A Real-Time PCR Array for Hierarchical Identification of *Francisella* Isolates

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**Abstract**

A robust, rapid and flexible real-time PCR assay for hierarchical genetic typing of clinical and environmental isolates of *Francisella* is presented. Typing markers were found by multiple genome and gene comparisons, from which 23 canonical single nucleotide polymorphisms (canSNPs) and 11 canonical insertion-deletion mutations (canINDELs) were selected to provide phylogenetic guidelines for classification from genus to isolate level. The specificity of the developed assay, which uses 68 wells of a 96-well real-time PCR format with a detection limit of 100 pg DNA, was assessed using 62 *Francisella* isolates of diverse genetic and geographical origins. It was then successfully used for typing 14 *F. tularensis* subsp. *holarctica* isolates obtained from tularemia patients in Sweden in 2008 and five more genetically diverse *Francisella* isolates of global origin. When applied to human ulcer specimens for direct pathogen detection the results were incomplete due to scarcity of DNA, but sufficient markers were identified to detect fine-resolution differences among *F. tularensis* subsp. *holarctica* isolates causing infection in the patients. In contrast to other real-time PCR assays for *Francisella*, which are typically designed for specific detection of a species, subspecies, or strain, this type of assay can be easily tailored to provide appropriate phylogenetic and/or geographical resolution to meet the objectives of the analysis.

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**Introduction**

The genus *Francisella* consists of three species: *F. philomiragia*, *F. novicida*, and the etiological agent of the zoonosis tularemia, *F. tularensis*. In addition, there are several soil bacteria, tick endosymbionts and fish parasites that are genetically closely related to *Francisella*, but are not (yet at least) assigned to the genus (Figure 1). Three subspecies of *F. tularensis* are recognized, of which *F. tularensis* subsp. *holarctica* cause severe, sometimes fatal, disease in humans. The third subspecies, *mediasiatica*, is rare and its virulence is described as moderate. *F. tularensis* subsp. *holarctica* has been isolated throughout the northern hemisphere, while *F. tularensis* subsp. *tularensis* and *mediasiatica* are geographically restricted to North America and Central Asia, respectively. The population structure of the two clinically relevant subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B), is highly clonal, a property that facilitates the design of genetic typing systems and deduction of evolutionary relationships among genetic subclades of *Francisella*, since mutations are mainly inherited vertically [1,2].

Tularemia is characterized by an acute course of infection, and mortality rates of *F. tularensis* subsp. *tularensis* infections historically reached 5 to 30% before effective antibiotic treatments were available. In contrast, *F. tularensis* subsp. *holarctica* infections are milder and may be fatal only to patients with an impaired immune system [3]. *F. tularensis* can infect humans, via aerosols or the skin, at doses as low as 10 cells [4,5] and is listed by the CDC as a major potential bioterror agent [6]. Cultivation of *F. tularensis* is often avoided, since it poses considerable risks of laboratory-acquired infections via aerosolization. Laboratory culture work requires biosafety-level 3 (BSL-3) conditions and primary cultivation from a clinical specimen may require a seven-day incubation before colonies visible to the naked eye appear. To shorten the time required for clinical diagnosis, PCR assays targeting 16S rDNA [7] or specific genes encoding outer membrane proteins such as *fopA* [8] and *lpnA* [9–11] have been used to detect *Francisella*, and several real-time PCR assays have been developed recently that appear to be more sensitive than conventional PCR [12–17]. However, a serious drawback of PCR-detection is that cross-reactivity with environmental non-pathogenic *Francisella* bacteria may occur [18–20]. Therefore there is a need to develop PCRs for distinguishing clinically relevant *Francisella* species from closely related non-pathogenic *Francisella* present in environmental sources.

In research laboratories, isolates of *F. tularensis* have been identified and classified using a variety of molecular typing methods, including amplified fragment length polymorphism (AFLP) analysis [21], pulse-field gel electrophoresis (PFGE) [22,23], insertion/deletion (INDEL) mutation analysis [24], multi-locus variable number of tandem repeats analysis (MLVA)
[25,26], multi-locus sequence typing (MLST) [2], and whole genome single-nucleotide polymorphism (SNP) analysis [1]. The highest typing resolution has been achieved by MLVA of rapidly mutating tandem repeats, but at a cost sometimes of incorrectly characterizing relationships among distantly related isolates.

