A multiplex real-time PCR assay for the detection and differentiation of *Francisella tularensis* subspecies

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*Francisella tularensis* is the aetiological agent of tularemia, a zoonotic disease with worldwide prevalence. *F. tularensis* is a highly pathogenic organism and has been designated a category A bioterrorism agent by the Centers for Disease Control and Prevention. Tularemia is endemic in much of the USA, Europe and parts of Asia. It is transmitted by numerous vectors and vehicles such as deer flies, ticks and rabbits. Currently, there are four recognized subspecies of *F. tularensis*: *tularensis* (type A), *holartica* (type B), *mediasiatica* and *novicida*. Within the type A classification there are two subclassifications, type A.I and A.II, each with a specific geographical distribution across the USA. *F. tularensis* subsp. *holartica* (type B) is found in both the USA and Europe. Because of virulence differences among subtypes, it is important that health departments, hospitals and other government agencies are able to quickly identify each subtype. The purpose of this study was to develop a multiplex real-time PCR assay for the identification and discrimination of type A.I, type A.II, type B and *novicida* subspecies of *F. tularensis*. The assay was validated using 119 isolates of *F. tularensis*, three of its nearest neighbours and 14 other bacterial pathogens. This assay proved to be ~98% successful at identifying the known subspecies of *F. tularensis* and could prove to be a useful tool in the characterization of this important pathogen.

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**INTRODUCTION**

*Francisella tularensis* is a Gram-negative facultative intracellular bacterial pathogen and is the causative agent of the zoonotic disease tularemia. As few as ten organisms can cause disease via the aerosol route (McCrum, 1961). Due to its high infectivity, ease of dissemination and ability to cause illness and death, *F. tularensis* has long been considered a potential bioweapon by Japan, the former Soviet Union and the USA (Dennis et al., 2001). With the advent of the Centers for Disease Control and Prevention (CDC) Select Agent Program (a series of rules and regulations governing the possession and transfer of organisms that could be used as bioweapons), *F. tularensis* has been classified as a category A potential agent of bioterrorism. It was estimated by the World Health Organization that 50 kg of *F. tularensis* dispersed as an aerosol over a highly populated area of five million people would result in 250,000 cases of tularemia with 19,000 deaths (Dennis et al., 2001).

*F. tularensis* is a member of the gamma subclass of the proteobacteria, currently consisting of three accepted subspecies: *tularensis* (type A), *holartica* (type B) and *mediasiatica*. The subspecies differ in their geographical distribution as well as in virulence (Johansson et al., 2004; Keim et al., 2007). Much of the scientific literature, including this work, refers to *Francisella novicida* as a fourth subspecies of *F. tularensis* (Svensson et al., 2005; Keim et al., 2007; Vogler et al., 2009). Types A and B are most associated with human disease, with type A being the more virulent. The *F. tularensis* subsp. *mediasiatica* is more commonly found in the Central Asian republics of the former Soviet Union and little is known about its ability to cause disease in humans (Broekhuysen et al., 2003). The *F. tularensis* subsp. *novicida* is more associated with water and rarely causes human disease (Hollis et al., 1989). The natural reservoir of *F. tularensis* remains largely unknown; though there is growing evidence that amoeba may play an important role in harbouring the bacterium (Gustafsson, 1989; Abd et al., 2003; El-Etr et al., 2009).

The genome of *F. tularensis* is highly conserved among the four subspecies. The 16S rRNA genes exhibit 98.5–99.9% similarity (Forsman et al., 1994). Even with this high degree...
of sequence similarity, each of the subspecies demonstrates notable differences in virulence. Within F. tularensis subsp. tularensis (type A), multi-locus variable-number tandem repeat analysis (MLVA) revealed a subdivision: type A.I and type A.II (Johansson et al., 2004). An apparent geographical separation exists between these two subtypes. Type A.I isolates are primarily found in the central and eastern USA, while type A.II isolates are generally found in the western portion (Staples et al., 2006). Molins-Schneekloth et al. (2008), using suppression subtractive hybridization, have successfully identified genetic markers used for the differentiation of type A.I and A.II isolates.

