *F. tularensis* ssp. *holarctica* strains resist serum killing in mice, hare and cattle. The attenuated LVS strain could evade the complement system of mice only. For the interactions the direct, specific binding of factor H on the cell surface is not needed in the examined animal hosts, or the pathogen might need a co-factor for the binding of factor H.

Ad 4. The *F. tularensis* ssp. *holarctica* genotype dominant in Western Europe is suggested to have moderately higher pathological potential, than the genotype dominant in Central and Eastern Europe.

Ad 5. Levofloxacin, ciprofloxacin and doxycycline are the recommended antibiotics for clinical use against tularaemia in Hungary. The effectiveness of tigecycline in the *in vitro* examinations suggests the potential of this antibiotic in the therapy of tularaemia. The use of linezolid and macrolides against tularaemia in the region should be avoided.

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10. Supplements

Table S1. Host, geographic origin, year of isolation of *Francisella tularensis* ssp. *holarctica* strains and examinations performed on the strains.

Strain ID	Host	City of origin	County of origin	Year of isolation	can SNP	MLVA	WGS	AB	H-P	V
FTH1/03	patas monkey	Szeged	Cs	2003	X	X		X		
FTH2/03	vervet monkey	Szeged	Cs	2003	X	X				
FTH3/07	brown hare	Alattyán	JNSz	2007	X	X		X		
FTH4/07	brown hare	Kengyel	JNSz	2007	X	X		X		
FTH5/07	brown hare	Békés	B	2007	X	X	X	X		
FTH6/07	brown hare	Battonya	B	2007	X	X	X	X		
FTH7/07	brown hare	Szarvas	B	2007	X	X		X		
FTH8/07	brown hare	Körösladány	B	2007	X	X		X		
FTH9/07	brown hare	Köröstarcsa	B	2007	X	X		X		
FTH10/07	brown hare	Csökmő	HB	2007	X	X		X		
FTH11/07	brown hare	Jászberény	JNSz	2007	X	X	X	X		
FTH12/08	brown hare	Kecel	BK	2008	X	X		X		
FTH13/08	brown hare	Jászárokszállás	JNSz	2008	X	X		X		
FTH14/08	brown hare	Hegyeshalom	GyMS	2008	X	X		X		
FTH15/08	brown hare	Jánossomorja	GyMS	2008	X	X	X	X		
FTH16/08	brown hare	Dévaványa	B	2008	X	X				
FTH17/08	brown hare	Dévaványa	B	2008	X	X				
FTH18/08	brown hare	Szegvár	Cs	2008	X	X		X		
FTH19/08	brown hare	Szegvár	Cs	2008	X	X				
FTH20/08	brown hare	Mindszent	Cs	2008	X	X				
FTH21/08	brown hare	Bucsa	B	2008	X	X				
FTH22/08	brown hare	Szeghalom	B	2008	X	X				
FTH23/08	brown hare	Püspökladány	HB	2008	X	X	X			
FTH24/08	brown hare	Orosháza	B	2008	X	X	X	X	X	X
FTH25/08	brown hare	Gerendás	B	2008	X	X				
FTH26/08	brown hare	Szeghalom	B	2008	X	X				
FTH27/08	brown hare	Orosháza	B	2008	X	X				
FTH28/08	brown hare	Csanádpalota	Cs	2008	X	X	X	X		
FTH29/08	brown hare	Bucsa	B	2008	X	X				
FTH30/09	brown hare	Alattyán	JNSz	2009	X	X	X			
FTH31/09	brown hare	Füzesgyarmat	B	2009	X	X				
FTH32/09	brown hare	Füzesgyarmat	B	2009	X	X				
FTH33/09	brown hare	Surjány	JNSz	2009	X	X		X		
FTH34/09	brown hare	Gyomaendrőd	B	2009	X	X				
FTH35/09	brown hare	Jászjákóhalma	JNSz	2009	X	X	X			
FTH36/09	brown hare	Gyomaendrőd	B	2009	X	X				
FTH37/09	brown hare	Törökszentmiklós	JNSz	2009	X	X		X		
FTH38/09	brown hare	Hegyeshalom	GyMS	2009	X	X				

