TaqMan Real-Time PCR Assays for Single-Nucleotide Polymorphisms Which Identify Francisella tularensis and Its Subspecies and Subpopulations

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Abstract

Francisella tularensis, the etiologic agent of tularemia and a Class A Select Agent, is divided into three subspecies and multiple subpopulations that differ in virulence and geographic distribution. Given these differences, there is a need to rapidly and accurately determine if a strain is F. tularensis and, if it is, assign it to subspecies and subpopulation. We designed TaqMan real-time PCR genotyping assays using eleven single nucleotide polymorphisms (SNPs) that were potentially specific to closely related groups within the genus Francisella, including numerous subpopulations within F. tularensis species. We performed extensive validation studies to test the specificity of these SNPs to particular populations by screening the assays across a set of 565 genetically and geographically diverse F. tularensis isolates and an additional 21 genetic near-neighbor (outgroup) isolates. All eleven assays correctly determined the genetic groups of all 565 F. tularensis isolates. One assay differentiates F. tularensis, F. novicida, and F. hispaniensis from the more genetically distant F. philomiragia and Francisella-like endosymbionts. Another assay differentiates F. tularensis isolates from near neighbors. The remaining nine assays classify F. tularensis-confirmed isolates into F. tularensis subspecies and subpopulations. The genotyping accuracy of these nine assays diminished when tested on outgroup isolates (i.e. non F. tularensis), therefore a hierarchical approach of assay usage is recommended wherein the F. tularensis-specific assay is used before the nine downstream assays. Among F. tularensis isolates, all eleven assays were highly sensitive, consistently amplifying very low concentrations of DNA. Altogether, these eleven TaqMan real-time PCR assays represent a highly accurate, rapid, and sensitive means of identifying the species, subspecies, and subpopulation of any F. tularensis isolate if used in a step-wise hierarchical scheme. These assays would be very useful in clinical, epidemiological, and/or forensic investigations involving F. tularensis.

Introduction

Francisella tularensis is a Gram-negative bacterium that causes the zoonotic disease tularemia in mammals [1] and is a rapidly emerging disease in humans in several regions of the world [2,3]. It is extremely infectious, with as few as ten organisms capable of causing disease [4]. Respiratory acquired infections are particularly dangerous, having a high mortality in the absence of antibiotic treatment [4]. Due to its virulence and potential for bioweapons development, F. tularensis is currently classified as a Class A Select Agent by the Centers for Disease Control and Prevention (CDC) [5]. F. tularensis is subdivided into three subspecies: tularensis, holarctica, and mediasiatica. F. novicida rarely causes disease and, thus, is rarely isolated [1,6], but it does share high levels of genomic and biochemical similarity to F. tularensis. For this reason, it is often considered a fourth subspecies of F. tularensis [7]. This classification remains controversial [8], however, and this paper will treat F. novicida as a separate species. The species membership within the Francisella genus is rapidly expanding and includes numerous fish pathogens [9,10], opportunistic human pathogens such as F. hispaniensis [7] and F. philomiragia [11–13], and non-pathogenic Francisella-like bacteria found in ticks [14] and soil samples [15].

The three subspecies of F. tularensis have distinct virulence, geographic distributions, and host/vector associations. F. tularensis subsp. tularensis (type A) causes a life-threatening form of tularemia and is found only in North America [1,16]. F. tularensis subsp. holarctica is further divided into type A.I and A.II subpopulations, which are correlated with geographically separate host and vector distributions [17]. These subpopulations also
appear to differ in virulence, with A.I associated with more severe disease than A.II [16,10]. Subpopulation A.I is further differentiated into numerous smaller subpopulations associated with differing disease severity [19,20] and geographical distribution [21]. F. tularensis subsp. holarctica (type B) is further separated into Japanese (sometimes referred to as biovar japonica) and non-Japanese groups due to biochemical and genetic differences distinguishing Japanese type B isolates from the other type B isolates found throughout the northern hemisphere [1]. F. tularensis subsp. mediasiatica is geographically restricted, having been isolated only from central Asia [1].