Many molecular methods have been used for the identification of F. tularensis such as PFGE, amplified fragment length polymorphism fingerprinting, 16S rRNA gene sequencing (García Del Blanco et al., 2002), RFLP (Thomas et al., 2003), MLVA (Johansson et al., 2004; Vogler et al., 2009) and PCR (Johansson et al., 2000; Versage et al., 2003; Kugeler et al., 2005, 2006). Many of these techniques can be labour intensive and cumbersome to perform, especially on a large number of samples. The previous PCR assays developed lack the convenience of real-time detection and are not performed in multiplex. Since tularemia is endemic in many areas of the USA and the potential exists for F. tularensis to be used as a bioweapon, rapid techniques are necessary to aid in the accurate identification and differentiation of F. tularensis subtypes. The goal of this study was to develop a multiplex real-time PCR (RT-PCR) assay for the rapid identification of F. tularensis isolates relevant to the subspecies commonly found in the USA and Europe.

**METHODS**

**Bacterial strains and culture conditions.** The isolates used in this study are a part of a select agent archive housed at Brigham Young University, Utah, USA, and maintained by Dr Richard Robison. The collection largely consists of isolates obtained from the State Health Departments of Utah and New Mexico over the past two decades. All F. tularensis isolates were grown on modified Mueller–Hinton agar (MMHA; Becton Dickinson) for 3–4 days with 5 % CO2 at 35 °C. The MMHA was prepared by autoclaving the Mueller–Hinton base, which was chocalotized by adding 5 % sheep blood while the medium was approximately 65 °C. After the medium cooled to 50 °C, 10 ml 10 % glucose and 20 ml IsoVitaleX were added to 1 l of the medium. For near neighbours, genomic DNA was obtained from the Critical Reagents Program (www.jpeocbd.osd.mil/packs/Default.aspx?pg=1205).

**DNA preparation.** Total genomic DNA was extracted from each isolate using the MagNA Pure System (Roche) and the MagNA Pure LC DNA Isolation Kit III (Roche), according to the manufacturer’s instructions. Briefly, cells grown on MMHA agar were suspended in 250 μl Tris/EDTA buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] containing 1.8 μg lysozyme μl−1 and incubated for 1 h at 37 °C. To this tube, 270 μl bacterial lysis buffer and 100 μl proteinase K were added and the tube was incubated for 10 min at 65 °C. DNA was eluted in a total volume of 100 μl. DNA concentration was measured using a PicoGreen assay (Invitrogen) and TBS–380 fluorometer (Turner Biosystems). For optimization purposes, DNA stock solutions were diluted to a concentration of approximately 50 ng μl−1.

**Primer and probe design.** Whole genome sequences of F. tularensis subsp. holarctica strains OSU18 (GenBank accession no. CP000437), LVS (AM233362) and FTNF002-00 (CP000803), subsp. novicida strain U112 (CP000439), subsp. tularensis strains WY96-3418 (CP000608), FSC198 (AM286280) and Schu S4 (AJ749949), and subsp. mediiasiatica strain FSC147 (CP000915) were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/; Table 1). These genomes were aligned to each other using the genome alignment tool Mauve (Darling et al., 2004). With the F. tularensis subsp. holarctica genomes set as the reference sequences, the genomes were analysed for regions of non-homology (Fig. S1, available in JMM Online). The process was repeated with each of the other genomes set as the reference and analysed. Only in the F. tularensis subsp. holarctica (nt 800 268–800 721 of strain FTNF002-00) and subsp. novicida (nt 1 579 889–1 580 210 of strain U112) genomes were unique regions identified using this method.

For the A.I and A.II subtypes, the RD8 (A.I) and RD5 (A.II) regions described by Molins-Schneekloth et al. (2008) were selected for TaqMan probe design. Putative sequences were then checked against known sequences in the National Center for Biotechnology Information database by using the BLAST engine (BLASTN) to confirm uniqueness. Once sequences were determined to be unique, primers and minor-groove-binder TaqMan probes (Table 2) were designed using Primer Express version 3.0 from Applied Biosystems. Primers and probes were also obtained from Applied Biosystems.

**PCR cycling conditions.** RT-PCR assays were performed on an ABI 7900 using TaqMan Universal Master Mix with UNG (Applied Biosystems). Total reaction volume was 25 μl, as recommended by the manufacturer. Individual assay mixes were as follows: 500 nM forward primer, 500 nM reverse primer, 250 nM probe, 20 ng target DNA and PCR-grade H2O (Quality Biological) to 25 μl. Thermal cycling conditions for individual reactions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 68 °C.
60 °C. The multiplex assay consisted of *F. tularensis* subsp. *tularensis* types A.I and A.II, and subspp. *holarctica* primer probe sets. Mixes for the multiplex reaction were as follows: 250 nM each forward primer, 250 nM each reverse primer and 125 nM each probe. Thermal cycling conditions for the multiplex assay were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 58 °C. A positive signal was determined by the crossing of a fluorescence threshold of 0.2 before cycle 40. Data analysis was performed using SDS v2.3 (Applied Biosystems). All tests were performed in at least triplicate to ensure reproducibility.