Strain ID	Host	City of origin	County of origin	Year of isolation	can SNP	MLVA	WGS	AB	H-P	V
FTH39/09	brown hare	Hegyeshalom	GyMS	2009	X	X		X		
FTH40/09	brown hare	Kevermes	B	2009	X	X				
FTH41/09	brown hare	Ópusztaszer	Cs	2009	X	X		X		
FTH42/09	brown hare	Gyomaendrőd	B	2009	X	X				
FTH43/09	brown hare	Szarvas	B	2009	X	X				
FTH44/09	brown hare	Szajol	JNSz	2009	X	X				
FTH45/09	brown hare	Szajol	JNSz	2009	X	X				
FTH46/09	brown hare	Szajol	JNSz	2009	X	X				
FTH47/09	brown hare	Okány	B	2009	X	X		X		
FTH48/09	brown hare	Okány	B	2009	X	X				
FTH49/09	brown hare	Kétegyháza	B	2009	X	X		X		
FTH50/09	brown hare	Püspökladány	HB	2009	X	X				
FTH51/09	brown hare	Dévaványa	B	2009	X	X		X		
FTH52/09	brown hare	Kétegyháza	B	2009	X	X				
FTH53/09	brown hare	Püspökladány	HB	2009	X	X				
FTH54/09	brown hare	Dévaványa	B	2009	X	X				
FTH55/09	brown hare	Békés	B	2009	X	X				
FTH56/09	brown hare	Békés	B	2009	X	X				
FTH57/10	brown hare	Báránd	HB	2010	X	X		X		
FTH58/10	brown hare	Püspökladány	HB	2010	X	X		X		
FTH59/10	brown hare	Battonya	B	2010	X	X				
FTH60/10	brown hare	Jászfelsőszent-györgy	JNSz	2010	X	X				
FTH61/10	brown hare	Jászfelsőszent-györgy	JNSz	2010	X	X		X		
FTH62/10	brown hare	Felsőszentiván	BK	2010	X	X				
FTH63/10	brown hare	Battonya	B	2010	X	X				
FTH64/10	brown hare	Szajol	JNSz	2010	X	X				
FTH65/10	brown hare	Szajol	JNSz	2010	X	X		X		
FTH66/10	brown hare	Báránd	HB	2010	X	X				
FTH67/10	brown hare	Felsőszentiván	BK	2010	X	X		X		
FTH68/10	brown hare	Szegvár	Cs	2010	X	X				
FTH69/14	red-handed tamarin	Szeged	Cs	2014	X	X				
FTH70/15	brown hare	Gyomaendrőd	B	2014	X	X				
FTH71/15	brown hare	Csökmő	HB	2014	X	X				
21851/ 2006	brown hare	Italy	NA	2006					X	X
FT6	NA	Spain	NA	NA					X	
LVS	NA	Russia	NA	NA	X	X		X	X	

Abbreviations are: NA: not available; canSNP: canonical single nucleotide polymorphism; MLVA: multi-locus variable number of tandem repeats analysis; WGS: whole genome sequencing; AB: antibiotic susceptibility; H-P: host-pathogen interactions; V: virulence comparison; B: Békés; BK: Bács-Kiskun; Cs: Csongrád; GyMS: Győr-Moson-Sopron; HB: Hajdú-Bihar; JNSZ: Jász-Nagykun-Szolnok.

Table S2. The number of pools and origin of examined tick species for the presence of Francisellaceae, with GenBank Accession numbers.

