Original Article

Genetic Relationship between *Francisella Tularensis* Strains from China and from Other Countries*

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Abstract

Objective To study the types of subspecies of Francisella tularensis from China and to investigate the genetic relationships between *F. tularensis* strains from China and from other countries.

Methods Ten strains of *F. tularensis* isolated from China were amplified by using typing primers C1/C4 and RD1. On the basis of the lengths of the polymerase chain reaction (PCR) products, it was concluded that these strains of *F. tularensis* belonged to the same subspecies. At the same time, the *fopA*, *tul4*, and *16S rRNA* genes of the 10 strains were amplified, and a three-gene based phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis software version 4.0.

Results The 10 strains of F. tularensis from China were all identified as belonging to subspecies *holarctica* (type B). We found no direct relationship between the genotypes of *F. tularensis* subsp. *holarctica* and the geographical area from where they were isolated.

Conclusion The *F. tularensis* strains isolated from North China mainly belong to subspecies *holarctica* (type B). The strains of *F. tularensis* subsp. *holarctica* from China may have evolved earlier than those from Europe and North America.

Key words: *Francisella tularensis*; Tularemia; Phylogenetic analysis; Genotyping, PCR

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INTRODUCTION

Tularemia, caused by the gram-negative intracellular pathogen *Francisella tularensis,* is a bacterial zoonotic disease prevalent in the northern hemisphere. *F. tularensis* is highly virulent in humans and in a range of animals such as rodents, hares, and rabbits. *F. tularensis* is an ideal organism to study because of its highly infectious nature and its relative stability in aerosols, which facilitates its dissemination. It is also at the center of rising concerns associated with the risk of bioterrorism, which can endanger public health and destabilize national economies. *F. tularensis* is one of two species in the genus *Francisella*; the other is *F. philomiragia.* The similarity between the 16S rRNA sequences of the two species is more than 98.3%[1]. Currently, *F. tularensis* is divided into 4 subspecies *(tularensis*, *holarctica*, *mediasiatica*, and *novicida*), each of which displays several distinct biochemical, epidemiological, and virulence characteristics[2]. Among the 4 subspecies, strains belonging to the subspecies *tularensis* (type A) are the most infectious pathogens.

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In the past, tularemia was a significant public health problem. In the USA, about 1 400 cases were reported between 1990 and 2000. In the former Soviet Union, about 67 000 cases were reported from the region surrounding Rostov-on-Don during the winter of 1941-1942. Tularemia occurs most frequently in Scandinavia, North America, Japan, and Russia[3-7] and is also seen in Turkey, Yugoslavia, Spain, Kosovo, and Switzerland[8-11]. However, tularemia has recently been reported in Costa Rica, Sweden, and Germany[12-14], indicating that it is even more widely distributed than what was previously thought.

In China, *F. tularensis* was first isolated in 1957 from *Citellus dauricus* in the Tongliao region of the Inner Mongolia municipality[15-16]. In 1959, the first human with tularemia was reported to be from Heilongjiang Province[17]. At present, the northern provinces and the autonomous regions of China that include Heilongjiang, Inner Mongolia, Xinjiang, Qinghai, and Tibet, are the natural foci of the disease, and *F. tularensis* has been isolated from humans, dead hares, and *Citellus dauricus* in these regions. Seropositive people infected with *F. tularensis* have also been found in Shandong, Shanxi, Gansu, and Ningxia provinces[18]. In 1986, there was an outbreak of the disease during the processing of hares in a cold storage plant in Jiaonan County of Shandong Province. Within 10 days, 31 (about 86%) of the workers in this plant were infected. Thereafter, no outbreaks of tularemia have been reported in China.

No reports about the genetic relationship between *F. tularensis* strains isolated from China and from other countries in the world are available. Previous research[19-22] used mainly multiple-locus variable-number tandem repeat analysis (MLVA) for the genotyping and phylogenetic analysis of *F. tularensis* strains. By selecting *fopA* and *tul4* genes that are unique to *F. tularensis* and with the *16S rRNA* gene that is used for identifying *Francisella*, we conducted a three-gene based phylogenetic analysis on the 10 *F. tularensis* strains isolated in China and the 8 *F. tularensis* strains that were already sequenced and published on the NCBI website. This analysis explores, for the first time, the genetic relationship between *F. tularensis* strains isolated from China and from other countries in the world.

