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# Identification of *Francisella tularensis* subsp. *tularensis* A1 and A2 infections by real-time polymerase chain reaction

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**Abstract**

*Francisella tularensis* subsp. *tularensis* (type A) is subdivided into clades A1 and A2. Human tularemia infections caused by A1 and A2 differ with respect to clinical outcome; A1 infections are associated with a higher case fatality rate. In this study, we develop and evaluate TaqMan polymerase chain reaction (PCR) assays for identification of A1 and A2. Both assays were shown to be specific to either A1 or A2, with sensitivities of 10 genomic equivalents. Real-time PCR results for identification of A1 and A2 were in complete agreement with results obtained by pulsed field gel electrophoresis analysis or conventional PCR when specimens from sporadic tularemia cases and a tularemia outbreak involving both A1 and A2 were tested. In addition, outbreak samples not previously typed to the clade level could be classified as A1 or A2. The assays described here provide new diagnostic tools with a level of sensitivity not previously available for identification of A1 and A2 infections.
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*Keywords:* *Francisella tularensis*; Tularemia; Type A, A1, A2; TaqMan

## 1. Introduction

*Francisella tularensis* is a Gram-negative bacterial pathogen that causes the zoonotic disease tularemia (Ellis et al., 2002). Two subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B), are of clinical relevance, and both can cause several distinct presentations of tularemia. Tularemia infections caused by *F. tularensis* subsp. *tularensis* are documented only in North America, whereas *F. tularensis* subsp. *holarctica* causes infections in North America, Europe, and Asia (Ellis et al., 2002; Sjostedt, 2005). In the United States, an average of 124 human cases of tularemia is reported each year (Centers for Disease Control and Prevention [CDC], 2002). *F. tularensis* subsp. *tularensis* has traditionally been considered more virulent than *F. tularensis* subsp. *holarctica* based on mean lethal dose studies in rabbits (Ellis et al., 2002). Infections caused by *F. tularensis* subsp *tularensis* are acquired by arthropod bites (ticks and deer-flies), handling of infected animal carcasses, and inhalation of infective aerosols. Outbreaks involving *F. tularensis* subsp. *tularensis* are rare. On Martha's Vineyard, cases caused by this subspecies have been reported between 2000 and 2006, with only two recognized outbreaks occurring in the last 22 years (1978 and 2000) (Feldman et al., 2001, 2003; Matyas et al., 2007).

Historically, *F. tularensis* subsp. *tularensis* strains have been considered equivalent with respect to virulence. Molecular methods, specifically multiple locus variable number tandem repeat analysis (MLVA), pulsed field gel electrophoresis (PFGE), and genome sequence comparisons, have separated this subspecies into 2 genetically distinct clades, A1 (also known as A.I and A-east) and A2 (also known as A.II and A-west) (Beckstrom-Sternberg et al., 2007; Farlow et al., 2005; Johansson et al., 2004; Larsson et al., 2007; Molins-Schneekloth et al., 2008; Nubel et al., 2006; Staples et al., 2006; Svensson et al., 2005). Recently, a difference in the severity of human illness caused by *F. tularensis* subsp. *tularensis* A1 and A2 has been reported (Staples et al., 2006). A1 isolates were recovered more frequently from the lung and blood as compared with A2

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isolates. In addition, case fatality rates were shown to differ significantly between infections caused by A1 and A2, at 14% and 0%, respectively. Despite this difference in clinical severity, the only methods currently available for differentiating *F. tularensis* subsp. *tularensis* clades A1 and A2 require a recovered isolate (PFGE and MLVA) or are lacking in sensitivity (conventional polymerase chain reaction [PCR] and MLVA) (Farlow et al., 2005; Molins-Schneekloth et al., 2008; Staples et al., 2006).