Numerous molecular typing methods are available but each has specific limitations due to either high cost (labor and expertise) and or a narrow range of discrimination within F. tularensis subspecies and principle populations. Molecular typing methods such as pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) [22], RD1 [23], and multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) [24] have been shown to be highly effective at F. tularensis species and subspecies differentiation [1]. However, these molecular methods are expensive, labor intensive, and often require extensive experience to perform [1], making them ill-suited for clinical diagnostics. Simple presence/absence PCR-based assays are effective at confirming F. tularensis from clinical [25–28] or environmental samples [14,29–32] and can be highly sensitive when a TaqMan fluorogenic real-time PCR platform is used [29,32–36], but they cannot further differentiate among the subspecies or subpopulations nor can they differentiate a negative result from a PCR failure. Other PCR assays do provide genetic differentiation at varying resolution [37,38], some between F. tularensis subspecies tularensis and holarctica [33,34,39,40] and another includes mediasiatica [41]. Other assays are capable of differentiating subpopulations of F. tularensis subsp. tularensis subpopulations A.I and A.II [16,42]. Several studies have presented SYBR green based real-time PCR assays that differentiate numerous genetic groups within F. tularensis [19,43,44]. However, due to the mild inhibitory properties of SYBR green [45], these assays are not typically highly sensitive to samples containing low level DNA amounts or trace PCR inhibitors, which are typical of environmental and clinical samples [46]. Although SYBR assays are ill-suited for environmental and clinical samples, Dual Probe TaqMan real-time PCR assays have been successfully used on such samples due to their sensitivity to low level DNA amounts [16,47] and tolerance for the trace amounts of PCR inhibitors. However, there is no simple assay or set of assays available, in the form of highly sensitive TaqMan real-time PCR assays, that are capable of distinguishing the species F. tularensis and then further differentiating F. tularensis isolates into all of the known subspecies and principle subpopulations. Such differentiation is highly desirable given the differences in virulence and geographic distributions among these major genetic groups [1,48]. Obtaining this information would be a necessary first step in any epidemiological or forensic investigation involving F. tularensis, which would likely involve environmental or clinical samples.

Single nucleotide polymorphisms (SNPs) can be highly effective as molecular markers for identifying genetic groups. In clonally reproducing bacterial populations with a low level of horizontal gene transfer, SNPs are highly stable and exhibit little to no homoplasy. Because of this, single SNPs known as canonical SNPs (canSNPs) can be effectively used to define different genetic groups, whether species, major clades (e.g., subspecies), or even individual strains [49]. These canSNPs are also amenable to a variety of high-throughput genotyping methods, including real-time PCR. This strategy has been successfully used for Brucella spp. [50], Yesinia pestis [51], and Bacillus anthracis [52] and is also appropriate for F. tularensis, given its clonal nature [53].

We present eleven rapid and highly-sensitive TaqMan real-time PCR canSNP genotyping assays that are diagnostic for major branches in the Francisella species and F. tularensis phylogenies, including: the separation of F. tularensis, F. novicida and F. hispaniensis from the more genetically distant F. philomiragia and Francisella-like tick endosymbionts; the branches leading to the three official F. tularensis subspecies; and numerous principal populations (Figure 1). We designed TaqMan real-time PCR assays for the canSNPs defining these lineages and tested the specificity of the canSNP-signatures by running the assays across a large genetically and geographically diverse DNA panel.

Methods

We identified SNPs that were potentially specific to subpopulations within F. tularensis and the closely related species F. philomiragia and the Francisella-like endosymbionts based on a survey of numerous publications [15,19,43,44,54] and an in-house sequencing effort on ParC gene from a single endosymbiont-infected tick submitted to GenBank (GQ303199). Eleven of these SNPs were used to develop TaqMan real-time PCR canSNP assays targeting the following Francisella genetic groups: (Figure 1) F. tularensis, F. novicida and F. hispaniensis (F. TNH), F. tularensis-specific (F.I-specific), F. tularensis subspecies tularensis and mediasiatica (F.I & M), F. tularensis subsp. mediasiatica (F.I. M), F. tularensis subsp. tularensis (F.I. A), F. tularensis subsp. tularensis subpopulation A.I (F.I. A.I), F. tularensis subsp. tularensis subpopulation A.Ia (F.I. A.Ia), F. tularensis subsp. tularensis subpopulation A.II (F.I. A.II), F. tularensis subsp. holarctica (F.I. B), Japanese F. tularensis subsp. holarctica (F.I. JB) and non-Japanese F. tularensis subsp. holarctica (F.I. nonJB).