MATERIALS AND METHODS

DNA Extraction and PCR Amplification

A total of 10 strains of *F. tularensis* were isolated from North China and used in this study. Strains 410 105 and 410 108 were isolated from Tibetan patients, and strains 410 111, 410 112, and 410 113 were isolated from *Dermacentor everestianus*; the sources of the other strains are unknown. The strains were inactivated for genomic DNA extraction; the extractions were performed according to the manufacturer's instructions of Nucleo Spin Tissue Kit (Macherey-Nagel, Germany).

The targets for the polymerase chain reaction (PCR) were the *fopA* and *tul4* genes, which encode outer membrane proteins, and the *16S rRNA* gene, which was amplified for the identification of *Francisella*. The typing primer C1/C4 was used for amplifying *PPI*-helicase (the intergenic region between the peptidyl-prolyl *trans*-isomerase) and RD1 (the region of difference) (Table 1). PCR was performed using Taq DNA polymerase, dNTPs, and 10× PCR buffer (Takara, Japan) and LabCycler thermal cycler (SensoQuest, Germany). The PCR products were electrophoresed, and the results were analyzed using Imaging systems Gel Doc XR (Bio-Rad, USA). Reagent preparation, DNA extraction, amplification, and agarose gel electrophoresis were performed in separate rooms to avoid contamination, and distilled water was used as the blank control during each reaction.

DNA Sequencing and Data Analysis

The amplification products of the 3 genes, *fopA*, *tul4*, and *16S rRNA*, from the 10 strains of *F. tularensis* were sequenced. The sequences of each of the amplification product were compared with the genome sequences of the 8 strains of *F. tularensis*, sourced from the NCBI website, using the Blast program (Information available from: http://www.ncbi.nlm.nih.gov/BLAST/). The identified sequences for the 3 genes in each of the 8 *F. tularensis* strains were then compared with the sequencing results of the amplification products of the 10 strains of *F. tularensis* from China, using Clustal W and the MegAlign module of DNAStar5 (DNASTAR, Inc., Madison, Wisconsin, USA). A 401 bp region of the *fopA* gene, a 410 bp region of the *tul4* gene, and a 1 044 bp region of the *16S rRNA* gene, were common to all 18 *F. tularensis* strains. When the common regions of the 3 genes were connected, the total length was about 1 855 bp long. Using the combined sequence, phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0[23]. We also generated an unrooted phylogenetic tree in MEGA4 by using the neighbor-joining method.

Table 1. Details of the PCR Amplification Reactions

Target of PCR	Primers	Sequence (5′–3′)	PCR Program	Fragment Size (bp)
fopA	FNA7L FNB1L	CTTGAGTCTTATGTTTCGGCATGTGAATAG CCAACTAATTGGTTGTACTGTACAGCGAAG	1 cycle: 95°C 3 min; 30 cycles: 95°C 15 s, 50°C 15 s, 72°C 30 s; 1 cycle: 72°C 5 min	401
tul4	TUL4-435 TUL4-863	GCTGTATCATCATTTAATAAACTGCTG TTGGGAAGCTTGTATCATGGCACT	1 cycle: 94°C 5 min; 30 cycles: 94°C 30 s, 55°C 30 s, 72°C 30 s; 1 cycle: 72°C 5 min	410
16S rRNA	F11 F5	TACCAGTTGGAAACGACTGT CCTTTTTGAGTTTCGCTCC	1 cycle: 95°C 5 min; 30 cycles: 95°C 1 min, 57°C 1 min, 72°C 1 min; 1 cycle: 72°C 10 min	1 044
PPI-helicase	C1 C4	TCCGGTTGGATAGGTGTTGGATT GCGCGGATAATTTAAATTTC	1 cycle: 94°C 5 min; 30 cycles: 94°C 30 s, 54°C 30 s, 72°C 30 s; 1 cycle: 72°C 5 min	180[a] or 150[b]
RD1	F R	TTTATATAGGTAAATGTTTTACCTGTACCA GCCGAGTTTGATGCTGAAAA	1 cycle: 95°C 3 min; 30 cycles: 95°C 30 s, 58°C 1 min, 72°C 1 min; 1 cycle: 72°C 5 min	1500[a] or 1 100/900[b] or 1 400[c] or 3300[d]

Note. [a]subspecies *tularensis*. [b]subspecies *holarctica*. [c]subspecies *mediasiatica*. [d]subspecies *novicida*.