Here, we develop and evaluate TaqMan PCR assays for the identification of *F. tularensis* subsp. *tularensis* clades A1 and A2. Two regions of difference (RD), RD-5 and RD-3, previously identified by suppression subtractive hybridization, were used for assay development (Molins-Schneekloth et al., 2008). RD-5 lies within a region spanning both gene-encoding regions (hypothetical protein and a pseudogene for methyltransferase) and an intergenic region. It is present in A2 strains and absent from A1 strains. RD-3 lies within a hypothetical protein and is present in A1 strains and absent from A2 strains. The A1 and A2 TaqMan assays demonstrated good sensitivity and specificity. Using samples from sporadic tularemia cases and a 2007 deer-fly–associated tularemia outbreak in Utah in which both A1 and A2 caused disease (Petersen et al., 2008), we show the use of these assays for identification of A1 and A2. This study provides new tools that enhance our ability to investigate *F. tularensis* subsp. *tularensis* outbreaks as well as offers clinical laboratories and researchers a rapid method for identification of *F. tularensis* subsp. *tularensis* A1 and A2.

## 2. Materials and methods

### 2.1. Bacterial isolates and DNA preparation

Representative strains of *F. tularensis*, *Francisella novicida*, and *Francisella philomiragia* were grown from frozen stocks on cysteine heart agar with 9% chocolatized sheep blood (CHAB) at 35 °C for 48 h, followed by subculture onto CHAB for 24 h at 35 °C. Other bacterial strains were grown as previously described (Versage et al., 2003). Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions or as previously described (Kugeler et al., 2006; Versage et al., 2003). Double-stranded DNA concentration was determined using PicoGreen and a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Waltham, MA). Aliquots of purified DNA were stored at −20 °C. Previously frozen DNA was verified amplifiable using the 16S rRNA gene.

### 2.2. Outbreak specimens

A total of 20 outbreak-associated specimens from human cases ($n$ = 11) and desiccated rabbit carcasses ($n$ = 8) were tested. Twelve samples were collected from 11 human tularemia cases; 5 culture-confirmed cases (5 *F. tularensis* isolates) (Petersen et al., 2008), and 6 culture-negative, PCR-positive cases (n=7; 5 wound swabs, 2 lymph node aspirates). The lymph node aspirates were drawn 3 weeks apart from the same patient. Eight bone marrow samples were collected from the rabbit carcasses. DNA was isolated from all 20 specimens as described above (culture-positive samples) or as previously described (Petersen et al., 2008) and tested by real-time PCR using the *F. tularensis* multitarget assay and the *F. tularensis* subsp. *tularensis* assay (Kugeler et al., 2006; Versage et al., 2003). Culture-positive specimens were previously identified as either A1 or A2 by *Pme*I PFGE typing (Petersen et al., 2008). Five culture-negative, PCR-positive specimens were previously identified as either A1 or A2 by conventional PCR (Petersen et al., 2008).

### 2.3. TaqMan primers and probes

Primers and probes were designed using ABI Primer Express Software v2.0 (Applied Biosystems, Foster City, CA) and synthesized by the CDC Biotechnology Core Facility (Atlanta, GA). Before synthesis, BLAST (blastn) analyses were performed to confirm specificity. The primers for the A1 assay were RD5RT-F (5′-CACTATCTTTACTTTAGCTTTGCCACAA-3′) and RD5RT-R-2 (5′-TGATCCTTGGCATAAGAAAAAACA-3′) and probe RD5RT-P (5′-CTGGACAAAAA″T″ATTATCAAAGATTAACAAGCCTACGC-3′). For the A2-specific assay, primers were RD3RT-F (5′-GACAATCATTTAAGCAAAACGCTACT-3′) and RD3RT-R (3′- GCAGGTAATGTAGTTTTAGCAAATGC-5′) and probe RD3RT-P (5′-TTCTAGGATAA'T"CATCTGCGATACCGTTGCC-3′). Probes were synthesized with an internal Black Hole Quencher I (bhq1-dT) at the position "T" and a 6-carboxyfluorescein reporter at the 5′ end.