In the present study, we developed a convenient real-time PCR assay based on robust genetic markers (SNPs and INDELs). A desired feature of the assay was that it should be able to distinguish between human pathogenic *F. tularensis* and the two genetically closely related species *F. novicida* and *F. philomiragia* which are of lower clinical relevance and often found in environmental sources. Moreover, the assay should be capable of identifying the subclades of *F. tularensis* (especially within *F. tularensis* subsp. *holarctica*, type B), and be compatible with standard real-time PCR machines that are now widely used in routine diagnostic laboratories. The developed assay meets all of these criteria, and can be tailored to match typing resolution requirements by adding or removing genetic markers as appropriate.

**Materials and Methods**

**Ethics Statement**

Ulcer specimens were collected as part of the routine clinical management of patients and the use of them for laboratory service improvement conducted in compliance with the regulation, policies and principles of the Swedish Public Health Service. Approval from an ethics committee was for that reason not sought after. The clinical routine for collecting specimens includes an open friendly verbal communication informing the patient that the sampling purpose is detecting the causative agent of tularemia. A verbal informed consent was required before submitting any sample to the laboratory. The specimens were de-identified and analyzed anonymously.

**Isolates and Clinical Specimens**

A panel of 62 *Francisella* isolates (listed in Table 1), spanning as much as possible of the known genetic diversity within the genus, was used to determine the specificity of all of the tested markers (listed in Tables 2 and 3). The final one plate-assay, including 34 genetic markers, was applied to 14 isolates and six patient ulcer specimens obtained in 2008 at Umeå University Hospital, Sweden (Table 4), and also to five additional isolates of global origins (Table 1). The new assay was evaluated along with the standard PCR assay that is used for diagnosis of human ulceroglandular tularemia [27]. Plate design and interpretation of assay results are exemplified in Figure 2 by the analysis of the Live Vaccine Strain (LVS).

**DNA Preparation**

*F. tularensis* isolates were re-cultured and a loopful of each isolate was suspended in phosphate buffered saline, heat-killed and DNA was prepared by phenol/chloroform extraction using Phase Lock Gel Light tubes (Eppendorf, Hamburg, Germany) or by a chaotropic salt method [27]. The latter was also used to prepare DNA from the clinical specimens. The concentration of DNA in each sample was determined using a BioPhotometer (Eppendorf, Hamburg, Germany) or NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), then adjusted to 2.5 ng/µl.

**Genetic Markers and Primers**

Phylogenetically informative SNPs and INDELs were identified by BLAST searches of available *Francisella* genomes and DNA
Table 1. Sixty-seven isolates of global origins used in this study.

