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

Brief Summary of Doctoral Thesis

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Introduction

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

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

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

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

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

Aims of the study

Aims of the study were:

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

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

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

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

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

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

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

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

Materials and Methods

Retrospective data collection

Retrospective data were collected from the databases of the local veterinary authorities and live hare export stations about the annual number of exported live hares from the different hunting areas of Hungary and the number of *F. tularensis* seropositive animals. The annual numbers of Hungarian human cases with their suspected exposure sites were obtained from the National Center for Epidemiology.

Serological methods

Slide and tube agglutination tests were performed according to the manufacturer's instructions of the used diagnostic kit (Bioveta Inc., Ivanovice na Hané, Czech Republic) utilizing inactivated bacteria.

Pathological methods

Carcasses (European brown hare, monkey, rodents) were necropsied on the day of collection. Four μm thick sections of formalin-fixed and paraffin-embedded tissue samples were stained with hematoxylin and eosin. Immunohistochemistry was applied for the demonstration of *F. tularensis* lipopolysaccharide antigen in tissue sections using mouse monoclonal antibodies (clones FB11 and T14, MAB8267; Chemicon International Inc., Southhampton, UK). Antibody binding was detected by a horseradish peroxidase-labelled polymer (EnVision^{TM}+ Kit; Dako Inc., Glostrup, Denmark).

Bacterological methods

Tularemic foci in parenchymal organs were excised, homogenized and injected subcutaneously into mice. After their death (7-10 days), heart blood and bone-marrow samples were inoculated on modified Francis agar plates (chocolate agar plate containing 1% glucose and 0.1% cysteine) and incubated at 37 ºC for 5 days in an atmosphere containing 6.5% CO_2. Culture, morphological and biochemical characteristics were examined using standard methods (Barrow and Feltham, 1993). A 96-well automated MicroLog MicroStation System with GN2 Microplates (Biolog Inc., Hayward, CA) was used to the characterization of carbon source utilization. Microplates were set up and analyzed with minor modification of the manufacturer's instructions. Dendrogram showing the metabolic relationships between the strains was created with a modified unweighted pair group method with arithmetic mean analysis (Biolog software).

Molecular methods

DNA was extracted from *F. tularensis* strain with the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA) while the X-tractor Gene (Corbett Robotics Pty. Ltd., Queensland, Australia) by the Total RNA Isolation Kit, Nucleospin 96 RNA (Macherey-Nagel GmbH & Co. KG, Düren, Germany), except for the DNase incubation step was used to extract DNA from tissue or tick pools.

A 1000 bp large part of the 16S rRNA gene was amplified using a polymerase chain reaction (PCR) system to identify the isolated *F. tularensis* strains (Relman, 1993). A 100 bp large fragment of the 17 kDa major membrane protein (*tul4*) precursor gene based TaqMan real-time PCR was used for screening tissue, water and insect samples (Versage et al., 2003). A 400 bp large sequence of the *tul4* gene was amplified using a conventional PCR (Sjöstedt et al., 1997) on the real-time PCR positive insect samples in order to amplify a larger part of the *tul4* gene for further direct cycle sequencing (ABI 373A automated DNA sequencer; Applied Biosystems Inc., Foster City, CA).

To resolve the phylogenetic structure of Hungarian isolates, we sequenced the WG of a single Hungarian strain (TUL07/2007, SRP003185.3) through Solexa (Illumina Inc., San Diego, CA) next-generation sequencing technology. Single nucleotide polymorphisms (SNPs) of the Hungarian WG were identified in a comparison to the WG SNPs of 5 other strains. SNPs were identified using an in-house bioinformatics pipeline (Auerbach, 2006; Vogler et al., 2009a). A maximum-parsimony WG SNP tree was constructed using the software package PAUP 4.0b10 (D. Swofford, Sinauer Associates, Inc., Sunderland, MA). We genotyped 19 unknown *F. tularensis* isolates sampled from Hungary (15) and Italy (4) using previously published canonical SNP (canSNP) assays known to be phylogenetically diagnostic for existing phylogroups (Svensson et al., 2009; Vogler et al., 2009a). We further resolved the phylogenetic structure of the Hungarian lineage (B.Br.TUL07/2007) by designing high-throughput SNP assays for the SNPs specific to the Hungarian strain as previously described (Papp et al., 2003). These assays were screened across 100 geographically diverse set of European isolates belonging to the B.Br.020/021 subclade under reaction and instrument conditions as previously described (Vogler et al., 2009a). The fifteen Hungarian isolates were screened with an 11-marker MLVA system (Vogler et al., 2009b) as well, in order to determine the level of genetic diversity within each identified canSNP subclade.

Results

Retrospective data collection

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

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

Seroprevalence of tularemia in the European brown hare population was 5.1% (10/197) with low titers (1/10 and 1/20). *F. tularensis* ssp. *holarctica* was isolated from four hares. *F. tularensis* was not detected by real-time PCR in any of the trapped 38 common voles (*Microtus arvalis*), 110 yellow-necked mice (*Apodemus flavicollis*), 15 stripped field mice (*Apodemus agrarius*) and a by-catch of 8 Eurasian pygmy shrews (*Sorex minutus*) and 6 common shrews (*Sorex araneus*). A total of 1106 *Ixodes ricinus* and 476 *Haemaphysalis concinna* ticks were collected from vegetation and 404 *I. ricinus*, 28 *H. concinna* ticks and 15 *Ctenophtalmus assimilis* and 10 *Nosopsyllus fasciatus* fleas were combed off small mammals. One *H. concinna* female and one nymph collected from the vegetation were infected with *F. tularensis* ssp. *holarctica* thus resulting a 0.42% (2/476) prevalence. *F. tularensis* was not detected in environmental water samples and the examined 100 sheep, 50 cows and 50 buffaloes, grazed in the study area, were all found seronegative.

