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Comparison of tul4, fopA, 16S rRNA and RD1 gene regions of Francisella tularensis strains isolated from Sivas, Turkey

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Abstract

Tularemia is a zoonotic disease that can infect animals and people. It is known that tularemia outbreaks in Turkey is water-borne and causative agent F. tularensis subsp. holarctica. Eight F. tularensis were isolated from the water samples in tularemia outbreaks observed in Sivas during 2011-2013. In this study; tul4, fopA, RD1 and 16S rRNA gene regions of eight F. tularensis isolates were amplified by PCR method and investigated by sequencing method. Sequence analysis of the gene regions were compared with each other and the samples found in GenBank. All samples were found to be similar in terms of the partial sequence of the tul4 and fopA gene region. In terms of 16S rRNA gene region, Belkent2012 and Belkent2013 isolates and Çiçekoğlu and Döllük isolates were detected similar among themselves. Bahçeiçi and Karaören isolates were found similar at RD1 gene regions. In the sequence comparison of GenBank in terms of examined gene regions, 94-100% similarity was determined with F. tularensis subsp. holarctica LVS and F. tularensis subsp. holarctica PHIT-FT049. As a result of our study; the tul4 and fopA gene regions of eight F. tularensis subsp. holarctica isolates were found to have identical and different base sequence origins in 16S rRNA and RD1 gene regions in tularemia outbreaks in Sivas. In order to reveal the phylogeography of F. tularensis' in Turkey, it is necessary to produce new isolates from epidemic regions and to investigate them with advanced molecular techniques.

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1. Introduction

Francisella tularensis, a gram-negative intracellular bacterium, causes tularemia in humans and animals. *F. tularensis* is transmitted to humans mainly by arthropod bites, direct contact with infected animals, infected animal tissues, contaminated water, food and inhalation of infected aerosols [1, 2]. *F. tularensis* is composed of 4 recognized subspecies: subsp. *tularensis* (type A), subsp. *holarctica* (type B), subsp. *novicida*, and subsp. *mediasiatica*. Only *F. tularensis* subsp. *tularensis* and subsp *holarctica* are considered clinically significant in humans [3].

F. tularensis subsp. *tularensis* (Type A) is one of the most infectious pathogens known and isolated from North America. It is transmitted to humans and animals by arthropods such as ticks, deer flies, or infected aerosols. *F. tularensis* subsp. *holarctica* (Type B) is found throughout the Northern Hemisphere, but has recently been detected in Australia [4, 5]. Type B tularemia cases caused by *F. tularensis* subsp. *holarctica* are associated with aquatic environments such as rivers, lakes, streams, and muskrat and beavers living in these environments. It has also been isolated from rabbits and other animals [1, 2]. *F. tularensis* subsp. *mediasiatica* have been reported only in a few Central Asian countries, and its virulence is similar to subsp. *holarctica*. The subspecies *novicida* causes infections in immunocompromised individuals [6].

Tularemia was first reported in Turkey in 1936 and then was sporadically reported for several decades [7]. Between 1988 and 2018, 28 tularemia outbreaks linked to consumption of contaminated water has been reported in Turkey [6]. Ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal and pneumonic types may be observed based on the entrance route to the body and location of the bacteria. Although the clinical presentation may vary, oropharyngeal tularemia is the most commonly seen clinical form in Turkey [8].

The first tularemia outbreak in Sivas, a central Anatolian city, was observed in 2009. *F. tularensis* was produced by culture method in water samples taken from the epidemic regions, and as a result of molecular studies, the agent was identified as *F. tularensis* subsp *holarctica* [9, 10]. At the same time, in 2011 and 2012

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years at Sivas province, *F. tularensis* was produced by culture method in water samples and it was confirmed that the agent was *F. tularensis* subsp. *holarctica* by PCR method [11].

In this study, it was aimed to investigate the phylogenetic relationship (regarding to tul4, fopA, RD1 and 16S rRNA gene regions) between *F. tularensis* subsp. *holarctica* strains isolated from Sivas, in Turkey and also other countries.