Primer design (Integrated DNA Technologies, San Diego, CA) and allele-specific TaqMan -minor groove binding (MGB) probes (Applied Biosystems, Foster City, CA) targeting each canSNP were designed using Primer Express software (Applied Biosystems) (Table 1). Each 5 μl TaqMan real-time PCR canSNP assay reaction contained 1× TaqMan Universal PCR master mix (Applied Biosystems), primers and probes (for concentrations see Table 1), and 1 μl diluted DNA template. Additionally, the F.I. A and F.I. A.I canSNP assays were supplemented with 0.025 U/μl of Platinum Taq DNA polymerase (Invitrogen) for improved efficiency. The TaqMan real-time PCR canSNP assays were run on an Applied Biosystems 7900HT Fast Real-Time PCR System with SDS software version 2.3 under the following conditions for all assays except F.I. JB: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec and 60°C for 1 min. The F.I. JB canSNP assay followed identical conditions except for an annealing temperature of 61.5°C.

We confirmed the specificity of all eleven SNP signatures by screening the canSNP assays across a panel of 506 genetically and geographically diverse Francisella DNAs, including: 32 F. tularensis subsp. tularensis subpopulation A.I, 33 F. tularensis subsp. tularensis subpopulation A.II, 446 F. tularensis subsp. holarctica (including 7 from Japan), 4 F. tularensis subsp. mediasiatica, and 21 genetic near-neighbor strains (8 F. novicida, 1 F. hispaniensis, 2 tick endosymbionts, and 10 F. philomiragia) (Table S1). The F.I.-specific and F.II.TNH-specific assays were also screened across a additional 7 environmental tick samples positive for endosymbionts (data not shown). The F.II.TNH-specific assay separates F. tularensis, F. novicida and F. hispaniensis from the more genetically distant F. philomiragia and Francisella-like tick endosymbionts. Two negative controls per canSNP assay.
were included with each experiment. The DNAs consisted of whole genome amplification (WGA) products (Qiagen, Valencia, CA) and genomic DNA from various types of DNA preparations (heat soaks, chloroform, and Qiagen DNA extractions). All of the WGA products were prepared from DNAs extracted from pure culture except for the two tick endosymbiont WGA products, which were amplified from starting DNA material that had been extracted from an *Francisella*-like endosymbiont infected tick (i.e., contained both tick endosymbiont and tick DNA). DNA templates were diluted prior to amplification in the canSNP assays at these ratios: 1:49 for WGA products, 1/10 for heat soak, and 1/100 for Qiagen or chloroform extractions. We tested the sensitivity of each canSNP assay by running it across four replicates of a serial ten-fold dilution series (10$^{-2}$–10$^{-10}$) of WGA products or genomic DNA from two DNA templates, one that possessed the targeted genetic group-specific (i.e. derived) allele and one that possessed the alternate (i.e. ancestral) allele.

**Results and Discussion**

All eleven canSNP assays displayed robust signal amplification from the perfect match probe with the mismatched probe signal either failing or showing weaker fluorescence. Six canSNP assays, *F.t.*-specific, *F.t.* M, *F.t.* A.I, *F.t.* A.II, *F.t.* JB, and *F.t.* nonJB showed some cross-hybridization with the mismatched probe but the delta Ct values of the match and mismatched probes were sufficient (≥ 4.7 Ct when analyzed at a threshold of 0.2) to provide clear genotyping (data not shown). Assay performance was equally robust regardless of the template DNA preparation method.