### 2.4. PCR conditions

PCR conditions were optimized using 1 ng of DNA from *F. tularensis* subsp. *tularensis* strains MA00-2987 (A1) and WY96-3418 (A2). A total volume of 20 μL was used for every PCR reaction. All reactions contained final concentrations of 1× LightCycler Fast Start DNA master hybridization probe mix (Roche Applied Sciences, Indianapolis, IN), 5 mmol/L $MgCl_2$, 0.75 μmol/L of each A1 primer or 0.5 μmol/L of each A2 primer, 0.5 U of uracil-DNA glycosylase (Roche), and 0.2 μmol/L probe. For outbreak specimens, the volume of sample tested ranged from 1 to 10 μL. Thermal cycling conditions were as follows: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 45 cycles of 95 °C for 10 s, and 60 °C for 30 s, followed by 1 cycle of 45 °C for 5 min. PCR amplification and detection were performed using the LightCycler 1.2 (Roche Applied Sciences). For each PCR run, a negative control (no DNA) and a positive control (1 ng of DNA from either strain MA00-2987 or WY96-3418) were included. $C_t$ values were determined by performing automatic quantification using the 2nd derivative maximum method with the $y$ axis set to F1/F3 (LightCycler software version 3.5).

### 2.5. Specificity and sensitivity determinants

Specificity testing was performed using 1 ng of DNA from *F. tularensis* subsp. *tularensis* clade A1 or A2, *F. tularensis* subsp. *holarctica*, *F. novicida*, *F. philomiragia*, or other bacterial species. All 46 *F. tularensis* subsp. *tularensis* strains were typed by *Pme*I PFGE as A1 or A2 using the previously described method (Staples et al., 2006). Genome equivalents (GEs) were calculated based on genome sizes of 1 892 819 and 1 898 476 bp for *F. tularensis* subsp. *tularensis* clade A1 strain SCHU S4 and *F. tularensis* subsp. *tularensis* clade A2 strain WY96-3418, respectively. One GE

Table 1
Evaluation of specificity using *F. tularensis* subsp. *tularensis* A1 and A2 isolates from sporadic and outbreak-associated tularemia cases