2. Materials and Methods

2.1. Bacterial strains and DNA isolation

In this study, a total of eight *F. tularensis* isolates were used. The isolates were obtained by culture method from water samples of regions where tularemia cases were observed in Sivas (Central Anatolia, Turkey) during 2011-2013. The isolates were confirmed to be *F. tularensis* subsp. *holarctica* by PCR using Tul4 and RD1 specific primers [11] (Table 1). The isolates stored in glycerol broth (16%) at -20 °C were revived in Glucose Cysteine Blood Agar (GCBA) medium under a 5% CO_2 environment. DNA isolation was performed from the isolates produced in the medium using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

Table 1. The names of the isolates used in the study and the regions where they were isolated

No	Isolate Name	Region of Isolation
1	Çiçekoğlu	Sivas-Gemerek-Çiçekoğlu village
2	Bahçeiçi	Sivas-Gürün-Bahçeiçi village
3	Karaören	Sivas-Gürün-Karaören village
4	Hüyük	Sivas-Şarkışla-Hüyük village
5	Maksutlu	Sivas-Şarkışla-Maksutlu village
6	Döllük	Sivas-Şarkışla-Döllük village
7	Belkent2012	Sivas-Şarkışla- Belkent fountain (2012)
8	Belkent2013	Sivas- Şarkışla- Belkent fountain(2013)

2.2. Target gene regions and PCR

The tul4, fopA, 16S rRNA and RD1 target gene region of the isolates used in the study were amplified by PCR method (Table 2). After confirmation of the isolates as *F. tularensis* by PCR with tul4 primers, another conventional PCR assay targeting the region of differentiation 1 (RD1) was performed in order to determine subspecies identification [12, 13]. *F. tularensis* subsp. *holarctica* LVS strain (NCTC 10857) was used as a positive control in the PCR study. Each PCR reaction was performed in a 50 µl volume; 1X PCR buffer, 1,25 U Taq DNA polymerase (GeneAll, Korea), 0,2 mM dNTP (GeneDireX, Inc., Miaoli County, Taiwan), 0,2 µM primer ve 5 µl DNA sample. GeneAmp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) thermal cycler was used in the study. The PCR products were electrophoresed and the results were visualized with the aid of gel imaging system.

Table 2. Details of the PCR Amplification Reactions

Target of PCR	Primers	Sequence (5´- 3´)	PCR Program	Fragment Size (bp)
tul4	TUL4-435 TUL4-863	GCTGTATCATCATTTAATAAACTGCTG TTGGGAAGCTTGTATCATGGCACT	1 cycle: 94°C 4 min; 40 cycles: 94°C 40 s, 64°C 30s, 72°C 45 s; 1 cycle: 72°C 5 min	410
fopA	FNA7L FNB1L	CTTGAGTCTTATGTTTCGGCATGTGAATAG CCAACTAATTGGTTGTACTGTACAGCGAAG	1 cycle: 94°C 4 min; 40 cycles: 94°C 40 s, 64°C 30s, 72°C 45 s; 1 cycle: 72°C 5 min	401
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	900/1100[a] 1500[b] ve 1400[c]
16S rRNA	F11 F5	TACCAGTTGGAAACGACTGT CCTTTTTGAGTTTCGCTCC	1 cycle: 94°C 3 min; 40 cycles: 94°C 30 s, 55°C 1 min, 72°C 35 s; 1 cycle: 72°C 5 min	1100