Our *F. TNH* canSNP assay detects *F. tularensis* and several members of the *Francisella* species that are common genetic near neighbors. The *F. TNH* canSNP assay differentiated *F. tularensis* and their closer genetic-near-neighbors, *F. novicida* and *F. hispaniensis*, from the more distant genetic near-neighbors the *Francisella*-like tick endosymbionts and *F. philomiragia* (Fig. 1). Specifically, the 565 *F. tularensis*, 8 *F. novicida*, and 1 *F. hispaniensis* DNAs displayed the derived (*F. TNH*-specific) allele and the genetic near-neighbors (9 tick endosymbionts and 9 of 10 *F. philomiragia*) DNAs displayed the ancestral (alternate) allele (Table S2). A single *F. philomiragia* DNA failed to amplify, likely due to additional SNPs within the primer/probe sites, although we were unable to confirm this due to a lack of available sequence. We did, however, confirm the ancestral SNP state for this DNA by using a different genotyping assay targeting the same SNP (data not shown). Differentiation among near neighbors appears to be based on the phylogenetic distance among strains but this hypothesis cannot be confirmed due to a lack of whole-genome sequence data for *F. hispaniensis* and the tick endosymbionts.

The *F.t.*-specific canSNP assay correctly differentiated *F. tularensis* species from their genetic near-neighbors *F. novicida* and *Francisella*-like tick endosymbionts (Figure 1). Specifically, the 565 *F. tularensis* DNAs displayed the derived (*F.t.*-specific) allele and the genetic near-neighbors (8 *F. novicida* and 5 tick endosymbionts) DNAs displayed the ancestral (alternate) allele (Table S2). The assay failed to amplify on some strains of *Francisella*-like tick endosymbionts (4 out of 9) and the other near-neighbor species, *F. hispaniensis* and *F. philomiragia* DNA samples. Amplification failure on these DNAs is likely due to additional SNPs within the primer/probe sites, although we were
| Specificity | Branch | Genome position | Primer Sequences (S' à³') | Probe Sequences (S' à³') | SNP identity | Annealing Tm °C | SNP source |
|-------------|--------|-----------------|---------------------------|---------------------------|--------------|----------------|-----------|
| F.TNH       | F.1    | 397,255         | F TTACWMMRATTACCT CATCARGTTCTCAATGG | D VIC-AATGGAGCAAAT:GCTAA | 0.20         | C 60          | this study |
|             |        |                 | R ATATTTTWWATCAGWGTTRATTYYTTGTGCC | A 6FAM-AATGGAGCAAAT:GCTAA | 0.20         | T             |           |
| F.t specific| T.1    | 1165688         | F CTAAGCCTAAGC CTTTCTCCTACCT    | D 6FAM-CTTTTGAAAC:CTTGACAT | 0.20         | G 60          | Svensson et al. 2009 |
| F.t         | M.1    | 75,124          | F GGACGGGGCATGCTCT    | D VIC-CAGGTTAA:TTAGCG | 0.20         | T 60          | this study |
| F.t A&M     | A/M.1  | 1,491,914       | R GCAACCACCTCC GTAGAAATATACA | A 6FAM-CAGGTTAA:TTAGCG | 0.20         | C             |           |
| **F.t. A    | A.1    | 397,639         | R GGCACTAGTGGTAAGTTGCAAAGATCG | A VIC-CAGGTTAA:TTAGCG | 0.20         | G             |           |
|             |        |                 | R CATAGCTTCTATGCTATGCT | *A VIC-CAGGTTAA:TTAGCG | 0.20         | T 60          | this study |
| F.t A.I     | A.I.1  | 75,109          | R ACCACTCCTCCAGTGAAGTGAGAATCTG | D VIC-TTACACATCG:CTGTATAG | 0.20         | A 60          | this study |
| F.t A1a     | A.I.12 | 142781          | R ACCTCTCTAGTTGAAGTAGTAAATCTGTTAGCTGATGCTCATGCAACGTG | A VIC-TTACACATCG:CTGTATAG | 0.20         | G             |           |
|             |        |                 | R ACCCTCATCTGAATATAGTAGTAAG | A 6FAM-TTACACATCG:CTGTATAG | 0.20         | G             |           |
| **F.t. A.II | A.I.12| 84,150          | R GTTGAAGAATGTGGGCTGCGGCGATGCTTACCGT | D VIC-TTACACATCG:CTGTATAG | 0.20         | T 60          | this study |
|             |        |                 | R CACATCTCTCGAAAGTGTTGGAAGTGATAGTAAATCTGTTAGCTGATGCTCATGCAACGTG | A 6FAM-TTACACATCG:CTGTATAG | 0.20         | C 60          | Pandya et al. 2009 |
| F.t B       | B.1    | 83,745          | R AGGAGAGAGACTCTATGCTGCTGATGGGCTGCTG | D VIC-AAGCTATACATGCTTACCTG | 0.20         | A 60          | this study |
| F.t JB      | B.16   | 608,245         | R CATATATATACGCTGCTGCTGCTGCTG | A 6FAM-AAGCTATACATGCTTACCTG | 0.20         | G             |           |
|             |        |                 | R ATATTTTWWATCAGWGTTRATTYYTTGTGCC | A 6FAM-AATGGAGCAAAT:GCTAA | 0.20         | T 61.5        | Svensson et al. 2009 |
Francisella detect the non-select agent forms of F. of its nearest genetic relatives. The ability of understanding of F. tularensis (data not shown). This net C T difference between mixed vs (possessing the ancestral allele state) (Table S2), thus making these possessing the derived allele state) or the alternate group (Table S1) to their known genetic group (subspecies or subpopulation canSNP assays correctly assigned F. tularensis DNA into a particular genetic group (represented as nonJB B.2 5,162 F AATTGAAGCACGCCAAAAATAG 0.9 *D VIC-CTGTAGTAA TACAAGGCT 0.20