genus	species	Number of DNA pools	Positive pools	origin	country of origin	Francisellaceae found	GenBank accession number 16S rRNA	tul4	sdhA
Amblyomma	*cohaerens*	100		cattle	Ethiopia				
	lepidum	2		cattle	Ethiopia				
	variegatum	118		cattle	Ethiopia				
Dermacentor	*marginatus*	55		environment	Hungary				
	reticulatus	74	12	environment (dog)	Hungary	F.t.holarctica(1) FLE(11)	JQ942364 JQ942365	JQ942367 JQ942368	n.e. n.e.
Haemaphysalis	*concinna*	90	2	environment (hamster, migratory bird)	Hungary	F.t.holarctica	JQ942363	JQ942366	n.e.
	inermis	64		environment	Hungary				
	punctata	9		environment	Hungary				
Hyalomma	*marginatum*	3		migratory birds	Hungary				
	rufipes	1	1	cattle	Ethiopia	FLE	KJ522773	-	KJ864964
Ixodes	*acuminatus*	36		hamster	Hungary				
	ricinus	290	1	migratory birds (environment, rodent)	Hungary	FLE	JQ740890	-	n.e.
Rhipicephalus	*decoloratus*	50		cattle	Ethiopia				
	evertsi	17		cattle	Ethiopia				
	praetextatus	8		cattle	Ethiopia				
	sanguineus	3		environment	Hungary				

Abbreviations are: FLE:*Francisella*-like endosymbiont; F.t.:*Francisella tularensis*; n.e.:not examined.

Table S3. Primers used in canSNP typing and their volumes in the melt-MAMA reaction mixtures (Chanturia *et al*., 2011, Gyuranecz *et al*., 2012a, Vogler *et al*., 2009a)

SNP	Genome SNP state (D/A)	melt-MAMA primer sequences[c]	Primer volumes (µl)	Melting T_m (°C)
B.Br.012	T/A[a]	cggggcggggcggggcggggSGGTTCAGCACGTCAATATCA	0.15	77,5
		ttttttttttttttttttttCSGGTTCAGCACGTCAATATTT	0.15	72,5
		YARKACAATYGCAAATGCAAATG	0.15	
B.Br.013	G/A[a]	cggggcggggcggggcggggGTATATTGGGTATGGGCGAATGC	0.15	73.5, 80[d]
		ttttttttttttttttttttGGTATATTGGGTATGGGCGAATTT	0.15	70
		GCAGCAGGTAGTTGTAATAACTCTAGTAATAAA	0.15	
B.Br.020	C/T[a]	gcgggcgcgggcagggcggcTCTGATGAAGAATATCTTAgAg	0.15	74,7
		gcgggcTCTGATGAAGAATATCTTAaAa	0.15	71,7
		ATTATGGCAAAACTATACCTT	0.15	
B.Br.021	T/C[a]	gcgggcACCAAGGTAGATTTGCAGCTtCa	0.15	75,9
		gcgggcgcgggcagggcggcACCAAGGTAGATTTGCAGCTcCg	0.15	78.1, 82.1[d]
		ATCCCTGTTGGGATATCCTCGACTAA	0.15	
B.Br.022	A/G[a]	TGAATACTCTACGCGATAAGtTa	0.3	73,6
		gcgggcgcgggcagggcggcTGAATACTCTACGCGATAAGgTg	0.15	76.2, 80.2[d]
		ATCAGACTTAGGTGTTAGATCAGAGTT	0.15	
B.Br.023	A/C[a]	TTACTACAAATTCGCCTCTtAt	0.15	72,8
		gcgggcgcgggcagggcggcTTACTACAAATTCGCCTCTgAg	0.15	77,3
		AGCAAAAGAGCTTACTAAACAATTTGA	0.15	
B.Br.024	C/A[a]	gcgggcgcgggcagggcggcTATCGCCAGGTTTAATTTGgTg	0.15	80,6
		gcgggcTATCGCCAGGTTTAATTTGtTt	0.15	75,8
		TCTGCAGCATCTATCCCATTAGCCTTA	0.15	
B.Br.025	A/G[a]	gcgggcTGTATCTAAGACAGCAGTGAtGt	0.15	73,5
		gcgggcgcgggcagggcggcTGTATCTAAGACAGCAGTGAgGc	0.15	76.6, 80.6[d]
		ATGGTAGCATAGTTCTAGGAATAAACT	0.15	
B.33	T/C[b]	ggggcggggcggggcATTGCTACTTCTATTTACGCCAAgAa	0.15	79,0
		ATTGCTACTTCTATTTACGCCAAcAg	0.15	74,3
		TGTGAACAACCAAGTTGGCTT	0.15	
B.34	A/G[b]	ggggcggggcggggcTAGCGAGCATTATTTGCTGGgTt	0.45	78,6
		GTAGCGAGCATTATTTGCTGGtTc	0.15	69,2
		ATAAAACTATAAATTTACATAAAATGAAAACTTCTC	0.15	
B.35	A/C[b]	ggggcggggcggggcGCCTTAATCTAGTATTTTCGCTTATCaCa	0.15	75,5
		GCCTTAATCTAGTATTTTCGCTTATCtCc	0.3	70,3
		CGGGCTCTAAAATAAGATTTAAGTTAGTAAGT	0.15	
B.36	A/C[b]	ggggcggggcggggcTATTATAGTTTCTAAAAACAGTCTAATTAATTgTt	0.15	73,9
		TATTATAGTTTCTAAAAACAGTCTAATTAATTtTg	0.45	69,0
		GTTCGACCATGACTACAGTGTTG	0.15	
B.37	T/C[b]	ggggcggggcggggcCATTTTAGGAACTCTACGATGATAAACTTgAt	0.15	75,9
		AACATTTTAGGAACTCTACGATGATAAACTTaAc	0.15	69,7
		GAAATATCTCAATGAAATCTAATTTAACTAAAATCAC	0.15	
B.38	C/T[b]	ggggcggggcggggcCCATCAGCCATTTACTACTCcCg	0.15	80,1
		ATGCCATCAGCCATTTACTACTCaCa	0.15	73,7
		CTTCCCTGATTTTCTAAGTTCTGCTTG	0.15	

