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Original Article

DEVELOPMENT OF A REAL-TIME PCR TEST SYSTEM FOR THE IDENTIFICATION OF *FRANCISELLA TULARENSIS*

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ABSTRACT

Tularaemia is caused by the Gram-negative bacterium *Francisella tularensis*, which has three subspecies: *holarctica*, *mediacia* and *tularensis*. Due to the high virulence of the pathogen, the wide range of susceptible animals and the presence of numerous vectors and natural reservoirs, the development of sensitive diagnostic methods for the epidemiological surveillance of tularemia is crucial. New opportunities for understanding the epidemiological situation of tularemia are opening up with the use of new molecular analysis technologies for typing *F. tularensis*. The development and use of molecular methods for the diagnosis of tularemia is important in this context. The present study developed a protocol for detection of *F. tularensis* by real-time polymerase chain reaction (qPCR). The selection of primers and a fluorescent probe was based on the aligned sequences of *ISFtu1* (multi-copy insertion element). qPCR conditions were optimised, including the determination of the optimal annealing and extension temperatures for the primers. Sensitivity was tested using successive 4-fold dilutions of DNA derived from *F. tularensis subsp. mediasiatica*, indicating a minimum sensitivity threshold of 15 genomic equivalents per reaction. Two subspecies of *F. tularensis* (*subsp. mediasiatica* and *subsp. holarctica*), 27 non-target bacterial species and 3 eukaryotic organisms were used to assess specificity.

Key words

Tularemia, Real-time PCR, Specificity, Pathogen detection, *Francisella tularensis,* Molecular diagnostics

INTRODUCTION

Tularemia is a zoonotic disease caused by the gram-negative bacterium *F. tularensis*, a facultative intracellular pathogen. Ten bacterial cells of *F. tularensis subsp. tularensis* are sufficient for the development of an infectious process with a mortality rate (without treatment) of up to 24%, making *F. tularensis* a potential biological weapon [1]. *F. tularensis* infects over 250 different species of animals and can be transmitted to humans in a variety of ways: by direct contact with an infected animal, by drinking contaminated water and food, by airborne droplets, and by bites from ticks, flies and mosquitoes [2]. According to the modern classification system, the species *F. tularensis* is divided into three subspecies: *tularensis* (with two subpopulations AI and AII), *holarctica* (with three biovariants: *japonica*, erythromycin-resistant *EryR* and erythromycin-sensitive *EryS*) and *mediasiatica* [3, 4, 5]. The subspecies *F. tularensis holarctica*, found in Europe, North America and Japan, is often associated with hare-like animals in Scandinavia, continental Europe and Japan, with ground voles in the former Union of Soviet Socialist Republics (USSR), and with beavers and muskrats in North America. This subspecies usually causes a less severe form of the disease in humans, often manifesting as glandular ulcer disease. The subspecies *F. tularensis mediasiatica* is moderately dangerous to rabbits and humans and is found only in the Central Asian republics of the former USSR [6]. *F. tularensis subsp. tularensis* is characterized by the highest mortality rate in humans and is found in North America [7]. The geographical origin and timing of the emergence of *F. tularensis* are unknown, and the population structure reflects the ability to spread over long distances and a very low mutation rate [8].

Due to the endemicity of tularemia in Kazakhstan, extensive fieldwork has been carried out over the past 70 years, allowing a number of natural foci of tularemia to be mapped from 1928 to 2018. Approximately 10,000 cases of tularemia infection have been reported in Kazakhstan [9]. The peak of infection was during labour migration of the unvaccinated population to endemic areas in the 1950s, with 100 to 2000 cases of human infection recorded in one outbreak [10]. Improvements in hygiene and routine vaccination of the population have reduced the number of cases to isolated cases. However, *F. tularensis* circulates in the regions of East Kazakhstan, Akmola, West Kazakhstan, Aktobe, North Kazakhstan and Pavlodar, as evidenced by periodic epizootics in rodents [11]. Epidemiological surveillance plays an important role in the control of tularemia in Kazakhstan. A wide variety of susceptible animals, the presence of vectors and natural reservoirs make methods based on direct detection of the agent the most important [12]. This factor increases the importance of the development and use of qPCR test systems in epidemiological surveillance, especially when working with complex environmental samples. Real-time PCR is a faster, more sensitive and more specific method for detecting pathogens [13].