F. tularensis DNA from our subspecies and subpopulation canSNP assays allowed for a statistically significant difference in geographic distribution and associated virulence [1,18–20,48]. For these reasons, F. tularensis-specific assays that can further differentiate a given F. tularensis sample into its subspecies and subpopulation classifications are especially useful for the study of this organism. Our nine F. t.-specific and F. tularensis presented as a mixture with F. t.-specific and F. TNH canSNP assays, with amplification of both the specific and alternate alleles (data not shown) with significantly diminished net C T difference when compared to homogenous DNA samples (data not shown). This net C T difference between mixed vs homogenous DNA samples allowed for a statistically significant means of identifying F. tularensis even in a background containing closely related tick endosymbionts. This ability makes the F. t.-specific and F. TNH canSNP assays, when used in combination, an accurate and simpler means of differentiating F. tularensis from tick endosymbionts.

F. tularensis isolates are comprised of numerous subpopulations [16,43,44] that have differences in geographic distribution and associated virulence [1,18–20,48]. For these reasons, F. tularensis-specific assays that can further differentiate a given F. tularensis sample into its subspecies and subpopulation classifications are especially useful for the study of this organism. Our nine F. t.-specific and subpopulation canSNP assays correctly assigned F. tularensis DNA from our F. tularensis diversity panel (n = 565; Table S1) to their known genetic group (subspecies or subpopulation) (Fig. 1 & Table S2). Each canSNP assay genotyped a F. tularensis DNA into a particular genetic group (represented as possessing the derived allele state) or the alternate group (possessing the ancestral allele state) (Table S2), thus making these
nine canSNP assays ideally suited to further subgroup *F. tularensis* DNA samples.