[a] SNP states are presented according to their orientation in the SCHU S4 reference genome (NC_006570)
[b] SNP states are presented according to their orientation in the LVS reference genome
[c] Primer tails and mismatch bases are in lower case, primers are in the order: derived, ancestral and consensus.
[d] Two melting temperatures reflecting differential dissociation of the product

Table S4. Primers and their volumes in the reaction mixtures used in MLVA and predicted ranges of amplicons (Vogler *et al*., 2009b).

Locus	Primer	Mixes for PCR	Mixes for fragment analysis	Volumes (µl) (10 pmol/µl)	Primer Sequence (5'-3')	Dye	Range of amplicon sizes (bp)	Size of repeats (bp)
Ft-M23	Ft-M23-2F	1A	1	0.5	gctggattattaaagcatatgacagacgagtagg	NED	326-349	23
	Ft-M23-2R			0.5	gttccctcaggtttatccaaagtttaatgttttatt	None		
Ft-M24	Ft-M24-2F			2	gaatcttaatccatacggtcctaataatattcctgtcaat	NED	379-416	21
	Ft-M24-2R			2	gttggtacttatggctatagcggatattatttcagt	None		
Ft-M03	Ft-M03-2F	1B		0.5	gcacgcttgtctcctatcatcctctggtagtc	HEX	240-654	9
	Ft-M03-2R			0.5	gaacaacaaaagcaacagcaaaattcacaaaa	None		
Ft-M20A	Ft-M20-2AF[a]	2	2	0.25	gtatatcttggaataagccggagttagatggttct	6FAM	306-486	12
	Ft-M20-2AFcold[a]			0.25	gtatatcttggaataagccggagttagatggttct	None		
	Ft-M20-2AR[b]			0.5	gcaataacttttatcaccctattgtagactgcttctgc	None		
Ft-M05	Ft-M05-2F			1.5	gtttgttacgccaataaacaaaaaagtgtaaataatg	NED	297-425	16
	Ft-M05-2R[c]			1.5	gctcagctcgaactccgtcatacctctctc	None		
Ft-M04	Ft-M04-2F	3A	3	0.5	gcgcgctatctaactaatttttatattgaaacaatcaaat	6FAM	216-236	5
	Ft-M04-2R			0.5	gcaaatataccgtaatgccacctatgaaaactc	None		
Ft-M20B	Ft-M20-2BF[b]			0.5	gggtgataaagttattgttaatggtgtgacttatgaa	None	149; 350-425	15
	Ft-M20-2BR			0.5	gtaactacttgaccgccagtatatgcttgacct	HEX		
Ft-M06	Ft-M06-2F			1.5	gtttttggtgaactgccaacaccataactt	NED	231-336	21
	Ft-M06-2R			1.5	gcaattcagcgaaaccctatcttagcctc	None		
Ft-M02	Ft-M02-2F	3B		0.5	gctgctgtggctgtaaatgttgctatgct	6FAM	338-752	6
	Ft-M02-2R			0.5	gcagggcacaattcttgaccatcagg	None		
Ft-M10	Ft-M10-2F	4A	4	0.5	gctaatttttcatatttatctccatttttacttttttgc	HEX	180-548	16
	Ft-M10-2R[c]			0.5	gctcagctcgaactccgtcatacctcttctc	None		
Ft-M22	Ft-M22-2F	4B		0.5	gtggaaatgcaaaaacaatatcgaggaacttta	6FAM	160-226	6
	Ft-M22-2R			0.5	gtttttctcgtccgctgttagtgatttacatc	None		