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

The serologic testing of 900 hamsters and the real-time PCR examination of 100 hamsters and 374 *I. acuminatus* ticks were collected from the animals yielded negative results. After a short period of apathy, the two infected animals died on the 8th and 9th days post infection. The pathological, histopathological and immunohistochemical examination contributed to the diagnosis of septicemia in both cases.

Pathology of tularemia in European brown hares

Lesions induced by *F. tularensis* were examined in 50 cases of naturally infected, seropositive European brown hares. Gross pathological examination revealed scant to numerous, grayish-white foci with a diameter of 0.1-1 cm in single (24 cases) or multiple organs (20 cases) in a total of 44/50 (88%) cases. Gross pathological lesions were found the most in the lungs 40/50 (80%), pericardia 14/50 (28%) and kidneys 10/50 (20%). These lesions were proven to be areas of granulomatous inflammation, frequently encompassing necrosis. *F. tularensis*

antigen was detected with immunohistochemistry in a total of 46/50 (92%) cases, while *F. tularensis* ssp. *holarctica* was isolated by culture and identified by PCR from 35/50 cases (70%).

Generalized tularemia in a vervet monkey and a patas monkey

Macroscopic lesions in each animal included disseminated, grayish-white foci in the lungs, lymph nodes, spleen, liver, and kidney. All focal lesions were characterized microscopically as purulent to pyogranulomatous to granulomatous inflammation with necrosis. *F. tularensis* ssp. *holarctica* strains were isolated from tissue samples and identified by carbon-source utilization test and PCR.

Establishing of a *F. tularensis* strain collection

Sixty-three strains were isolated from European brown hares originating from different parts of Hungary and two strains from Austria. Two further strains were isolated from the patas monkey and the vervet monkey from Szeged Zoo.

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

The Biolog system was already able to identify the examined 15 (2 monkeys, 13 hares) strains after 4 hours of incubation, instead of the standard 24 hours. The Biolog software failed to distinguish the highly virulent *F. tularensis* ssp. *tularensis* and the moderately virulent *F. tularensis* ssp. *holarctica.* As none of the studied strains was able to use glycerol they could be identified as *F. tularensis* ssp. *holarctica*. The dendrogram based on the metabolic relationship of the strains showed that the isolates are very similar to each other.

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

WG SNP comparisons among the 6 genomes resulted in the identification of ~820 putative SNPs and among these, 20 SNPs were specific to the Hungarian strain which grouped closely to the FSC 200 strain found in Sweden but differed from it by ~60 putative SNPs. All 15 Hungarian isolates belonged to the B.Br.013 lineage (subclade found in Central and Eastern Europe). Among the four isolates collected in Italy, the three native isolates belonged to the B.Br.FTNF002-00 subclade (subclade found in Western Europe) while the isolate collected in Italy from an imported hare of Central European origin belonged to the B.Br.013 lineage. By designing high-throughput SNP assays for the 20 SNPs specific to the Hungarian strain and screening these assays across 100 geographically diverse set of European isolates resulted the identification of 6 new phylogenetic subclades within the B.Br.013 lineage. MLVA showed further genetic diversity within some canSNP subclades.

Discussion

Retrospective data collection

The number of human cases documented by the National Center for Epidemiology is in agreement with the official reports of the human health service (Epinfo). Contrary, the percentage of *F. tularensis* seropositive hares makes likely that the official reports (2002: 14 cases, 2003: 2 cases, 2004: 6 cases /WAHID/) are a gross under representation of the true incidence of tularemia.

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

It can be hypothesized that during interepizootic periods *F. tularensis* ssp. *holarctica* persists only in the European brown hare – *H. concinna* cycle. *H. concinna* may not serve exclusively as an arthropod vector but it might also harbor bacteria for three to four years through multiple life stages and act as an important reservoir of *F. tularensis*. Rodent species probably do not play as true reservoir hosts of *F. tularensis*. The modification of the diagnostic tube agglutination titer 1/80 of the used kit was presumed.

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

The results confirmed previous findings that common hamsters are highly sensitive to *F. tularensis*. It was concluded that although septicemic hamsters could pose substantial risk to humans during tularemia outbreaks, hamsters in interepizootic periods do not act as a significant reservoir of *F. tularensis*.

Pathology of tularemia in European brown hares

Infection by respiratory route was presumed by the presence of tissue lesions in the thoracic organs in 44/50 (88%) cases. These results emphasize the importance of the European brown hare as a reservoir of *F. tularensis*.

Generalized tularemia in a vervet monkey and a patas monkey

The pathological pattern suggests that the most likely route of infection may have been through inhalation. Zoo primates regularly hunt and consume small prey species such as rodents and birds that enter their enclosures. This could be a potential source for contracting *F. tularensis* from free-ranging wildlife. The current report indicates that infected nonhuman primates may be potential sources of zoonotic disease for animal keepers and visitors and appropriate hygienic measures should be taken.

Establishing of a *F. tularensis* strain collection

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

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

The results show that carbon source utilization is a reliable method of characterization and identification of *F. tularensis* strains and the Biolog system can be used to the identification and comparative examination of this bacterium species. Extending the database using our date is recommended as the Biolog software/database does not distinguish between *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica*, though differentiation based on glycerol utilization is absolutely necessary because of their different virulence. *F. tularensis* strains recovered from hares and monkeys were found to be very similar based on carbon source utilization. The cladogramm, derived from the metabolic profile of the strains, supported the notion of the conservative genetic character of *F. tularensis* ssp. *holarctica*.

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

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

New scientific results

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

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

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

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

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

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

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

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

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

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

Scientific publications

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