Nucleotide Sequence Accession Numbers

The partial sequences of the *fopA*, *tul4*, and *16S rRNA* genes of the 10 *F.tularensis* strains used in this study have been deposited in the GenBank database with accession numbers HM371332-HM371361.

RESULTS

Results of the PCR Amplifications Performed Using Typing Primers

Using *fopA*-, *tul4*-, and *16S rRNA*-specific primers to amplify the nucleic acids of the 10 *F. tularensis* strains, we obtained fragments of the amplified parts. Using the specific primer C1/C4, we obtained a 150-bp amplified fragment (Figure 1A), indicating that the 10 strains of *F. tularensis* belong to the subspecies *holarctica*[24]. Similarly, using the RD1-specific primer, we found that the amplification products from 3 strains were slightly longer than 1000 bp, and those from the remaining 7 were slightly less than 1 000 bp (Figure 1B). These 10 strains of *F. tularensis* were again identified as belonging to subspecies *holarctica*[25].

Phylogenetic Analysis

After comparing the 3 gene fragments (1 855 bp) of the 10 *F. tularensis* strains that were isolated from China and the 8 strains sourced from the NCBI website, a three-gene based phylogenetic analysis using the MEGA4 software was performed on the subset of the 18 *F. tularensis* strains. It was found that all the 10 *F.tularensis* strains that belong to the subspecies *holarctica* clustered in the clade with a node value 63. Three strains of *F. tularensis*, namely, 410 108, 410 109, and 410 111, were in one subclade, and the remaining 7 were in another; a further 3 strains of foreign *F. tularensis* subsp. *holarctica* were clustered in another subclade (Figure 2). *F. tularensis* subsp. *novicida* U112 and 13 strains of subspecies *holarctica* were clustered together in one clade and *F. tularensis* subsp. *mediasiatica* FSC147 and 3 strains of *F. tularensis* subsp. *tularensis* were in another.

DISCUSSION

All the *F. tularensis* strains used in this study were found to be from North China and all belong to the subspecies *holarctica* (type B), in agreement with an earlier study by Zhang et al[26]. Amplifications were performed using 2 typing primers. Using the RD1 primer, we obtained amplification fragments of 3 strains of *F. tularensis* with lengths similar to the fragments obtained from the FSC075 (1 135 bp) subspecies *holarctica* from Japan. The lengths of the amplification fragments from the other 7 strains of *F. tularensis* were similar to the fragments from the live vaccine strain (LVS)[25]. The phylogenetic tree based on the 3 genes, *fopA*, *tul4*, and *16S rRNA*, showed that the sequence similarity among the isolates of the *F. tularensis* subspecies was extensive. The 10 strains of *F. tularensis* isolated from China and the 3 strains isolated from Europe and North America were all clustered within a clade with a node value of 63.

We found no correlation between the genotype and the geographical area from which the *F. tularensis*

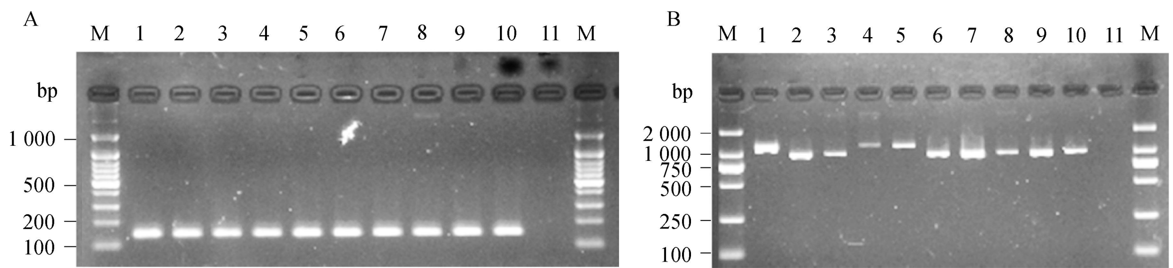

Figure 1. The amplification results for the 10 strains of *F. tularensis* using 2 specific primers. Lanes 1 to 10 are *F. tularensis*. Lane 11 is a blank control. Molecular sizes are indicated in the left and right lanes. A) Amplification results obtained using the C1/C4 primer. The fragment length in lanes 1 to 10 is 150 bp. B) Amplification results obtained using the RD1 primer. The fragment lengths in lanes 1, 4, and 5 are slightly more than 1 000 bp, and those in lanes 2, 3, and 6-10 are slightly less than 1 000 bp.