[a]Equal amounts of 6FAM-labeled and unlabeled (cold) Ft-M20-2AF were added to PCR Mix 2 to decrease signal strength for multiplexing.
[b]Ft-M20-2AR and Ft-M20-2BF have overlapping primer sequences and so cannot be run in the same PCR.
[c]Ft-M05-2R and Ft-M10-2R have the same primer sequence.

Table S5. Antibiotics and concentration ranges used in susceptibility examinations of *F. tularensis* ssp. *holarctica* strains

antibiotic group	antibiotic	concentration range on test strip (mg/L)
Aminoglycosides	Gentamicin (Cn)	0.016 - 256
	Streptomycin (S)	0.064 - 1024
Tetracyclines	Doxycycline (Dx)	0.016 - 256
	Tetracycline (Te)	0.016 - 256
Quinolones	Ciprofloxacin (Cip)	0.002 - 32
	Levofloxacin (Lev)	0.002 - 32
Macrolides	Erythromycin (E)	0.016 - 256
Rifampin	Rifampicin (Rd)	0.016 - 256
Phenicols	Chloramphenicol (C)	0.016 - 256
Oxazolidinons	Linezolid (Lnz)	0.016 - 256
Glycylcyclines	Tigecyclin (Tgc)	0.016 - 256

Table S6. CanSNP profiles of the 70 *Francisella tularensis* ssp. *holarctica* strains examined.

Strain ID	Br 012	Br 013	FtB 23M	FtB 24M	FtB 25M	FtB 20M	FtB 21M	FtB 22M	BBr 33	BBr 34	BBr 35	BBr 36	BBr 37	BBr 38
FTH1/03	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH2/03	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH3/07	der	der	**der**	anc	anc	anc	anc	anc	anc	anc	anc	anc	anc	anc
FTH4/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH5/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH6/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH7/07	der	der	anc	anc	anc	der	anc	anc	der	der	der	der	der	**der**
FTH8/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH9/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH10/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH11/07	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH12/08	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH13/08	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH14/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH15/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH16/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH17/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH18/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH19/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH20/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH21/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH22/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH23/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH24/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH25/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH26/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH27/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH28/08	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH29/08	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH30/09	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH31/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH32/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH33/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH34/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH35/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH36/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH37/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH38/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH39/09	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH40/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH41/09	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH42/09	der	der	anc	anc	anc	der	anc	anc	der	der	der	**der**	anc	anc

Strain ID	Br 012	Br 013	FtB 23M	FtB 24M	FtB 25M	FtB 20M	FtB 21M	FtB 22M	BBr 33	BBr 34	BBr 35	BBr 36	BBr 37	BBr 38
FTH43/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH44/09	der	der	anc	anc	anc	der	anc	anc	der	der	**der**	anc	anc	anc
FTH45/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH46/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH47/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH48/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH49/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH50/09	der	der	anc	anc	anc	der	anc	anc	der	der	**der**	anc	anc	anc
FTH51/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH52/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH53/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH54/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH55/09	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH56/09	der	der	anc	anc	anc	der	anc	anc	der	der	der	der	**der**	anc
FTH57/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH58/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH59/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH60/10	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH61/10	der	der	anc	anc	anc	**der**	anc	anc	anc	anc	anc	anc	anc	anc
FTH62/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH63/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH64/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH65/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH66/10	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH67/10	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
FTH68/10	der	der	anc	anc	anc	der	anc	anc	der	**der**	anc	anc	anc	anc
FTH69/14	der	der	anc	anc	anc	der	anc	anc	**der**	anc	anc	anc	anc	anc
LVS	der	der	der	**der**	anc	anc	anc	anc	anc	anc	anc	anc	anc	anc

Abbreviations are: anc=ancestor; der=derived. The strains classification into canSNP groups are according to the last derived primers (highlighted).