In contrast to their excellent performance on *F. tularensis* DNA samples, our nine *F. tularensis* subpopulation canSNP assays are not ideally suited to be used on genetic near neighbors of *F. tularensis* (*F. hispaniensis*, tick endosymbiont and *F. philomiragia*). These nine assays were designed accounting for the sequence variation found among *F. tularensis* genomes and not near neighbors. The genetic distance between *F. tularensis* and near neighbors [15] increases the chances of additional SNPs in the primer or probe sequence regions targeted by the assays. Therefore, it was not surprising to find confounding results such as amplification failures, loss of probe specificity, and/or homoplasies when these assays were screened on near-neighbor samples. Particular issues appeared to be specific to assays and the tested near-neighbor strain DNA sample (Table S2). All the assays, except *F.t.* A.II, failed to amplify the endosymbiont-infected tick samples (Table S2), *F.t.* A & M, *F.t.* B, and *F.t.* JB failed to amplify the *F. hispaniensis* sample. *F.t.* A & M consistently failed to amplify *F. philomiragia* samples although all other assays displayed sporadic amplification for this distant genetic group. Among the sporadically amplifying assays, PCR amplification of *F. philomiragia* was significantly compromised as indicated by delays in PCR amplification (data not shown). *F.t.* AI, *F.t.* B, and *F.t.* nonJB display a loss of probe specificity, which appears as the reporting of both alleles or a conflict of allele calls among replicates of amplified *F. philomiragia* samples. Four assays resulted in homoplasmic assignment of near neighbor strains by genotyping them as the derived allele state (genetic group-specific). *F.t.* A.II, *F.t.* B, and *F.t.* nonJB homoplastically genotyped the *F. philomiragia* samples and *F.t.* M homoplastically genotyped the *F. hispaniensis* sample (Table S2). In addition, when we in silico genotyped the *F. philomiragia* whole genome sequenced strain 25017 (GeneBank accession NC_010336), we also observed homoplasmic genotyping results for the *F.t.* A&M, *F.t.* M, *F.t.* A.II and *F.t.* B canSNP assays, although *F.t.* M amplified as ancestral allele state in vitro albeit in a sporadic manner. This conflict between in silico data and in vitro result could be due to sequencing errors under the probe site or homoplasmic genotyping by *F.t.* M due to neighboring base mismatches near the SNP site. The sporadic amplification of *F.t.* M assay on *F. philomiragia* samples suggest the later. Based on our extensive validation study, our nine subpopulation canSNP assays will provide accurate data on only *F. tularensis* samples. Therefore, we recommend to use the nine *F.t.* subpopulation canSNP assays only once the DNA sample is confirmed as *F. tularensis*.

The above results suggest that the eleven canSNP assays presented here could be used in a step-wise fashion to identify any unknown sample potentially containing *F. tularensis* or its genetic near-neighbors. A sample that possesses the derived allele (*F.t.*-specific) could subsequently be screened across our nine *F. tularensis* subspecies and subpopulation canSNP assays. Our validation data suggest that this would result in the accurate classification of the confirmed *F. tularensis* sample into subspecies and subpopulation. If all eleven assays are used concurrently on an unknown sample for efficiency purpose, then the validity of nine *F. tularensis* subpopulation assays will be dependent on the *F. tularensis* status of the examined sample. It is critical that only samples that are derived for *F.t.*-specific assay be examined on the nine *F. tularensis* subspecies and subpopulation canSNP assays because these assays were designed accounting for *F. tularensis* genomes and not near neighbors. Also, our validation study showed sporadic amplification, loss of probe specificity and homoplasmic results when these assays were screened on genetic near-neighbors. Samples that result as possessing the ancestral allele (alternate) or fail altogether on *F.t.*-specific canSNP assay can be screened using our *TNH* canSNP assay to determine the presence of other Francisella species and possibly differentiate among the *F. TNH*-specific and alternate near-neighbor Francisella species. The sporadic amplification, loss of probe specificity, and homoplasmic results described among the nine *F. tularensis* subspecies and subpopulation canSNP assays also illustrate the importance of validating potential canSNPs across a large panel of isolates that includes genetic near-neighbors, particularly genetic near-neighbors that might be found in close proximity to the target.

