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Comparative characterisation of members of the family Francisellaceae

Brief Summary of Ph.D. thesis

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Introduction

The family Francisellaceae is rapidly expanding with several new members described in the last few decades. Francisellaceae family involves six *Francisella* species and several *Francisella* variants. Members of the family Francisellaceae show high genetic homology (Keim *et al*., 2007).

Francisella-like endosymbionts (FLEs) are detected in both soft and hard ticks from all over the world (Barns *et al*., 2005). Due to the close relatedness, FLEs may lead to misidentification of the pathogen *F. tularensis* by conventional PCR techniques in routine diagnostics (Kugeler *et al*., 2005).

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 of the bacterium are present in the continent, which occur in distinct geographic regions. Genotype B.FTNF002-00 is dominant in Western European countries and genotype B.12 in Central and Eastern European countries (Gyuranecz *et al*., 2012a, Vogler *et al*., 2009a).

F. tularensis can infect more than 300 animal species, which show different susceptibility to the disease (Keim *et al*., 2007). The European brown hare (*Lepus europaeus*) is considered to be the main reservoir host of the bacterium in Central and Eastern Europe. In the area, tularaemic hares usually show sub-acute or chronic pathological changes (Gyuranecz *et al*., 2010b). However, signs of acute clinical course are observed in hares died of tularaemia originating from Western European regions (Decors *et al*., 2011, Rijks *et al*., 2013).

F. tularensis may manifest six clinical forms in humans in a wide spectrum from mild glandular to generalized septicaemic forms. Tularaemic patients can be treated by the administration of the appropriate antibiotics (Ellis *et al*., 2002).

In Hungary, besides the potential threat to public health tularaemia is also important economically, as the hare's tularaemia free status is crucial in the country's brown hare export (Somogyi, 2006).

Aims of the study

The aims of the study were:

Ad 1. to investigate the occurrence 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;

Ad 3. to discover host-pathogen interactions among various *F. tularensis* ssp. *holarctica* strains and the complement system of animal species with different susceptibility to tularaemia;

Ad 4. to compare the pathogenicity of *F. tularensis* ssp. *holarctica* strains from the two dominant genetic clade endemic in Europe;

Ad 5. to characterize the antimicrobial susceptibility profile of the Hungarian *F. tularensis* ssp. *holarctica* strains.

Materials and methods

F. tularensis ssp. *holarctica* strains and tick collection

A collection of 69 *F. tularensis* ssp. *holarctica* strains originating from six counties of Hungary, isolated between 2003 and 2014 from 67 brown hares and three zoo monkeys was examined. Two Western European strains (from Italy and Spain), and the live vaccine strain (LVS, NCTC 10857) was also included in the examinations.
A total of 5806 ticks of 16 species were examined for the presence of Francisellaceae. In Hungary, ticks were collected from the environment and migratory birds. In Ethiopia, ticks were collected from cattle.

Molecular methods

Francisellaceae specific conventional PCRs were performed for the detection of *F. tularensis* and FLEs in ticks targeting the 16S rRNA gene, the *tul4* gene and the *sdhA* locus (Barns *et al*., 2005, Long *et al*., 1993, Sjöstedt *et al*., 1997). Identification and phylogenetic analyses of the *Francisella* variants was based on sequence analysis. For the specific detection of *F. tularensis* and for the discrimination of the genotypes B.FTNF002-00 and B.12 the *tul4* gene and the region of genomic difference 23 (RD23) were amplified, respectively (Dempsey *et al*., 2007, Versage *et al*., 2003). Canonical single nucleotide polymorphism (canSNP) typing was performed using melt analysis of mismatch amplification mutation assays for the genetic analysis of the 69 Hungarian *F. tularensis* ssp. *holarctica* strains (Chanturia *et al*., 2011, Gyuranecz *et al*., 2012a, Vogler *et al*., 2009a). To further resolve genetic relationships the multi-locus variable number of tandem repeats analysis (MLVA) was performed (Vogler *et al*., 2009b).
Whole genome (WG) sequence of nine selected strains was determined by next generation sequencing.

Examination of host-pathogen interactions

The survival of *F. tularensis* ssp. *holarctica* strains of different virulence and genotypes was examined in the sera of selected animal hosts of distinct susceptibility to tularaemia in complement sensitivity assays. Wild, virulent strains of the two main genotypes prevalent in Europe (B.FTNF002-00 /*n*=2/ and B.12 /*n*=1/) and the attenuated LVS were examined. Sera of mice, European brown hares and cattle were used to represent hosts that are highly or moderately (reservoir) sensitive or resistant to tularaemia, respectively. The binding of the complement regulator factor H to bacterial proteins was also examined in Western blot and pull-down assays.

Artificial infection

The virulence of two *F. tularensis* ssp *holarctica* strains of different genotypes (B.FTNF002-00 and B.12) was compared in experimentally infected Fischer 344 rats. Three concentrations of the bacteria solutions were injected intraperitoneally in the rats. The animals were monitored daily for 21 days. Necropsy, histological and immunohistochemical examinations and slide agglutination tests were performed on all animals.

Antibiotic susceptibility examinations

From the collection of *F. tularensis* ssp. *holarctica* strains originating from Hungary 29 isolates were systematically chosen for antibiotic susceptibility examination considering their geographic origin, host species and genetic characteristics. Commercially available test strips were used to determine the minimum inhibitory concentrations (MIC) of the antibiotics on solid medium. MICs of erythromycin, streptomycin, gentamicin, ciprofloxacin, levofloxacin, tetracycline, doxycycline, tigecycline, rifampicin, linezolid and chloramphenicol were determined. The breakpoints were interpreted according to the standards of the Clinical and Laboratory Standards Institute standards (CLSI, 2009).