Table S7. MLVA profiles of the 70 *Francisella tularensis* ssp. *holarctica* strains examined.

Strain ID	FtM22	FtM03	FtM23	FtM24	FtM04	FtM02	FtM20B	FtM06	FtM20A	FtM10	FtM05
FTH1/03	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH2/03	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH3/07	172	**348**	326	416	226	338	149	**273**	306	196	297
FTH4/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH5/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH6/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH7/07	172	**330**	326	416	226	338	149	**273**	306	196	297
FTH8/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH9/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH10/07	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH11/07	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH12/08	172	**303**	326	416	226	338	149	**357**	306	196	297
FTH13/08	172	**303**	326	416	226	338	149	**357**	306	196	297
FTH14/08	172	**312**	326	416	226	338	149	**273**	306	196	297
FTH15/08	172	**303**	326	416	226	338	149	**336**	306	196	297
FTH16/08	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH17/08	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH18/08	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH19/08	172	**321**	326	416	226	338	149	**294**	306	196	297
FTH20/08	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH21/08	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH22/08	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH23/08	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH24/08	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH25/08	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH26/08	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH27/08	172	**321**	326	416	226	338	149	**294**	306	196	297
FTH28/08	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH29/08	172	**303**	326	416	226	338	149	**336**	306	196	297
FTH30/09	172	**294**	326	416	226	338	149	**273**	306	196	297
FTH31/09	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH32/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH33/09	172	**321**	326	416	226	338	149	**315**	306	196	297
FTH34/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH35/09	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH36/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH37/09	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH38/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH39/09	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH40/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH41/09	172	**294**	326	416	226	338	149	**315**	306	196	297
FTH42/09	172	**312**	326	416	226	338	149	**294**	306	196	297

Strain ID	FtM22	FtM03	FtM23	FtM24	FtM04	FtM02	FtM20B	FtM06	FtM20A	FtM10	FtM05
FTH43/09	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH44/09	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH45/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH46/09	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH47/09	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH48/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH49/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH50/09	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH51/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH52/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH53/09	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH54/09	172	**303**	326	416	226	338	149	**294**	306	196	297
FTH55/09	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH56/09	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH57/10	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH58/10	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH59/10	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH60/10	172	**312**	326	416	226	338	149	**357**	306	196	297
FTH61/10	172	**312**	326	416	226	338	149	**357**	306	196	297
FTH62/10	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH63/10	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH64/10	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH65/10	172	**303**	326	416	226	338	149	**315**	306	196	297
FTH66/10	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH67/10	172	**312**	326	416	226	338	149	**315**	306	196	297
FTH68/10	172	**312**	326	416	226	338	149	**294**	306	196	297
FTH69/14	172	**312**	326	416	226	338	149	**315**	306	196	297
LVS	172	**357**	326	416	226	338	149	**315**	318	196	297

The profiles are given by the sizes of the amplicons in base pairs. The most variable loci are highlighted.

Table S8. *In vitro* activity of 11 antibiotics against 29 Hungarian *F. tularensis* ssp. *holarctica* clinical strains