Our eleven canSNP assays also showed considerable sensitivity in detecting and genotyping low concentrations of WGA DNA. Results from ten-fold serial dilution experiments indicated that these canSNP assays are able to consistently detect and genotype *F. tularensis* WGA DNA at dilutions as low as 10\(^{-3}\) to 10\(^{-7}\), depending on the assay. Sporadic amplification was observed at dilutions 10\(^{-4}\) to 10\(^{-9}\), depending upon the canSNP assay (data not shown). All eleven assays completely failed on negative water controls, furthering our confidence in the specificity and sensitivity of our assays to detect low concentrations of DNA targets. WGA products possess unequal copies of different loci across the genome due to amplification bias inherent to the WGA process [58]. Therefore, the total DNA concentration or copy number of a locus in a WGA sample cannot be accurately calculated. However, in our serial dilution experiments, the C\(T_2\) values ranged from 29–48 in the dilutions where low level but consistent amplification was observed, depending upon the canSNP assay. These C\(T_2\) values are comparable to the C\(T_2\) values for other assays run against templates containing 100 fg of DNA [50,51]. This comparison suggests that our *F. tularensis* canSNP assays have the same level of sensitivity as other TaqMan MGB real-time PCR dual probe assays [50,51] and likely have consistent detection of ∼100 fg and sporadic detection of ∼10 fg of target genomic DNA. The ability to detect and genotype low levels of target DNA makes these canSNP assays highly valuable diagnostic tools for use in clinical, epidemiological, and/or forensic investigations where samples often suffer from low DNA concentrations and/or limited quantities.

The eleven TaqMan real-time PCR canSNP assays presented here provide a simple means of obtaining highly accurate and sensitive typing scheme that classifies unknown isolates to species, subspecies, and subpopulation level when used or examined in a progressive, step-wise fashion. The real-time platform is amenable for high throughput screening at a rapid pace, permitting classification within 3 hours. Our eleven assays, as a collection, provide a comprehensive genotyping scheme that overcomes specific limitations that burden other published individual typing schemes. As mentioned previously, the virulence and geographic distribution differences among *F. tularensis* genetic groups [1,19,20] would make such identification a logical first step in any investigation. In clinical investigations, species, subspecies, and subpopulation identification would be useful for diagnosis and predicting disease outcome as well as for assessing risk associated with handling clinical isolates [59]. These genetic tools will also assist epidemiological investigations, particularly within the United States, where *F. tularensis* subsp. *tularensis* subpopulations A.I, A.I.12 and A.II and *F. tularensis* subsp. *holarctica* all occur [1,17–19,21]. The precise ecology of these different *F. tularensis* genetic groups is still relatively unknown [1] and epidemiological investigations focusing on transmission patterns would benefit from a simple means of differentiating subspecies and subpopu-
lutions when analyzing potential source isolates and comparing them to clinical isolates. The Class A Select Agent status of *F. tularensis* also means that any suspected tularemia case may be subject to bioterrorism assessment and a forensic investigation in which molecular subtyping will likely play a significant role [60]. Identifying the species and subpopulation of any forensic isolate will be a necessary first step in any such forensic investigation. Given that we confirmed that these canSNP assays accurately genotyped complex clinical samples (data not shown), consistent with similar successes in other studies [50,61], we do not foresee problems in adapting these canSNP assays to a clinical or forensic setting. In support of this, F. t.-specific and F. T'N assays showed success when directly screened on tick environmental samples. Here, endosymbiont DNA was detected despite high concentration of background Tick DNA (data not shown). In summary, these canSNP assays should have broad applicability for clinical, epidemiological, and forensic applications involving *F. tularensis*.

**Supporting Information**

Table S1 Strain Table. (XLS)

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**Author Contributions**

Conceived and designed the experiments: DNB AJV DMW PSK. Performed the experiments: DNB JB AC EK AN ED. Analyzed the data: DNB. Contributed reagents/materials/analysis tools: DMW PSK. Contributed to the writing of the manuscript: DNB AJV DMW PSK.

**Table S2 canSNP Table.** (XLSX)

**Acknowledgments**

We thank numerous contributors of DNA to our *Francisella* collection, including the Arizona Department of Health Services, National Center for Disease Control and Public Health from the Country of Georgia, Bundeswehr Institute of Microbiology, Istituto Zooprofittico Sperimentale della Lombardia e dell’Emilia Romagna “Bruno Ubertini,” Department of Microbiology and Infectious Diseases Veterinary Science Szent István University, California Department of Health Services, Centers for Disease Control and Prevention, Oklahoma State University, Ana Montiel from the Universidad Nacional Autónoma de México, and the Swedish Defence Research Agency. We thank James Schupp for providing invaluable technical support.

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