| *F. tularensis* subsp. *tularensis*[a] | CDC accession no. | Source | Year[b] | Geographic origin | A1 assay ($C_t$ value)[c] | A2 assay ($C_t$ value)[c] |
|---|---|---|---|---|---|---|
| Clade A1 | MA00-2987 | Human | 2000 | Massachusetts | 20.87 | – |
| | AR99-3448 | Human | 1999 | Arkansas | 20.57 | – |
| | MO02-1911 | Human | 2002 | Missouri | 21.15 | – |
| | NE03-1457 | Human | 2003 | Nebraska | 21.14 | – |
| | NY04-2565 | Human | 2004 | New York | 21.06 | – |
| | OK01-2528 | Human | 2001 | Oklahoma | 21.12 | – |
| | SCHU S4 | Human | 1940s | Ohio | 20.49 | – |
| | SD00-3147 | Human | 2000 | South Dakota | 20.62 | – |
| | VA00-1000 | Human | 2000 | Virginia | 20.52 | – |
| | ND00-0952 | Human | 2000 | North Dakota | 20.62 | – |
| | KS00-0948 | Cat | 2000 | Kansas | 20.92 | – |
| | NC99-3990 | Rabbit | 1999 | North Carolina | 21.00 | – |
| | AR00-0028 | Human | 2000 | Arkansas | 20.73 | – |
| | NC97-3057 | Rabbit | 1997 | North Carolina | 20.35 | – |
| | MO01-1907 | Human | 2001 | Missouri | 20.86 | – |
| | OK00-2731 | Human | 2000 | Oklahoma | 20.75 | – |
| | UT98-3134 | Human | 1998 | Utah | 21.27 | – |
| | NC01-5379 | Cat | 2001 | North Carolina | 20.93 | – |
| | AR98-2146 | Rabbit | 1998 | Arkansas | 21.17 | – |
| | OK00-4337 | Human | 2000 | Oklahoma | 20.72 | – |
| | AR01-1117 | Human | 2001 | Arkansas | 21.13 | – |
| | GA02-5501 | Human | 1982 | Louisiana | 21.22 | – |
| | IL01-3022 | Human | 2001 | Illinois | 20.88 | – |
| | NY04-2787 | Rabbit | 2004 | New York | 21.28 | – |
| | UT07-4262 | Human | 2007 | Utah | 21.21 | – |
| | UT07-4263 | Human | 2007 | Utah | 21.19 | – |
| | UT07-4265 | Human | 2007 | Utah | 20.44 | – |
| Clade A2 | WY96-3418 | Human | 1996 | Wyoming | – | 18.72 |
| | AZ01-4999 | Human | 2001 | Arizona | – | 19.50 |
| | CA02-0099 | Human | 2002 | California | – | 18.28 |
| | GA02-5453 | Human | 1993 | Wyoming | – | 18.94 |
| | IDO4-2687 | Human | 2004 | Oregon | – | 19.44 |
| | UT02-1927 | Human | 2002 | Utah | – | 19.28 |
| | WY01-3847 | Human | 2001 | Wyoming | – | 18.97 |
| | WY01-3911 | Human | 2001 | Wyoming | – | 19.80 |
| | WY03-1228 | Human | 2003 | Wyoming | – | 20.15 |
| | NM99-0295 | Rabbit | 1999 | New Mexico | – | 20.03 |
| | CO01-2364 | Cat | 2001 | Colorado | – | 19.02 |
| | CO01-3713 | Rabbit | 2001 | Colorado | – | 19.61 |
| | CO04-2154 | Rabbit | 2004 | Colorado | – | 19.27 |
| | OR02-0978 | Cat | 2002 | Oregon | – | 19.40 |
| | UT01-2936 | Human | 2001 | Utah | – | 19.50 |
| | NV87-3695 | Human | 1987 | Nevada | – | 19.59 |
| | NM99-1823 | Human | 1999 | New Mexico | – | 20.03 |
| | WY01-3650 | Human | 2001 | Wyoming | – | 19.72 |
| | OR00-3117 | Human | 2000 | Oregon | – | 19.58 |
| | CO81-0728 | Human | 1981 | Colorado | – | 19.63 |
| | AZ87-2825 | Human | 1987 | Arizona | – | 19.47 |
| | CO02-1841 | Cat | 2002 | Colorado | – | 19.65 |
| | UT07-4632 | Human | 2007 | Utah | – | 19.68 |
| | UT07-4633 | Human | 2007 | Utah | – | 19.95 |

[a] Clade information was known for all isolates by *Pme*I PFGE genotyping.
[b] Isolates (not outbreak associated or a reference strain) were obtained from sporadic tularemia cases occurring for a 26-year period.
[c] A negative $C_t$ value (−) represents no logarithmic amplification detected within 41 amplification cycles.

of either A1 or A2 was estimated at approximately 2 fg of DNA. Tenfold serial dilutions of DNA ranging from 1 to 100 000 GEs were prepared in nuclease-free water and tested in duplicate for each assay. Standard curves were generated using the LightCycler software version 3.5.

### 2.6. Inhibition testing

A1 (SCHU S4) or A2 (WY96-3418) DNA was diluted to 1000 GEs, 100 GEs, and 10 GEs and mixed with DNA from the opposing *F. tularensis* subsp. *tularensis* clade at ratios of 1:0, 1:1, 1:2, 1:5, and 1:10. All mixtures of A1 and A2 were tested in duplicate.

## 3. Results

### 3.1. Development and optimization

The A1- and A2-specific assays were targeted against RD-5 and RD-3, respectively. The A1-specific assay targeted RD-5, which is present in A2 and absent from A1. The A2-specific targeted RD-3, which is present in A1 and absent from A2. These 2 RDs were chosen for development of TaqMan PCR assays because they showed the most nucleotide variability within the regions flanking the RD. In both cases, the TaqMan probe was designed to hybridize to the junction because this region discriminates A1 from A2. The amplification size for the A1 and A2 PCR products is 101 and 111 bp, respectively.