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

2.3. Sequencing of PCR products and phylogenetic analysis

Sequence analysis of the amplification products of the four gene regions (tul4, fopA, 16S rRNA and RD1) of *F. tularensis* isolates was performed by a commercial company (MG Bioinformatic, Turkey). The amplified product was purified using the QIAquick Extraction Kit (Qiagen GmbH). Purified DNA was sequenced using the BigDye Terminator V3.1 CycleSequencing Kit (Applied Biosystems, Foster City, CA). Automated fluorescence sequencing was performed with an Applied Biosystems™ 3730xl DNA Analyzer device. Nucleotide sequences were evaluated using the program BLAST (National Center for Biotechnology Information, www.blast.ncbi.nlm.nih.gov/Blast). The gene sequences of the izolates were compaired using Molecular Evalutionary Genetic Analysis (MEGA) software version 10.0.5. The phylogenetic tree was produced according to the neighbor-joining method after Kimura 2-parameter correction in the MEGA using bootstrap analyses with 1000 replicates [14]. The partial sequences of 16S rRNA gene was deposited in the GenBank under the accession numbers MK249699-706.

3. Results and Discussion

Tularemia is a highly infectious zoonotic disease caused by the bacterium *F. tularensis* that affects humans and other animals. The disease was first defined by George McCoy and Chapin in Tulare County, California in 1911 [6]. In Turkey, tularemia has been known since the 1930's. The first published tularemia epidemic in Turkey had been reported in 1936 from Thrace region (Lüleburgaz town), and the second was in 1945 again in the same location. In recent years, tularemia outbreaks were reported from various regions of Turkey [8, 15]. It is known that tularemia outbreaks in Turkey are caused by contaminated water and food, and are mostly seen in oropharyngeal form and the factor is *F. tularensis* subsp. *holarctica* [6, 8].

In this study, *F. tularensis* strains isolated from water samples taken from tularemia outbreak regions in Sivas province were used. The tul4 gene region encoding the 17 kDa outer membrane proteins of *F. tularensis*, the fopA gene region encoding the 43 kDa outer membrane protein, the 16S rRNA gene amplified for the identification of *F. tularensis* and the RD1 target gene region was amplified by classical PCR method and sequence analysis was performed. The PCR test targeting the *tul4* gene which is common in *F. tularensis* species was found positive in all isolates investigated in this study (Figure 1). For the determination of the subspecies of *Francisella tularensis* strains, the region of difference 1 (RD1) subspecies-specific PCR test was employed (Figure 1). All study isolates (n=8) yielded RD1 fragments of 900-1000 bp that corresponds to the RD1 size of *F. tularensis* subsp. *holarctica.* All isolates were identified as *F. tularensis* subsp. *holarctica* in agreement with earlier study by Ataş [11]. In the PCR study of the fopA gene region of *F. tularensis* bacteria, a band of approximately 400 bp was observed in all isolates. Similarly, using the 16S rRNA specific primer, we detected that the amplification products of all isolates approximately 1100 bp (Figure 1).

As a result of the sequencing analysis of the tul4 and fopA gene regions of the *F. tularensis* subsp. *holarctica* isolates, no difference was found and the tul4 and fopA gene regions of all samples were found to be identical. According to BLAST analysis, our samples were found to be 100% similar to the *F. tularensis* subsp. *holarctica* LVS (accession number CP009694) and *F. tularensis* subsp. *holarctica* PHIT-FT049 (accession number CP007148) samples in terms of tul4 gene region. *F. tularensis* subsp. *holarctica* PHIT-FT049 (accession number CP007148) was isolated from water sample in Turkey and was identified biovar *japonica* [16]. In terms of fopA gene region, it was determined that it was 100% similar to *F. tularensis* subsp. *holarctica* LVS (accession number CP009694) and 99.45% with *F. tularensis* subsp. *holarctica* PHIT-FT049 (accession number CP007148).

It is possible to distinguish *F. tularensis* subspecies from each other by PCR directed to the RD1 gene region. As a result of PCR directed to this gene region, *F. tularensis* subsp. *tularensis* in the 1500 bp region, subsp. *holarctica* in the 900/1100 bp region, subsp. *mediasiatica* gives bands in the region of 1400 bp [12]. In our study, as a result of the PCR made for the RD1 gene region, a band was observed in the region slightly less than 1000 bp (Figure 1). With this study for determination of subspecies, it was determined that the samples belong to the subspecies *holarctica*. As a result of the sequence analysis of the RD1 gene region of the *F.tularensis* isolates, the sequenced 835 bp was compared and it was found that the Bahçeiçi and Karaören isolates were the same in terms of this gene region. Both of Bahçeiçi and Karaören samples have been isolated from tularemia outbreaks in Gürün district. In terms of RD1 gene region, the other six samples were found to be different from Bahçeiçi and Karaören isolates and from each other in terms of base distribution.