While an *F. tularensis* subsp. *novicida* specific primer probe set was initially designed and tested singularly, it was later determined that it could be omitted from the multiplex reaction. Rather than use all four assays in the multiplex reaction, shared genomic markers among the subspecies allowed a scoring matrix (Table 3) that was used to differentiate the subspecies using only three of the assays.

**MLVA analysis.** MLVA was performed as described previously by Johansson *et al.* (2004) and Vogler *et al.* (2009). Briefly, the 11 marker MLVA was set up in five PCR mixes. Mix 1A contained three labelled primers, mix 1B contained one labelled primer, mix 2 contained three labelled primers, mix 3A contained three labelled primers and mix 3B contained one labelled primer. After amplification of the targets by PCR, mixes 1A and 1B were combined with water in a final ratio of 2 : 1:97 to produce mix 3. The resulting three mixes were subjected to capillary electrophoresis on a 16-capillary 3130xl (Applied Biosystems). Each sample was run with 2.25 µl MapMarker 1000 (Bioventures) per well as a nucleic acid size standard. Resulting peaks (Fig. S2) were compared with an MLVA database, kindly provided by Paul Keim, Northern Arizona University, for subspecies identification. Positive identifications were called if a minimum of 8 of 11 peaks were present with at least one uniquely identifying peak to distinguish the isolate from others.

**RESULTS**

The goal of this work was to address some of the shortfalls of previous PCR assays for *F. tularensis* and develop a multiplex RT-PCR assay useful for the rapid identification and characterization of *F. tularensis* isolates commonly found in the USA and Europe. Markers unique to each of the *F. tularensis* subspecies could not be identified by us or others (Molins-Schneekloth *et al.*, 2008). Molins-Schneekloth *et al.* (2008) identified a genetic marker that was unique to *F. tularensis* subsp. *tularensis* type A.I, subsp. *holarctica* and subsp. *novicida*, and another marker unique to subsp. *tularensis* type A.II, subsp. *holarctica* and subsp. *novicida*. Based on these differences, we found it more reasonable and economical to use a multiplex assay with a scoring matrix to type the four different types of *F. tularensis* (Table 3).

The development of this RT-PCR assay has broad application across the fields of medical surveillance and CDC select agent detection. The different types and subspecies of *F. tularensis* differ not only in their capacity to cause disease but also in their geographical distribution (Keim *et al.*, 2007).

**Assay validation**

For the validation of the multiplex PCR assay, results from 119 *Francisella* isolates were compared with those from other PCR assays (Kugeler *et al.*, 2006) and the MLVA data from this study (Table S1). On 32 (~27%) of the isolates, insufficient MLVA data were obtained for an identification. The experiment was repeated three times, with the same

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**Table 2. Primer and probe sequences**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer/probe</th>
<th>Sequence (5′→3′)*</th>
<th>Start position 5′</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tularensis</em> A.I</td>
<td>Forward</td>
<td>AGCTTATGCATCGGATGTTAATTT 100 855 (Schu S4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAAGCTGGACGCTCAAAGGT 100 921 (Schu S4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>6FAM-ATGATAGATCCTTTGGCCCTGA-MGBNFQ 100 881 (Schu S4)</td>
<td></td>
</tr>
<tr>
<td><em>tularensis</em> A.II</td>
<td>Forward</td>
<td>CGAGATTTCGACGCTTCTTCT 427 942 (WY96-3418)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTGCCCAAGACAGCAAGGT 428 002 (WY96-3418)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>VIC-AACCTTATGCTAAAGGTCG-MGBNFQ 427 964 (WY96-3418)</td>
<td></td>
</tr>
<tr>
<td><em>holarctica</em></td>
<td>Forward</td>
<td>TTGCGCATCAATTACTCAGCTTAG 800 609 (LVS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAAGCGGTGCTGGCTTGGATAA 800 670 (LVS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TET-CCTGGCCAGTATT-MGBNFQ 800 635 (LVS)</td>
<td></td>
</tr>
<tr>
<td><em>novicida</em></td>
<td>Forward</td>
<td>TCAATGTTGCTAAAGCTCTGGAGTT 1 580 042 (U112)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AATGATGATATAAAGAAGTGAGGCTT 1 580 141 (U112)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>NED-TGTAAAGCCATATAAGG-MGBNFQ 1 580 092 (U112)</td>
<td></td>
</tr>
</tbody>
</table>

*MGBNFQ, Minor groove binder, non-fluorescent quencher.