The aim of these studies is to develop a real-time PCR protocol for the detection of *F. tularensis.*

MATERIALS AND METHODS

DNA samples

The work used a DNA sample isolated from the collection strains of *F. tularensis subsp. holarctica* and *F. tularensis subsp. mediasiatica* deposited at the RSE at the National Scientific Center of Especially Dangerous Infections named after

Masgut Aikimbayev. To test specificity, 27 DNA samples from unrelated bacteria and 3 samples from higher eukaryotes (cattle, small cattle and a white laboratory mouse) were used. The species identity of the bacterial strains was determined by analysing the nucleotide sequence of the 16S rRNA gene [14].

The DNA concentration was measured by a fluorometric method using a Qubit 2.0 fluorimeter and the Qubit dsDNA HS Assay Kit (Invitrogen, USA) according to the manufacturer's instructions.

Primer Selection

Primers and a fluorescent probe were selected for aligned Insertion sequence elements *ISFtu1* sequences of the following strains: *F. tularensis subsp. mediasiatica* FSC147, *F. tularensis subsp. tularensis* strain WY96, *F. tularensis* strain T01, *F. tularensis subsp. holartica* strain 2015321842, *F. tularensis* strain NR-28537, *F. tularensis subsp. holarctica* PHIT-FT049, *F. tularensis subsp. tularensis* TI0902, *F. tularensis* strain Schu4 *F. tul* Mut-127, *F. tularensis subsp. tularensis* strain Scherm, *F. tularensis subsp. tularensis* isolate FSC237, *F. tularensis* strain 2017317779, *F.. novacida* PA10-7858, *F. tularensis subsp. holarctica* srain FSC021. The alignment was performed using the BioEdit sequence alignment editor software (Hall T.A) [15].

Optimization of Qpcr Conditions

For the qPCR reaction, a ready-to-use reaction mix BioMaster HS-PCR (2×) (Biolabmix, Russia) was used. Primer annealing temperature optimization was performed on a CFX96 thermocycler (Bio-Rad, USA). The qPCR program included an initial denaturation step at 95°C for 5 minutes; 10 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 54-61°C for 1 minute; followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 54-61°C for 1 minute, taking into account the fluorescence results at the annealing/elongation step. Data analysis was performed using the BioRad CFX Manager software. During optimization, DNA from *F. tularensis subsp. mediasiatica* and F. *tularensis subsp. holarctica* (1 ng per reaction) were used. DNA from *Moraxella bovis* (15 ng per reaction) was used as a heterologous species. The qPCR mixture contained 1× BioMaster HS-PCR reaction mixture, 400 nM of forward and reverse primers, 400 nM of fluorescent probe, and one of the bacterial species in a volume of 25 µL. After determining the optimal primer annealing temperature, primer concentration in the reaction mixture was optimized in the range of 200 to 800 nM, with a standard concentration of 400 nM for the fluorescent probe.

Sensitivity and Specificity Assessment

The sensitivity assessment was conducted by employing a series of 4-fold dilutions of *Francisella tularensis subsp. mediasiatica* DNA (ranging from 1,000,000 to 0.2 genome equivalents (GE) per reaction) in triplicate. DNA concentration was converted to the number of GE using the online resource: https://www.technologynetworks.com/tn/tools/copy-numbercalculator.

To test the specificity of the developed qPCR protocol, DNA from *F. tularensis subsp. mediasiatica* and *F. tularensis subsp. holarctica* was used at a concentration of 1 ng per reaction, along with DNA from 27 bacterial species not belonging to the *Francisella* genus (Table 1) at 15 ng per reaction, and DNA samples from 3 eukaryotic organisms at 50 ng per reaction (*Bos taurus*, *Ovis aries*, *Mus albus officinarum*).

RESULTS

Selection of primers

Alignment of *ISFtu1* sequences from 12 strains of *F. tularensis* and 1 strain of *F. novacida* showed that this region is highly conserved across the analyzed genomes. An optimal region was selected for designing the forward primer isftu1_F_54, reverse primer isftu-1_R_189, and fluorescent probe isftu-2_Probes_108 (Figure 1). The probe was labeled with FAM at the 5' end and quencher BHQ1 at the 3' end.