Both assays were optimized for primer concentration, $MgCl_2$ concentration, and annealing temperature (data not shown). The optimal concentration of each primer was determined to be 0.75 μmol/L for the A1-specific assay and 0.5 μmol/L for the A2-specific assay. A final $MgCl_2$ concentration of 5 mmol/L was found to give the best results for both assays. An annealing temperature of 60 °C was optimal for performing both assays simultaneously (data not shown).

### 3.2. Specificity and sensitivity

Specificity of the 2 TaqMan assays was evaluated using DNA from 24 *F. tularensis* subsp. *tularensis* A1 and 22 *F. tularensis* subsp. *tularensis* A2 isolates obtained from sporadic tularemia cases occurring in different geographic locations for a 26-year period (Table 1). The A1-specific assay identified all 24 A1 strains with no cross-reactivity to A2 strains. Conversely, the A2-specific assay recognized all 22 A2 strains with no cross-reactivity to A1 strains. In addition, identification of the strains as A1 or A2 by real-time PCR was in complete agreement with *Pme*I PFGE genotyping, validating the ability of these assays to accurately differentiate A1 and A2 (Table 1). The $C_t$ value for 1 ng of DNA ranged from 20.35 to 21.28 and from 18.28 to 20.15 for the A1 and A2 assays, respectively (Table 1).

A panel of 20 *F. tularensis* subsp. *holarctica* strains, 7 *F. novicida* strains, 10 *F. philomiragia* strains (Table 2), and 13 other bacterial species (20 strains total) were also tested for cross-reactivity with the A1 and A2 assays. The other bacterial species included *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Moraxella* spp., *Haemophilus influenzae*, *Enterococcus cloacae*, *Escherichia coli*, *Acinetobacter* spp., *Proteus* spp., *Serratia* spp., *Streptococcus faecalis*, *Legionella* spp., *Yersinia pestis*, and *Yersinia enterocolitica*. No cross-reactivity was detected with any of the *Francisella* (*F. tularensis* subsp. *holarctica*, *F. novicida*, and *F. philomiragia*) (Table 2) or other bacterial species tested (data not shown).

Sensitivity testing indicated that both assays reproducibly detected 10 GEs of *F. tularensis* DNA. The average $C_t$ value for the A1 assay for 100 000, 10 000, 1000, 100, and 10 GEs was 23.92, 27.94, 30.97, 34.75, and 38.12, respectively. The average $C_t$ value for the A2 assay for 100 000, 10 000, 1000, 100, and 10 GEs was 21.52, 25.13, 28.84, 32.26, and 35.71, respectively. For both assays, the $C_t$ value for 1 GE was >41.00. Both TaqMan assays showed good linear log correlation with a regression coefficient of 1.00 and a slope of −3.587 for the A1-specific assay and −3.551 for the A2-specific assay (data not shown).

### 3.3. Competitive inhibition

The sensitivity of the A1-specific assay in the presence of A2 DNA and the A2-specific assay in the presence of A1 DNA was tested because degenerate primer binding sites for the respective assays are present in both A1 and A2. The A1-specific assay showed no inhibition in the presence of 5-fold excess A2 DNA; a sensitivity of 10 GEs (average $C_t$ value of 38.15) was maintained under these conditions. When A2 DNA was added at 10 times the concentration of A1 DNA, a slight inhibitory effect was noted; the A1-specific assay displayed a sensitivity of 100 GEs, with an average $C_t$ value of 34.59. The A2-specific assay showed no signs of inhibition in the presence of 10 times excess A1 DNA; a sensitivity of 10 GEs (average $C_t$ value of 35.46) was maintained under these conditions. Thus, the real-time assays provide identification of A1 and A2 in the presence of 10-fold excess DNA of the opposing clade, demonstrating the ability of these assays to detect potential A1/A2 coinfections.