**Table 3. Scoring matrix for triplex assay**

<table>
<thead>
<tr>
<th><em>F. tularensis</em> type</th>
<th>Assay</th>
<th>A.I</th>
<th>A.II</th>
<th>holarctica</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.I</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.II</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td><em>holarctica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>novicida</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
results. Even the U112 strain, which is widely known to be an *F. tularensis* subsp. *novicida* isolate, was inconclusive using the MLVA technique. This illustrates the need for additional typing methodologies.

Of the 87 isolates for which the MLVA was successful, 85 (~98 %) isolates had the same subspecies identification as the multiplexed PCR assay. The assay was also tested against three near neighbours, *Wolbachia persica*, *Francisella philomiragia* 25015 and *F. philomiragia* 25016; each with a negative result. The assay also showed no cross-reactivity with other laboratory species (Table 4).

Multiplex PCR results for isolates 70000907 and 6168 did not match up with the MLVA data. While the multiplexed PCR assay categorized isolate 70000907 as *F. tularensis* subsp. *novicida*, the MLVA analysis grouped it as type A.I. This is consistent with another PCR assay (Kugeler et al., 2006), which classified this isolate simply as type A. The multiplex PCR data classified isolate 6168 as type B, while both the MLVA and the Kugeler et al. (2006) PCR assay classified it as *F. tularensis* subsp. *novicida*. The reasons for these discrepancies are not immediately clear but may be due to genomic rearrangement. These discrepancies are a focus of ongoing research.

### Assay sensitivity

Serial tenfold dilutions of genomic *F. tularensis* DNA were assayed to estimate the detection limits when used in singleplex and multiplex reactions. In singleplex reactions, *F. tularensis* type A.II was the most sensitive at 25 fg. *F. tularensis* type A.I, type B and subsp. *novicida* all had the same detection limit at 250 fg (Fig. S3). Assuming a genome weight of 2.2 fg, limits of detection were ~11 organisms for *F. tularensis* type A.II and ~141 organisms for type A.I, type B and subsp. *novicida* assays. In the multiplex reactions, limits of detection were reduced by 1 log10 step; *F. tularensis* types A.I, A.II and subsp. *novicida* all had limits of detection at 250 fg, while type B was at 2.5 pg (Fig. 1). Again, with a genome weight of 2.2 fg, this translates to ~114 organisms for subtypes A.I and A.II, and *novicida* assays, and ~1136 organisms for the type B assay. Each of the assays were run in triplicate with identical results.

### Characterization of isolates

We used this multiplex assay to characterize *F. tularensis* isolates, the majority of which were natural isolates from Utah and New Mexico. Of all the isolates tested (Table S1), 5.0 % were *F. tularensis* type A.I, 76.5 % were *F. tularensis* type A.II, 6.7 % were identified as *F. tularensis* type B and 11.8 % were identified as *F. tularensis* subsp. *novicida*.

### DISCUSSION

Many PCR assays for *F. tularensis* and its subspecies have been developed. An assay developed by Broekhuising et al. (2003) is capable of discriminating the four subspecies of *F. tularensis*. However, it does not have the convenience of real-time detection and necessitates running the products on a gel to visualize amplicons up to 3 kb in length. Furthermore, the assay is not multiplexed, nor can it discriminate between *F. tularensis* types A.I and A.II. Kugeler et al. (2006) developed an RT-PCR assay for *F. tularensis* that was able to distinguish *F. tularensis* type A from type B, but it could not differentiate subtype A.I from subtype A.II, nor could it identify the *novicida* subspecies. Finally, Molins-Schneekloth et al. (2008) were able to develop a multiplex PCR assay that could differentiate *F. tularensis* subtype A.I from subtype A.II, but the assay was not real time, nor could it differentiate among *F. tularensis* type B or the *novicida* subspecies.

This work describes the first, according to our knowledge, multiplexed RT-PCR assay for the characterization of the major types of *F. tularensis* found in the USA and around the world: type A.I, type A.II, type B and subspecies *novicida*. Since *F. tularensis* type A.I is usually found on the East Coast of the USA, an outbreak of type A.I on the West Coast could be an indication of an intentional release. This multiplex assay could help law enforcement agencies to identify possible bioterrorism events as well as guide the administration of therapeutics by health officials.