During primer testing for secondary structure formation, no hairpins or primer dimers were detected. Primer checking in BLAST [16] showed specific annealing only to *F. tularensis* genomes.

Table 1. List of bacteria used to test specificity

Family *Lactobacillaceae:* *Lactobacillu sparaplantarum, Lactobacillus paracasei, Lactobacillus pontis, Pediococcus acidilactici.*	Family *Nocardiaceae: Rhodococcus kroppensteti,Rhodococcus corynebacterioides.*	Family *Pseudomonadaceae*: *Pseudomonas silesiensis, Pseudomonas putida, Pseudomonas peli, Pseudomonas mucidolens*
Family Bacillaceae: *Bacillus wiedmannii, Bacillus aerius, Bacillus velezensis.*	Family *Enterococcaceae:* *Enterococcus durans.*	Family *Enterobacteriaceae*: *Klebsiella oxytoca, Enterococcus faecalis,* *Klebsiella pneumoniae, Shigella sonnei, Shigella flexneri, Escherichia fergusonii.*
Family *Alcaligenaceae*: *Bordetella bronchiseptica.*	Family *Pasteurellaceae*: *Pasteurella multocida.*	Family *Streptococcaceae*: *Streptococcus criceti.*
Family *Oxalobacteraceae:* *Massilia putida.*	Family *Erwiniaceae*: *Erwini aendophytica, Pantoea agglomerans.*	Family *Paenibacillaceae*: *Brevibacillus borstelensis.*

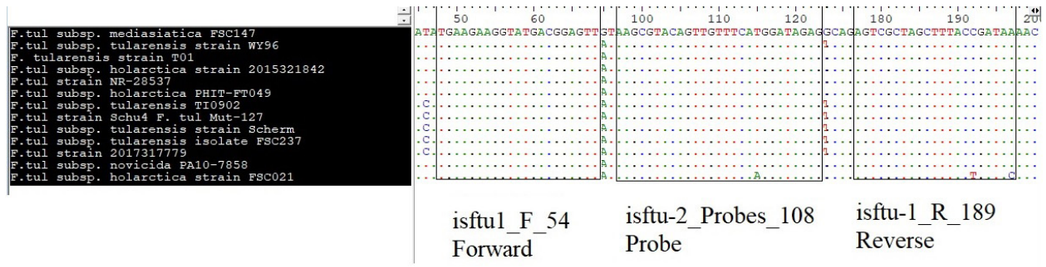
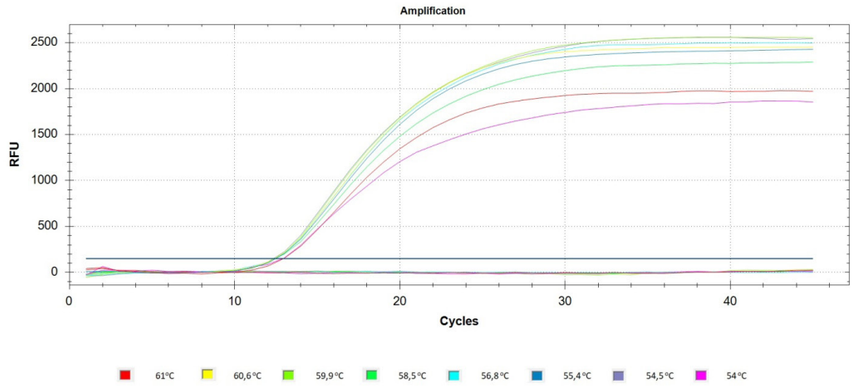