### 3.4. Evaluation and application using outbreak specimens

To evaluate the ability of the TaqMan assays to identify *F. tularensis* subsp. *tularensis* A1 or A2 infections, we tested DNA extracted from samples collected during a deer-fly–associated tularemia outbreak in Utah in 2007. Results for the 5 culture-confirmed human cases obtained with the A1 and A2 TaqMan assays matched those obtained by *Pme*I PFGE (Table 3). Two isolates were identified as A2 (UT07-4632 and UT07-4633), and 3 isolates were identified as A1 (UT07-4262, UT07-4263, and UT07-4265). Similarly, when 5 bone marrow DNA samples, extracted from rabbit carcasses (UT07-5152, UT07-5156, UT07-5157, UT07-5159, and UT07-5161) collected at the

Table 2
Evaluation of specificity using other *Francisella* strains

| *Francisella* spp. | CDC accession no. | Source | Geographic origin | A1 assay ($C_t$ value)[a] | A2 assay ($C_t$ value)[a] |
|---|---|---|---|---|---|
| *F. tularensis* subsp. *holarctica* | LVS | Rat | Russia | − | − |
| | KY99-3387 | Human | Kentucky | − | − |
| | AZ00-1324 | Squirrel | Arizona | − | − |
| | IL00-3633 | Human | Illinois | − | − |
| | CN98-5979 | Human | Canada | − | − |
| | MO01-1673 | Human | Missouri | − | − |
| | OH01-3029 | Prairie dog | Ohio | − | − |
| | KY00-1708 | Human | Kentucky | − | − |
| | IN98-3055 | Rat | Indiana | − | − |
| | UT00-2098 | Human | Missouri | − | − |
| | SP98-6120 | Rabbit | Spain | − | − |
| | CO96-1243 | Vole | Colorado | − | − |
| | CA99-0837 | Human | California | − | − |
| | IN00-2758 | Human | Indiana | − | − |
| | NM00-2642 | Human | New Mexico | − | − |
| | SP98-2108 | Human | Spain | − | − |
| | CA99-3992 | Monkey | California | − | − |
| | AZ00-1325 | Rat | Arizona | − | − |
| | JAP5-3-11 | Human | Japan | − | − |
| | KO97-1026 | Human | Korea | − | − |
| *F. novicida* | GA99-3548 | Human | Louisiana | − | − |
| | GA99-3549 | Human | California | − | − |
| | GA99-3550 | Water | Utah | − | − |
| | UT01-4992 | Human | Utah | − | − |
| | AS02-0814 | Human | Australia | − | − |
| | FX1 | Human | Texas | − | − |
| | FX2 | Human | Texas | − | − |
| *F. philomiragia* | GA01-2796 | Human | California | − | − |
| | GA01-2799 | Human | Connecticut | − | − |
| | GA01-2800 | Human | Connecticut | − | − |
| | GA01-2801 | Human | New York | − | − |
| | GA01-2802 | Human | California | − | − |
| | GA01-2803 | Human | New Mexico | − | − |
| | GA01-2804 | Human | Virginia | − | − |
| | GA01-2806 | Human | Massachusetts | − | − |
| | GA01-2807 | Human | Unknown | − | − |
| | GA01-2811 | Water | Utah | − | − |

[a] A negative $C_t$ value (−) represents no logarithmic amplification detected within 41 amplification cycles.

outbreak site, were tested with the A1 and A2 real-time assays, the results matched those obtained using conventional PCR (Table 3). Thus, the A1 and A2 real-time PCR assays provide results in complete agreement with 2 independent methods, PFGE and conventional PCR, demonstrating the ability of these assays to accurately