A major hurdle for creating *F. tularensis* PCR assays specific for subspecies is that their genomes are highly similar. The 16S rRNA genes that are typically used for identification of micro-organisms exhibit 98.5–99.9 % similarity across all subspecies, which equates to a difference of 2–23 nt (Forsman et al., 1994). A recent study further highlighted the similarities of *F. tularensis* subspecies. The results showed that a pairwise alignment of sequenced draft genomes of low and high virulence subspecies exhibited over 95 % similarity (Champion et al., 2009). Using various regions of difference identified by suppression subtractive hybridization (Molins-Schneekloth et al., 2008) and comparative genomic data...
using Mauve software (Darling et al., 2004), we were able to identify genomic regions conducive to the creation of RT-PCR assays.

To validate the assays, we tested them against other laboratory strains (Table 4) in addition to two near-neighbours from the same genus: F. philomiragia and the nearest neighbour of F. tularensis, W. persica (Forsman et al., 1994). The results showed no cross-reactivity among these other species, demonstrating that the identified regions were unique to the subspecies of F. tularensis. Furthermore, the subspecies identification based on the scoring matrix (Table 3) shows a
successful identification of ~98% of the isolates compared with the MLVA data. This discrepancy was not an artefact of the multiplex assay, as it was also seen in the singleplex reaction. Despite these slight differences, we believe that this multiplex assay remains a powerful, rapid, presumptive screening test for the subspecies of *F. tularensis*. In addition, this work further underscores the need for multiple identification assays to definitively identify these closely related subspecies.

In singleplex reactions, we demonstrated a sensitivity based on serial tenfold dilutions of stock DNA of 11 organisms for the type A.II, and 114 organisms for the type A.I, type B and *novicida* assays. These sensitivities are within an order of magnitude of other published *F. tularensis* RT-PCR assays (Versage *et al.*, 2003; Kugeler *et al.*, 2006). In the triplex reactions, the type A.I, type A.II and *novicida* assays yielded a sensitivity of 114 organisms and 1136 organisms for the type B reaction. The sensitivities remained the same for the type A.I and *novicida* assays, with an order of magnitude decrease in sensitivity for the type A.II and type B assays. Another multiplex RT-PCR assay for *F. tularensis* demonstrated sensitivities as low as 0.5 genome equivalents (Tomaso *et al.*, 2003). This increased sensitivity may be explained by two factors: 1) the assay targeted the 16S rDNA, of which multiple copies are present in the genome; and 2) the multiplex assays only included two assays, thus reducing the chance of competitive PCR problems. When Tomaso *et al.* (2003) included three assays in their multiplex assay, no amplification curves were observed.

In larger multiplex reactions, it is not uncommon to see a decrease in sensitivity. In a quadruplex RT-PCR assay for *Yersinia pestis*, Stewart *et al.* (2008) saw a tenfold decrease in the sensitivity when their assay was moved from singleplex to quadruplex: from 150 pg to 1.5 ng. The results for our triplex assay are similar to these results, showing a tenfold decrease in sensitivity for two of the assays when used in a multiplex format: from 114 to 1136 organisms.

This multiplex assay was used to characterize a large collection of *F. tularensis* isolates. Many of the natural isolates were obtained from the Utah and New Mexico Departments of Health. Some standard laboratory strains of *F. tularensis*, such as Schu S4, LVS, U112 and other *novicida* variants, were included for reference. The assay, including all isolates except the standard strains and *novicida* variants, returned 104 isolates characterized. Of those, the vast majority (91 isolates or ~88%) were identified as type A.II. Four isolates (~3.8%) were identified as type A.I, seven isolates (~6.7%) were identified as type B and one isolate (~0.9%) was identified as *novicida*. This one isolate that was identified as *novicida*, however, is likely a misidentification because another PCR assay (Kugeler *et al.*, 2006), as well as the MLVA data, identified it as type A.

It is not surprising that the vast majority of the tested isolates were type A.II, since most of the isolates used in this study were obtained from the Utah and New Mexico Departments of Health. This distribution of isolates is consistent with previous data (Staples *et al.*, 2006; Keim *et al.*, 2007), suggesting that *F. tularensis* type A.II is predominant in western USA and that *F. tularensis* type A.I is predominantly found in eastern USA.

*F. tularensis* is classified as a category A select agent by the CDC. Due to its potential use as a bioterrorism weapon, its endemic status in various locations around the world and differences in virulence among the various types of *F. tularensis*, it is important that government agencies, health departments and hospitals are able to rapidly identify each subtype. The assay presented in this work is a rapid, single-tube, multiplex RT-PCR assay that can be used to quickly screen individual samples or adapted for high-throughput applications in either 96- or 384-well formats. We believe that this assay will be an invaluable tool in the presumptive identification and characterization of *F. tularensis* isolates during outbreaks of disease or possible bioterrorism events.

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