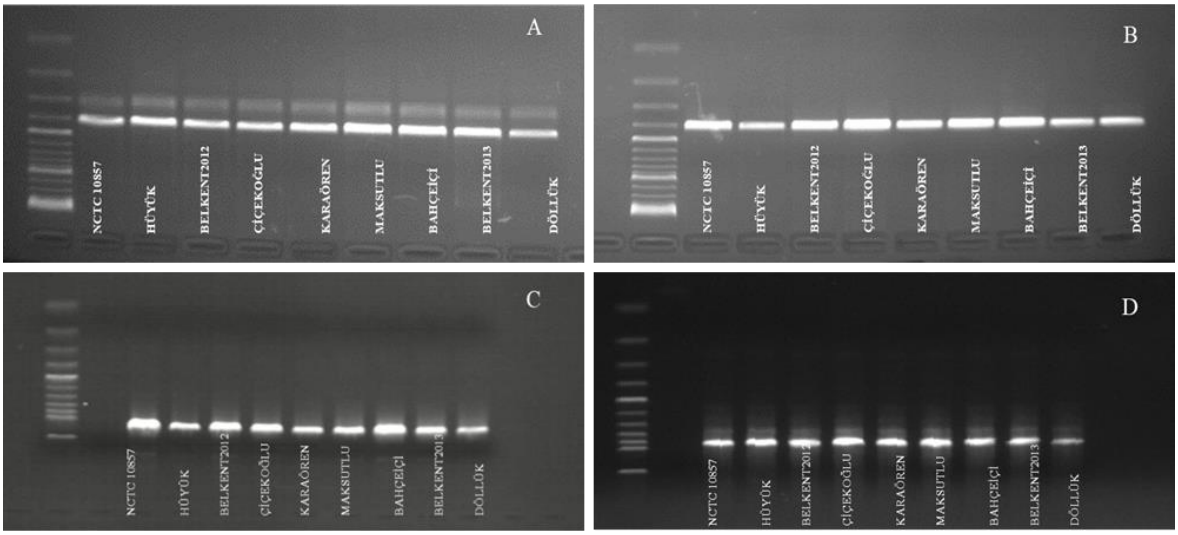

Figure 1. The amplification results of the isolates. *F. tularensis* subsp. *holarctica* LVS strain (NCTC 10857) was used as a positive control. A 100 bp-ladder (Promega) was used as a marker. A) tul4 (410 bp) B) fopA (401 bp) C) 16S rRNA (1100 bp) D) RD1 (900-1000 bp).

In comparison with GenBank, our samples were found to be 99.75% similar to *F. tularensis* subsp. *holarctica* PHIT-FT049 (accession number CP007148) and 94.64 to 95.10% with *F. tularensis* subsp. *holarctica* LVS (accession number CP009694) in terms of RD1 gene region.

According to the 16S rRNA gene region sequence analysis results, the base sequences of Belkent 2012 and 2013 isolates, Çiçekoğlu and Döllük isolates were determined to be identical. Belkent2012 and Belkent2013 strains were isolated from water samples taken from Belkent fountain of Şarkışla district 2012 and 2013 years, respectively. The other four isolates (Bahçeiçi, Karaören, Hüyük and Maksutlu) were found to have different base sequences in terms of 16S rRNA gene region. Our samples were found to be 97.74 - 98.49% similar to *F. tularensis* subsp. *holarctica* LVS (accession number CP009694) and 97.56 - 98.31% to *F. tularensis* subsp. *holarctica* PHIT-FT049 (accession number CP007148) in terms of 16S rRNA gene region (Figure 2). In a study conducted in Spain, 42 *F. ularensis* subsp. *holarctica* isolates were found to be similar in terms of 16S rNA gene region. The sequence detected in this study shared 99% similarity to those of strains from another Spanish outbreak of tularemia [17]. As a result of three gene-based phylogenetic analysis (tul4, fopA and 16S rRNA gene regions) of 10 *F. ularensis* subsp. *holarctica* strains isolated from China, the correlation not found between the genotype and the geographical area from which the bacteria were isolated [13].