Table 3
Comparison of results for *Pme*I PFGE typing and conventional PCR versus A1 and A2 TaqMan assays using outbreak samples

| Sample (CDC accession no.) | Source | Sample type | *Pme*I PFGE[a] | Conventional PCR | A1 TaqMan assay ($C_t$ value)[b] | A2 TaqMan assay ($C_t$ value)[b] | Clade identified by A1/A2 TaqMan assay |
|---|---|---|---|---|---|---|---|
| UT07-4632 | Human | Isolate | A2 | NT[c] | − | 19.68 | A2 |
| UT07-4633 | Human | Isolate | A2 | NT | − | 19.95 | A2 |
| UT07-4262 | Human | Isolate | A1 | NT | 21.21 | − | A1 |
| UT07-4263 | Human | Isolate | A1 | NT | 21.19 | − | A1 |
| UT07-4265 | Human | Isolate | A1 | NT | 20.44 | − | A1 |
| UT07-5152 | Rabbit | Bone marrow | NT | A1 | 23.43 | − | A1 |
| UT07-5156 | Rabbit | Bone marrow | NT | A1 | 26.83 | − | A1 |
| UT07-5157 | Rabbit | Bone marrow | NT | A1 | 18.40 | − | A1 |
| UT07-5159 | Rabbit | Bone marrow | NT | A1 | 19.58 | − | A1 |
| UT07-5161 | Rabbit | Bone marrow | NT | A2 | − | 21.45 | A2 |

[a] Data from previously published study (Petersen et al., 2008).
[b] A negative $C_t$ value (−) represents no logarithmic amplification detected within 41 amplification cycles.
[c] NT represents assays that were not tested.

Table 4
Identification of A1 and A2 infections in *F. tularensis* subsp. *tularensis* PCR-positive, culture-negative specimens

| Sample (CDC accession no.) | Source | Specimen type | *F. tularensis* TaqMan PCR | *F. tularensis* subsp. *tularensis* TaqMan PCR | A1 TaqMan Assay[a] (+ or −/$C_t$ value) | A2 TaqMan Assay[a] (+ or −/$C_t$ value) | Clade identified using A1/A2 TaqMan assays |
|---|---|---|---|---|---|---|---|
| UT07-6575 | Human | Wound | + | + | +/36.98 | −/− | A1 |
| UT07-6576 | Human | Wound | + | + | −/− | +/36.83 | A2 |
| UT07-6577 | Human | Wound | + | + | −/− | +/38.55 | A2 |
| UT07-6578 | Human | Wound | + | + | +/37.00 | −/− | A1 |
| UT07-6579 | Human | Wound | + | + | +/38.74 | −/− | A1 |
| UT07-6580 | Human | Lymph node | + | + | −/− | +/33.05 | A2 |
| UT07-6581 | Human | Lymph node | + | + | −/− | +/34.46 | A2 |
| UT07-5153 | Rabbit | Bone marrow | + | + | +/35.96 | −/− | A1 |
| UT07-5158 | Rabbit | Bone marrow | + | + | −/− | +/38.77 | A2 |
| UT07-5163 | Rabbit | Bone marrow | + | + | −/− | +/35.02 | A2 |

[a] A positive $C_t$ value was logarithmic amplification detected within 41 amplification cycles; a negative $C_t$ value was no logarithmic amplification detected within 41 amplification cycles.

identify A1 and A2 infections by a rapid non–labor-intensive method (Table 3).

To determine if the A1 and A2 real-time assays could be used for identification of A1 and A2 infections in culture-negative, *F. tularensis* subsp. *tularensis* PCR-positive samples, we tested DNA from 6 human cases and 3 rabbit carcasses (Table 4). Conventional PCR was not previously able to identify A1 or A2 infections in these specimens because of limits in sensitivity (Petersen et al., 2008). In contrast, the A1 and A2 real-time assays were able to identify infections as either A1 or A2 for all 6 human cases; 3 were positive for A1 (UT07-6575, UT07-6578, and UT07-6579) and 3 were positive for A2 (UT07-6576, UT07-6577, UT07-6580) (Table 4). In addition, 2 independent samples drawn 3 weeks apart from the same patient (UT07-6580 and UT07-6581) were both positive for A2. The A1 and A2 real-time assays also identified the infecting type A clade in rabbit carcasses; 1 was positive for A1 (UT07-5153) and 2 were positive for A2 (UT07-5158 and UT07-5163). Thus, the real-time assays were able to identify either A1 or A2 as the source of infection in primary samples, even in the absence of a culture.