mg/L	CN	S	DX	TE	CIP	LEV	E	RD	C	LNZ	TGC
LVS	0.094	0.38	0.25	0.19	0.008	0.006	>256	0.094	1.0	6.0	0.064
FTH1/03	1.0	8.0	1.0	0.5	0.047	0.023	>256	1.0	1.0	32.0	0.125
FTH3/07	0.75	4.0	0.5	0.75	0.032	0.016	>256	1.0	0.75	32.0	0.094
FTH4/07	0.5	4.0	0.75	0.38	0.047	0.016	>256	1.0	1.5	48.0	0.125
FTH5/07	0.75	6.0	1.0	0.5	0.047	0.012	>256	1.0	1.0	24.0	0.125
FTH6/07	0.5	6.0	1.0	0.5	0.047	0.012	>256	1.0	1.5	24.0	0.125
FTH7/07	1.0	4.0	0.75	0.38	0.047	0.023	>256	1.0	1.5	32.0	0.125
FTH8/07	0.75	3.0	1.0	0.38	0.032	0.016	>256	1.0	1.0	24.0	0.125
FTH9/07	0.75	4.0	0.75	0.38	0.047	0.016	>256	0.75	1.5	16.0	0.19
FTH10/07	0.75	3.0	1.0	0.25	0.032	0.012	>256	1.0	1.0	24.0	0.125
FTH11/07	0.5	6.0	0.75	0.38	0.047	0.016	>256	0.75	0.5	24.0	0.125
FTH12/08	0.38	3.0	1.0	0.5	0.032	0.012	>256	1.0	0.75	32.0	0.125
FTH13/08	0.5	4.0	1.0	0.5	0.023	0.023	>256	0.75	0.75	32.0	0.125
FTH14/08	0.5	4.0	0.75	0.38	0.047	0.016	>256	0.75	0.75	32.0	0.125
FTH15/08	0.5	3.0	0.5	0.38	0.047	0.016	>256	1.0	2.0	16.0	0.125
FTH18/08	0.47	3.0	0.5	0.19	0.023	0.004	>256	1.0	0.75	12.0	0.19
FTH24/08	0.5	4.0	1.5	0.38	0.012	0.004	>256	0.5	1.0	32.0	0.125
FTH28/08	0.5	4.0	0.75	0.38	0.032	0.016	>256	1.0	1.5	32.0	0.125
FTH39/09	0.5	4.0	0.75	0.38	0.047	0.016	>256	1.0	2.0	24.0	0.19
FTH41/09	0.5	4.0	0.75	0.38	0.032	0.008	>256	1.0	0.5	12.0	0.19
FTH44/09	0.5	3.0	0.125	0.5	0.012	0.006	>256	0.5	1.0	24.0	0.125
FTH47/09	0.5	4.0	0.75	0.19	0.032	0.008	>256	0.5	0.75	12.0	0.094
FTH37/09	0.5	3.0	0.75	0.38	0.047	0.016	>256	0.75	1.0	48.0	0.125
FTH51/09	0.75	4.0	0.5	0.5	0.047	0.016	>256	1.0	1.0	12.0	0.094
FTH52/09	0.5	4.0	1.5	0.5	0.047	0.006	>256	0.75	1.5	32.0	0.19
FTH57/10	0.5	4.0	0.75	0.38	0.047	0.016	>256	1.0	2.0	24.0	0.094
FTH58/10	0.5	4.0	0.75	0.75	0.047	0.016	>256	2.0	1.5	32.0	0.19
FTH61/10	0.5	3.0	1.0	0.75	0.032	0.012	>256	1.0	0.75	12.0	0.125
FTH65/10	0.5	4.0	1.0	0.25	0.032	0.023	>256	1.0	1.5	12.0	0.125
FTH67/10	0.75	4.0	1.5	0.25	0.032	0.016	>256	0.75	1.5	12.0	0.125
MIC range (mg/L)for clinical strains	0.38 - 1.0	3.0 - 8.0	0.125 - 1.5	0.19 - 0.72	0.012 - 0.047	0.004 - 0.023	>256	0.5 - 2.0	0.5 - 1.5	12.0 - 48.0	0.094 - 0.19
MIC50	0.5	4.0	0.75	0.38	0.032	0.016	>256	1.0	1.0	24.0	0.125
MIC90	0.75	6.0	1.0	0.5	0.047	0.023	>256	1.0	1.5	32.0	0.19

Abbreviations are: CN: gentamicin; S: streptomycin; DX: doxycycline;TE: tetracycline; CIP: ciprofloxacin; LEV: levofloxacin; E: erythromycin; RD: rifampicin; C: chloramphenicol; LNZ: linezolid; TGC: tigecycline.

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