| Species (no. isolates) | origin | FSC no.a | NAU IDb | Alternative designations | Vogler et al. 2009 subclade | Johansson et al. 2004 groupc | Table 5 geno-type | Figure 3 subclade |
|------------------------|--------|----------|---------|--------------------------|---------------------------|-----------------------------|-------------------|-------------------|
| *F. philomiragia* (5)  | Water, Bear River Refuge, UT | 037 | F0047 | ATCC 25016 | – | – | 1 | PATC25017 |
|                        | Water, Bear River Refuge, UT | 038 | F0048 | ATCC 25017 | – | – | 1 | PATC25017 |
|                        | Water, Ogden Bay Refuge, UT | 039 | F0049 | ATCC 25018 | – | – | 1 | PATC25017 |
|                        | Moribund muskrat (*Ondatra zibethicus*), 1959, Brigham City, UT | 144 | F0045 | ATCC 25015 | – | – | 1 | PATC25017 |
|                        | Atlantic cod (*Gadus morhua*), 2008, Norway | 775d | DSM18777 | – | – | – | 1 | PATC25017 |
| *F. novicida* (5)      | Water, 1950, UT | 040 | F0050 | ATCC 15482, U112 | N | N | 2 | N.U112 |
|                        | Human blood, 1991, Houston, TX | 156e | F0051 | fx1 | N | N | 3 | N.FSC156 |
|                        | Human blood, 1991, Houston, TX | 159 | F0052 | fx2 | N | N | 3 | N.FSC156 |
|                        | Human blood, 2003, Spain | 454 |  |  |  |  |  |  |
|                        |  |  |  |  |  |  |  |  |
| *F. tularensis* subsp. *mediasiatica* (4) | Experimental isolate, cap-, Rostov, Russia | 122 | F004 | TTC-R)6-4-1 | M.Br.FSC 147 | M | 6 | M.FSC147 |
|                        | Midday gerbil (*Meriones meridianus*), 1965, Kazakhstan | 147e | F0011 | GIEM 543 | M.Br.FSC 147 | M | 6 | M.FSC147 |
|                        | Hare, 1965, former USSR, Central Asia | 149 | F0012 | 120 | M.Br.FSC 147 | M | 6 | M.FSC147 |
|                        | Tick, 1982, former USSR, Central Asia | 148 | F0013 | 240 | M.Br.FSC 147 | M | 6 | M.FSC147 |
| *F. tularensis* subsp. *tularensis* (11) | 1960 (Eigelsbach) | 013 | F0006 | FAM standard | – | – | 7 | A1.3/[4,5] |
|                        | Tick, 1935, British Columbia, Canada | 041 | F0005 |  | Vavenby | A1.Br.001/002 | A1 | 7 | A1.3/[4,5] |
|                        | Squirrel, Georgia, USA | 033e |  |  | SnMF | – | – | 8 | A1.FSC033 |
|                        | Human pleural fluid, 1940, Fox Downs, Ohio, USA | 046 | F0008 |  |  | A1.Br.SCHU S4 | A1 | 9 | A1.SCHU54 |
|                        | Human, 1941, Ohio, USA | 237 | F0567 | Schu S4 | A1.Br.SCHU S4 | – | 9 | A1.SCHU54 |
|                        | Mites, 1988, Slovakia | 199 | F0007 | SE-221/38 | A1.Br.SCHU S4 | A1 | 9 | A1.SCHU54 |
|                        | Lab acquired when handling Nevada 14 | 053 | F0009 | F.tul AC | A1.Br.001/002 | A2 | 10 | A2 |
|                        | Hare, 1953, Nevada, USA | 054 | F0010 | Nevada 14 | A1.Br.001/002 | A2 | 10 | A2 |
|                        | Hare, Canada | 042 | F0296 |  | Utter | A1.Br.003/004 | A2 | 10 | A2 |
|                        | Human, 1920, Utah, USA | 230 | F0419 | ATCC 6223 | A1.Br.ATCC 6223 | A2 | 10 | A2 |
|                        | 1959, USA | 604 | Rki 03-1300, 8859 |  | – | – | 10 | A2 |
| *F. tularensis* subsp. *holarctica* (42) | Human lymphnode, 1926, Japan | 017 | F0016 | 5-2 | B.Br.001/002 | B5 | 11 | B5.FSC022 |
|                        | Hare, 1954, Oniwa, Japan | 020 | F0292 |  | – | B5 | 11 | B5.FSC022 |
|                        | Human, 1958, Tsuchiya, Japan | 021 | F0014 |  | B.Br.001/002 | B5 | 11 | B5.FSC022 |
|                        | Human, 1950, Ebina, Japan | 022 | F0015 |  | B.Br.001/002 | B5 | 11 | B5.FSC022 |
|                        | Tick, 1954, Fukushima, Japan | 023 | F0293 | TH | – | B5 | 11 | B5.FSC022 |
|                        | Yerma, Japan | 024 | F0294 |  | – | B5 | 11 | B5.FSC022 |
|                        | Tick, 1957, Jima, Japan | 075 | F0017 |  | B.Br.001/002 | B5 | 11 | B5.FSC022 |