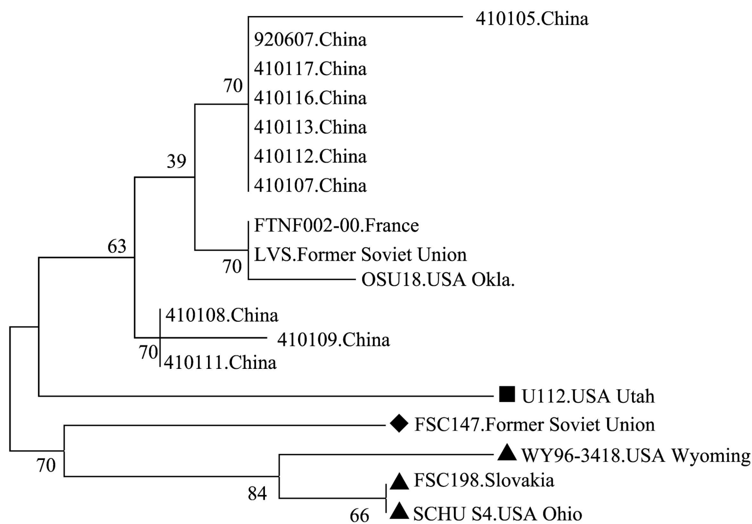

Figure 2. Genetic relationship between the *F. tularensis* strains from China and other countries. The distance matrix was calculated using the Maximum Composite Likelihood method. The support of each branch was determined using 1 000 bootstrap samples and is indicated by the value at the node. Triangles represent the subspecies *tularensis;* diamonds represent the subspecies *mediasiatica*; squares represent the subspecies *novicida*; and strains without symbol represent the subspecies *holarctica*.

bacteria were isolated. Compared to other genomic sequences, single nucleotide variations exhibit slow mutation rates, making them valuable for phylogenetic analysis[27]. The 3-gene-based phylogenetic tree of the *F. tularensis* strains that were isolated from China and other countries shows that the 13 strains of the subspecies *holarctica* were grouped into 3 distinct clades. Strains 410 108, 410 109, and 410 111 from China belonged to one genotype that we called B1; strain 410 109 was slightly different from the other two. The remaining 7 strains of *F. tularensis* belonged to another genotype (B2); among these, strain 410 105 was significantly different from the other 6 strains. The 3 strains of *F. tularensis* from France, the former Soviet Union, and the USA belonged to one genotype (B3). Of the 3 genotypes that we identified, B2 and B3 have a closer genetic affinity. In other words, the 7 strains of *F. tularensis* from China and FTNF002-00, LVS, and OSU18 from France, the former Soviet Union, and the USA, respectively, have a closer genetic relationship than the 2 genotypes (B1 and B2) in the 10 strains of *F. tularensis* isolated from China. Thus, the genotypes of *F. tularensis* subsp. *holarctica* are not directly related to the geographical area from which they were isolated. This result is different from the results of an earlier study which concluded that, after using the molecular typing method for *Yersinia pestis* strains isolated from China, the *Y. pestis* strains in one ecotype (isolated from one geographical area) mostly belong to the same genotype[28].

F. tularensis subsp. *holarctica* strains from China may have evolved earlier than those from Europe and North America. Vogler et al. suggested that *F. tularensis* subsp. *holarctica* may have originated in Asia[29]. Our results also indicate that among the 4

subspecies of *F. tularensis*, subspecies *holarctica* and subspecies *novicida*, and subspecies *mediasiatica* and subspecies *tularensis* have close genetic relationships. At present, tularemia is not a notifiable infectious disease in China; therefore, the isolation of *F. tularensis* is not routinely performed in the Centers for Disease Control and Prevention (CDCs). Furthermore, the culturing of *F. tularensis* requires fastidious conditions; hence, relatively small numbers of *F. tularensis* have been obtained in China. A limitation of the present study is that a relatively small number of strains have been used. In a future study, we plan to isolate the pathogens from the spleen of rodents in northern China to obtain more strains of *F. tularensis* for a more rigorous investigation of the phylogenetic character of *F. tularensis* from China.

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