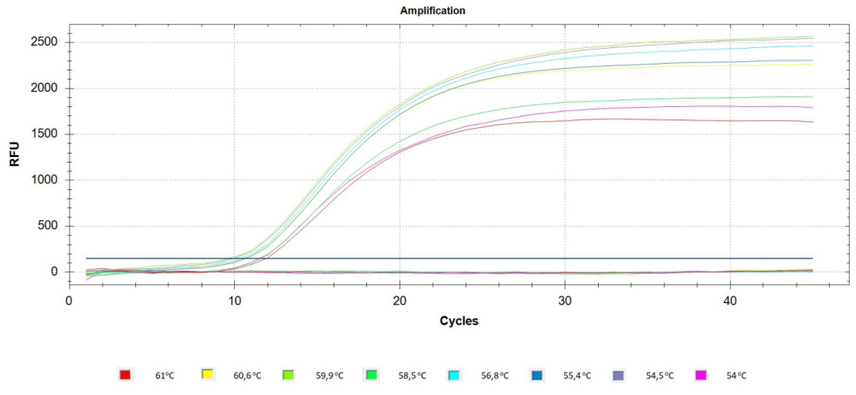
Figure – 1. Aligned *ISFtu1* sequences

qPCR optimization

During the optimization stage, the annealing and elongation temperature of 60ºC was chosen as the optimal temperature. At this temperature, wells containing DNA from the target pathogen exhibit one of the minimum threshold cycle (Ct) values and a high fluorescence level at the plateau. Increasing the temperature results in an increase in the threshold cycle value and a lower fluorescence level at the plateau (Figure 2). Meanwhile, in wells containing a high concentration of internal control DNA (*Moraxella bovis*), an increase in fluorescent signal is not detected at any temperature.



2.A



2.B

Figure - 2. Growth of the amplification curve at temperatures ranging from 54°C to 61°C for *F. tularensis subsp. holarctica* (A) and *F. tularensis subsp. mediasiatica*

Adjusting the primer concentrations in the reaction mixture from 200 to 800 nM allowed us to establish that at concentrations of 400 nM for the forward primer (ISFtu1_F_54), 600 nM for the reverse primer (ISFtu1_R_189), and 400 nM for the fluorescent probe (ISFtu1-2_Probes_108) per reaction, the minimum threshold cycle value is observed.

Sensitivity and specificity testing

Sensitivity and specificity testing were conducted under optimized conditions. Sensitivity testing on a series of 4-fold dilutions of *F. tularensis subsp. mediasiatica* DNA revealed a minimum detection threshold of 15 genome equivalents per reaction (Figure 3). Clear fluorescence signal registration with threshold cycle values ranging from 15 to 35 was observed in the range from 1 million to 15 genome copies per reaction, respectively. Lower copy numbers did not result in fluorescent signal accumulation.

Specificity testing was confirmed using DNA from two *subsp*ecies of *F. tularensis* (*subsp. mediasiatica* and *subsp. holarctica*), 27 non-target bacterial species, and 3 eukaryotic organisms. A specific signal was observed only in samples of the target organisms, without amplification occurring in the DNA samples of the 3 eukaryotic organisms (Figure 4).

DISCUSSION

Regular epidemiological monitoring of highly dangerous pathogens is a key element in the control system for preventing the spread and interrupting the chain of infection transmission. In Kazakhstan, regular monitoring is conducted, making the use of rapid direct pathogen detection methods a relevant task [17].

In our current research, we targeted the multicopy insertion element *ISFtu1*, which has a frequency of occurrence in the *F. tularensis* genome reaching up to 50 copies [18]. *ISFtu1* belongs to the IS630 Tc-1 mariner family. The transposases of this family recognize the TA sequence for insertion, but the insertion frequency depends on the surrounding sequences. It is presumed that the target sequence IS630 - 5'-CTAG-3' creates a TA duplicate at the insertion site. Members of this family contain the DDE (or DDD) motif, which is necessary for catalytic activity. These transposases utilise cut-and-paste transposition, leaving behind 2-base pair inverted repeats after staggered DNA breaks, which are then sealed by cellular repair processes. *ISFtu1* contains a sliding heptamer (AAAAAAG) for reading frame shifting-1, allowing translation of the entire transposase [19, 20]. The use of a multicopy insertion element allowed us to develop a highly sensitive protocol with a minimal sensitivity limit of 15 genomic equivalents per reaction mixture with 100% specificity verified on DNA from 27 bacterial species and 3 eukaryotic organisms.