Identification of *Francisella*
| Species (no. isolates) | origin | FSC no. | NAUID | Alternative designations | Vogler et al. 2009 subclade | Johansson et al. 2004 group | Table 5 geno-type | Figure 3 subclade |
|------------------------|--------|---------|-------|--------------------------|---------------------------|-----------------------------|---------------------|------------------|
| Human blood, 1989, Norway |       | 089     | F0038 | N1/89 (4SF2)             | B.Br.OSU18                 | B2                          | 12                  | B2.OSU18         |
| Human blood, 1994, Bergen, Norway |       | 158     | F0301 | CCLU 33391               | B.Br.OSU18                 | B2                          | 12                  | B2.OSU18         |
| Beaver, 1976, Montana, USA |       | 035     | F0018 | B423A                    | B.Br.OSU18                 | B2                          | 12                  | B2.OSU18         |
| Hare, 1997, Austria |       | 584     | F30   | –                        | –                          | –                           | 12                  | B2.OSU18         |
| Human ulcer, 2005, Ljusdal, Sweden |       | 641     | 05-32-85 | –                      | –                          | –                           | 12                  | B2.OSU18         |
| Human, 2000, Orebro, Sweden |       | 285     | F0212 | A073-46/00               | B.Br.007/008               | 84                          | 13                  | B4.Ftnf49/18    |
| Tick, 1941, Montana, USA |       | 012     | F0291 | 423 F4G                  | –                          | –                           | 13                  | B4.Ftnf49/18    |
| Human ulcer, 2004, Orebro, Sweden |       | 519     | 04-32-23 | –                      | –                          | –                           | 13                  | B4.Ftnf49/18    |
| Human, 2004, Umeå, Sweden |       | 663     | –     | –                        | –                          | –                           | 13                  | B4.Ftnf49/18    |
| Human, 2000, Uppsala, Sweden |       | 274     | F0228 | R63/00                   | B.Br.010/011               | Spain, France, & Sweden     | 13                  | B4.Ftnf49/18    |
| Human, 1993/94, Vosges, France |       | 247     | F0020 | T-20                     | B.Br.FTNF002-00            | Spain, France, & Sweden     | 14                  | B4.FTNF002-00   |
| Hare, 1952, Chateauroux, France |       | 025     | F0295 | 061-1                    | B.Br.FTNF002-00            | Spain, France, & Sweden     | 14                  | B4.FTNF002-00   |
| Hare, Castilla y León, Spain |       | 455     | FT1   | –                        | –                          | –                           | 14                  | B4.FTNF002-00   |
| Human skin lesion, Castilla y León, Spain |       | 456     | FT7   | –                        | –                          | –                           | 14                  | B4.FTNF002-00   |
| Human, 1995, Ockelbo, Sweden |       | 162     | F0162 | –                        | B.Br.012/013               | B3                          | 15                  | B3.19(20,23)    |
| Human, 1995, Ockelbo, Sweden |       | 178     | F0044 | B.Br.012/013             | B3                          | 15                          | B3.19(20,23)       |
| Water, 1980, Crimea, Ukraine |       | 115     | F0021 | B.Br.013/014             | B3                          | 15                          | B3.19(20,23)       |
| Norwegian rat (Rattus norvegicus), 1988, Rostov, Russia |       | 150     | F0029 | –                        | B3                          | 15                          | B3.19(20,23)       |
| Human blood, 1996, Raah, Finland |       | 250     | F0164 | B.Br.013/014             | B3                          | 16                          | B3.23(24,25)       |
| Human lymph node, 2005, Summi Admin area, Ukraine |       | FDC079  | –     | –                        | –                           | –                           | 16                  | B3.23(24,25)    |
| Live vaccine strain, Russia |       | 155     | F0566 | B.Br.LVS                 | B3                          | 17                          | B3.LVS              |
| Tick (Dermacentor pictus), 1949, Moscow area, Russia |       | 257     | F0019 | GEM 503/840              | B.Br.013/014               | B3                          | 18                  | B3.Rc503        |
| Tick (Dermacentor reticulatus), 1995, Lanzhot, Czech Republic |       | 184     | F0191 | T-35                     | –                           | B1                          | 19                  | B1.20/21         |
| Tick (Dermacentor reticulatus), 1995, Lanzhot, Czech Republic |       | 185     | F0192 | T-38                     | B.Br.013/014               | B1                          | 19                  | B1.20/21         |
| Tick (Dermacentor reticulatus), 1995, Lanzhot, Czech Republic |       | 186     | F0193 | T-44                     | B.Br.013/014               | B3                          | 19                  | B1.20/21         |
| Tick (Ixodes ricinus), 1995, Lanzhot, Czech Republic |       | 187     | F0194 | T-60                     | B.Br.013/014               | B1                          | 19                  | B1.20/21         |
| Bank vole (Clethrionomys glareolus), 1977, Seneca district, Slovakia |       | FDC010  | –     | –                        | –                           | 19                          | B1.20/21           |
| Brown hare (Lepus europaeus), 1964, Viedek district, Slovakia |       | FDC014  | –     | –                        | –                           | 19                          | B1.20/21           |
| Water, 1983, Rostov region, Russia |       | 121     | F0025 | 12267                    | B.Br.013/014               | B1                          | 19                  | B1.20/21         |
| Human, 1995, Čiánk, Finland |       | 249     | F0163 | 1468                    | B.Br.013/014               | B1                          | 19                  | B1.20/21         |
| Water, 1990, Odessa region, Ukraine |       | 124     | F0027 | 14588                   | B.Br.013/014               | B1                          | 20                  | B1.21/22         |
| Water, 1990, Odessa region, Ukraine |       | 119     | 14592 | –                        | B1                          | 20                          | B1.21/22           |
Table 1. Cont.