To look for evidence of A1/A2 coinfection in the outbreak samples, 10 μL of purified DNA was tested from the 7 human and 8 rabbit culture-negative, PCR-positive specimens using A1 and A2 real-time assays. No evidence of coinfection was found; all specimens tested positive ($C_t$ values ranged from 19.23 to 37.66) for either *F. tularensis* subsp. *tularensis* clade A1 or *F. tularensis* subsp. *tularensis* clade A2 under these conditions.

## 4. Discussion

In this study, we developed 2 real-time TaqMan PCR assays for rapid identification of *F. tularensis* subsp. *tularensis* A1 and A2 infections by targeting genomic regions differing between A1 and A2 strains. The 2 real-time assays were shown to be specific for either A1 or A2 and showed no cross-reactivity to other *Francisella* or other bacterial species tested. The sensitivity of both assays was determined to be 10 GEs, falling in line with the current real-time TaqMan PCR assays used for identification of *F. tularensis* and *F. tularensis* subsps. *tularensis* and *holarctica* (Kugeler et al., 2006; Versage et al., 2003).

Identification of A1 and A2 infections by real-time PCR was shown to directly correlate with results obtained by both PFGE genotyping and conventional PCR (Petersen et al., 2008) when specimens from sporadic cases and an outbreak of tularemia were tested. PFGE and conventional PCR were used successfully to describe the 2007 Utah outbreak as the first documented tularemia outbreak involving both A1 and A2 strains; however, limitations in detection were apparent for both assays. PFGE was only useful for culture-positive samples, and conventional PCR had a limit in sensitivity as demonstrated by identification of the *F. tularensis* subsp. *tularensis* infective clade for only 5 of the 15 PCR-positive, culture-negative samples collected. Here, using the real-time assays, the infecting clade was identified as either A1 or A2 within all 20 (culture-positive and PCR-positive, culture-negative) human and rabbit specimens associated with the outbreak. Thus, the real-time TaqMan PCR assays have the advantage, in comparison with PFGE, MLVA, and conventional PCR, of being not only rapid and gel free but also more sensitive, allowing for identification of A1 and A2 in both culture-positive and culture-negative samples. Both real-time PCR assays show promise for identification of A1 and A2 in primary specimens; however, because primary specimens from only a single outbreak were evaluated in this study, limitations to the data may exist.

Competitive inhibition testing of the A1 and A2 real-time assays demonstrated the ability of these assays to detect both A1 and A2 in a single sample. Currently, it is unknown whether coinfections with A1 and A2 may occur in human, animal, or vectors. To date, the only *Francisella* coinfection studies documented have focused on *F. novicida*, with studies finding that *F. novicida* can suppress the growth of *F. tularensis* subsp. *holarctica* strain LVS by as much as 100-fold using a rat model (Cowley et al., 1997). The co-occurrence of A1 and A2 infections in a localized deer-fly–

associated outbreak of tularemia (Petersen et al., 2008) led us to question whether coinfection with A1 and A2 may have occurred during this outbreak. When the outbreak samples were tested with A1 and A2 real-time PCR assays, no evidence of coinfection was found. The implications of this finding are unclear but could suggest that coinfection with A1 and A2 does not occur or that the deer-flies propagating the outbreak were only singly infected with either A1 or A2. Because no *F. tularensis*-positive deer-flies were recovered (Petersen et al., 2008), it is not possible to distinguish between these 2 possibilities.

Given that A1 infections are associated with a higher case fatality rate than A2 infections (14% fatality rate for A1 infections as compared with 0% fatality rate for A2 infections) (Staples et al., 2006), the real-time assays described here provide a new rapid and sensitive diagnostic tool for clinical identification of A1 and A2 infections. In addition, the 2 assays developed offer a rapid, sensitive, and accessible tool for the research community to use for classification of *F. tularensis* subsp. *tularensis* strains.

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