Results

Francisellaceae in ticks from Hungary and Ethiopia

F. tularensis ssp. *holarctica* was detected in two *Haemaphysalis concinna* pool and in one *Dermacentor reticulatus* pool collected in Hungary, representing a minimum prevalence (calculating with only 1 infected tick per pool) of 0.27% within the examined tick species.

The same FLE was found in 11 pools of *D. reticulatus* questing ticks, showing a minimum prevalence of 3%. The FLE of *D. reticulatus* was described previously in Europe. A novel FLE was described in a new tick host, *Ixodes ricinus* collected from a migratory bird in Hungary. In a *Hyalomma rufipes* originating from Ethiopia a FLE was found, which was previously detected in other tick species also.

Genotyping of *F. tularensis* ssp. *holarctica* strains from Hungary

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 into 8 subclades, and 89.85% (62/69) of the strains belonged to the B.33/34 subclade or derivated subclades. Three subclades determined by canSNP typing were further resolved with MLVA into 14 subgroups. Isolates of different genotypes were involved in tularaemia outbreaks in several regions. Strains originating from zoo monkeys from the 2003 and 2014 tularaemia outbreaks in Szeged Zoo showed identical MLVA profiles. WG sequencing confirmed the relatively high diversity of the Hungarian strains.

Examination of host-pathogen interactions

All wild, virulent strains showed similar resistance to the selected hosts' complement system. No differences were observed in the complement sensitivity of the examined genotypes. The attenuated LVS was resistant to mouse serum, but was killed in hare and cattle serum.

No specific, direct binding was observed between factor H and bacterial proteins.

Comparison of pathogenicity of genotypes B.12 and B.FTNF002-00

Experimentally infected rats showed clinical signs of tularaemia between days 4 and 12 post infection. No correlations were observed between challenge dose and severity of clinical signs or number of death. More rats showed severe clinical signs infected with the B.FTNF002-00 genotype, although the difference was not significant ($p=0.066$). A total of 33% (6/18) of the animals died of tularaemia infected with B.FTNF002-00 group, while only 11% (2/18) succumbed to the infection caused by B.12 genotype.

Antibiotic susceptibility examinations

Resistance to erythromycin (>256 mg/L) and linezolid (32 mg/L) was detected in all examined *F. tularensis* ssp. *holarctica* strains. According to the MIC values that inhibited the growth of 90% of the strains, 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 the strains.

Discussion

Francisellaceae in ticks from Hungary and Ethiopia

The results of the study confirm the role of ticks in the ecology of tularaemia and highlights that ticks carrying the pathogen could pose a threat to public health (Gyuranecz *et al*., 2010d). In the case of the FLE species harboured by *D. reticulatus* in Europe host adaptation and a host species–linked evolution could be assumed. The identification of a FLE in *I.ricinus* was the first molecular evidence of their occurrence in *Ixodes* spp. Close relatedness among endosymbionts of hard ticks from Europe and Africa was observed. The detected homology of FLEs of different tick species supports the hypothesis, that most FLEs had independent evolution from their tick hosts (Scoles, 2004).

Genotyping of *F. tularensis* ssp. *holarctica* strains from Hungary

The genetic analyses of Hungarian strains demonstrated the relative diversity of *F. tularensis* ssp. *holarctica* in the country. Involvement of several genotypes in tularaemia outbreaks supports the suggestion that epidemics are triggered by ecological factors rather than the increased infectivity of a specific *F. tularensis* clone (Gyuranecz *et al*., 2012b, Johansson *et al*., 2014). The finding of the same genotype in a region over a period of a decade presents an example for long-term environmental phase of the pathogen, which is to be considered in the prevention of human infections as well.

Examination of host-pathogen interactions

The comparison of host-pathogen interactions in the *in vitro* experiments showed differences in the resistance of the wild and attenuated strains to serum killing. Previously, the binding of factor H to the surface of *F. tularensis* has been described in humans (Ben Nasr and Klimpel, 2008). In the present study, the role of binding factor H on bacterial cell surface was not confirmed in the host–pathogen interactions among *F. tularensis* ssp. *holarctica* strains and the complement system of the examined animal species.

Comparison of pathogenicity of genotypes B.12 and B.FTNF002-00

While clear differences are described among *F. tularensis* ssp. *tularensis* subpopulations, little or no information is available about the subpopulations of the widespread *F. tularensis* ssp. *holarctica* (Keim *et al*., 2007). In the study, differences were revealed in the pathogenic potential of the *F. tularensis* ssp. *holarctica* strains belonging to the two genotypes dominant in Europe. The results support the hypothesis that B.FTNF002-00 genotype from Western Europe is moderately more virulent than the B.12 genotype from Eastern Europe.

Antibiotic susceptibility examinations

On the basis of *in vitro* examinations, quinolones are recommended as first choice in the therapy of tularaemia in Hungary. The use of aminoglycosides, tetracyclines and chloramphenicol is also appropriate against *F. tularensis* in Hungary. 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.

Overview of the new scientific results

Ad 1. Ticks possess epidemiologic importance in the ecology 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.

Scientific publications

In peer-reviewed journals

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