In the research conducted by Jessica L. Versage and her colleagues, they reported on the development of a real-time TaqMan multiplex PCR system. This system utilized the mobile element *ISFtu2*, along with genes encoding *fopA* and *tul4* [21]. The method demonstrated specificity towards *F. tularensis*, with the highest sensitivity at 44 genome equivalents (GE) when primers targeting the *fopA* gene were used. Successful utilization of *fopA* was also demonstrated in the work by Osama Fujima and co-authors, who developed a PCR test

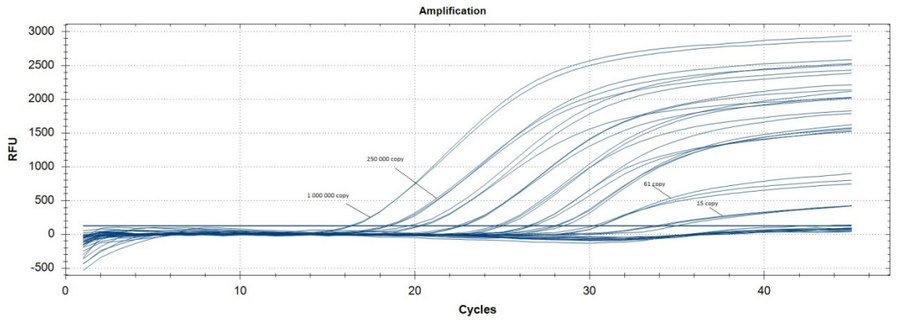


Figure - 3. Rise of the amplification curve across concentrations ranging from 0.2 to 1,000,000 GE in reactions

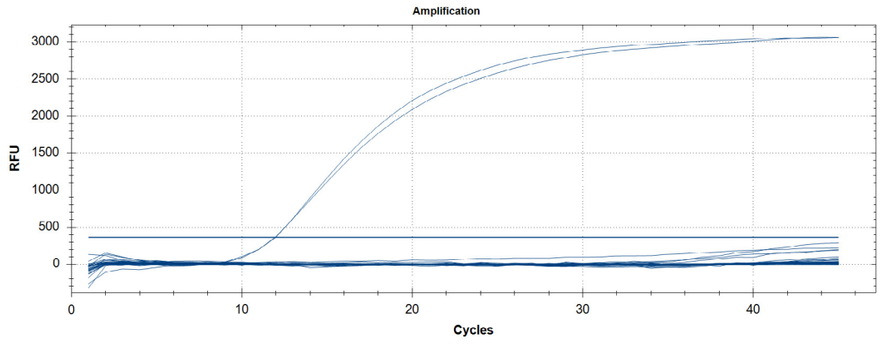


Figure 4. Specificity testing. Thirty DNA samples were used (27 prokaryotic DNA samples and 3 eukaryotic DNA samples), along with DNA from F. *mediasiatica* and F. *holarctica*.

with sensitivity ranging from 20 fg to 1 ng (from 10 copies to 500 thousand) [22]. Jan L. Mitchell and co-authors utilized unique genes for three *subsp*ecies of *F.* (*tularensis*, *holarctica*, and *mediasiatica*) - FTT0376 and FTT0523. The minimum sensitivity for the PCR test FTT0376 was approximately 80 GE, and for the PCR test FTT0523 - about 20 genomic GE [23]. The use of the mobile element *ISFtu2* for *F. tularensis subsp. holarctica* allowed for an increase in sensitivity to 10 GE [24].

In terms of sensitivity, the real-time PCR assay developed by us is comparable to counterparts published in the literature and potentially can be utilized for testing DNA extracted from natural reservoirs, vectors, and environmental samples.

PCR is successfully applied in the examination of samples from rodents and ticks in China, Japan [25, 26]. Real-time PCR (PCR) targeting *ISFtu2* was recently employed to register the first cases of tularemia in Cape hares (*Lepus capensis*) and wild rabbits (*Oryctolagus cuniculus*) on the African continent [27].

CONCLUSION

The conducted research has led to the development of a real-time PCR test for the detection of *F. tularensis*. The sensitivity at 15 genomic equivalents and high specificity, verified on a collection of DNA from various bacterial species and higher eukaryotic organisms, including rodents, allow it to be considered as a highly sensitive and specific test for monitoring studies on tularemia.

GRATITUDE

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