Figure 2. Phylogenetic tree (Neighbor-joining, Kimura 2-parameters) based on 16S rRNA gene sequences comparing the *F. tularensis* subsp. *holarctica* strains of Sivas with other known GenBank records. Bootstrap percentage values from 1,000 replicates are located at nodes of the tree. Sequence alignments and tree generation were conducted in MEGA10.

During the tularemia epidemic in Sivas province between 2009 and 2010, *F. tularensis* DNA in lymph node samples of two patients was found positive by PCR method and it was stated that the outbreaks were waterborne. In this epidemic, 75.9% of the disease was observed in the oropharyngeal form [9]. The first cases of tularemia were observed in Tokat, which is adjacent to Sivas province, in 2005 and the disease is reported to be waterborne [18]. Two cases of tick-borne tularemia have been reported from Yozgat, another neighboring city of Sivas [19]. However, the presence of *F. tularensis* could not be detected by PCR method in 2054 ticks collected from vegetation in Sivas province (unpublished data). In a study conducted in Kayseri province, which is approximately 200 km away from Sivas, 1477 tick samples and 6203 mosquito samples were examined for the presence of *F. tularensis*. Francisella-like endosymbionts (FLEs) and *F. tularensis* were not detected in any genomic DNA pools constructed from ixodid ticks and mosquitos [20]. The first *F. tularensis* isolation from Sivas region with culture method was carried out in 2009. Şimşek et al. isolated *F. tularensis* from water samples taken from Sivas, Çorum and Samsun regions by culture method, and it was found that these samples were 100% similar to *F. tularensis* subsp. *holarctica* LVS in the 16S rRNA sequence analysis [10]. *F. tularensis* subsp. *holarctica* strains isolated from water samples in the outbreaks observed in Sivas and Çorum were found similar with the Whole Genome Sequencing method [21]. In a study conducted in our country, Gürcan et al. isolated two *F. tularensis* subsp. *holarctica* strain from lymph node aspirates of patients in Yazıkara and Nuhören villages in Gerede, Bolu. This strains were found similar to strains isolated from Bulgaria as a result of MLVA analysis [22].

F. tularensis subsp. *holarctica* has three biovars, biovar I, biovar II and biovar japonica. Biovars are divided according to erythromycin resistance, glucose and glycerol fermentation properties [23]. In Turkey, 250 *F. tularensis* subsp. *holarctica* strains were investigated and 249 strains were typed as biovar II and one strain as biovar *japonica* [16]. Çelebi et al. examined 776 samples isolated from various sources and in their study they typed 764 samples as *F. tularensis* subsp. *holarctica* and 12 samples as *F. tularensis* subsp. *holarctica* biovar *japonica* [24]. Kılıç et al. studied 40 samples with the "single nucleotide polymorphisms (SNP)" method and found that the samples were *F. tularensis* subsp. *holarctica*. In this study, the canSNP method was used and *F. tularensis* subsp. *holarctica* isolates are divided into 3 main groups and also subgroups [7]. As a result of our study, it was determined that *F. tularensis* isolates obtained from water samples in Sivas region belong to *holarctica* subspecies, were similar in terms of tul4 and fopA gene regions, and showed polymorphism in terms of 16S rRNA and RD1 gene regions. A limitation of this study is that relatively few strains were used. In the future, it is planned to isolate new strains from possible epidemic areas in our region and to conduct more comprehensive studies.

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Conflicts of interest

The authors state that did not have conflict of interests.

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