| Species     | NAUID   | Alternative designation |
|-------------|---------|-------------------------|
| 50 F0178    | T-1002  | Human, 2001, Uppsala, Sweden |
| 50 F0134    | 3001MA  | Human, 2001, Uppsala, Sweden |
| 50 F0133    | R42/95  | Human ulcer, 1995, Uppsala, Sweden |
| 50 F0133    | R42/95  | Human ulcer, 1995, Uppsala, Sweden |

All isolates were typed with the final assay and were part of a set of 62 isolates used in the development stage to confirm the typing accuracy. The isolates FSC017 (B5), FSC033 (A1), FSC147 (M), FSC156 (N), FSC257 (B3), and FSC641 (B2) were typed with the final one-plate assay, and were part of the set of 62 isolates used in the development stage.

Real-time PCR

In the final assay, real-time PCR amplifications of 34 genetic markers were performed using an iCycler (BioRad) with 5 ng DNA, or a Mastercycler instrument (Eppendorf) with 2 ng DNA, in both cases in 25 μl reaction mixtures in 68 wells of a 96-well plate (one primer pair per well was used). The reaction mixture for SNP detection consisted of 5 pmol of each primer (MWG-Biotech), 3U of AmpliTaq DNA Polymerase Stoffel Fragment, 2 mM MgCl₂, 50 μM dNTP, 20x SYBR Green I, 4% dimethyl sulfoxide (DMSO), and 2% glycerol. Two master mixes were prepared in which each of the allele-specific primers were added. The amplification conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The SNP in each sample was determined by inspecting the amplification curves. Amplification appeared earlier in reaction mixtures containing the forward primers with a matching 3′-base. A positive result was assigned when there was a one cycle or more difference between the time of appearance of PCR-products, and the number of cycles did not exceed 35. For INDEL analysis, Power SYBR Green PCR Mastermix was used with the same cycling conditions as for SNPs. The presence of a deletion was detected by failure of the reaction mixture with one primer in the deleted sequence to yield a detectable amplification product, while the control reaction with primer pairs surrounding the deletion succeeded. For cases where one primer overlapped a small deletion a minimum detection threshold of a five-cycle difference in time of appearance between the control and test reactions was set.

Quality Controls

The final 68-well assay included one PCR reaction per well (no multiplexing). To evaluate the typing accuracy of the assay, a test blinded to the investigator was performed on a subset of six isolates previously used in the development stage and representing the MLVA genetic groups F. novicida (N), F. t. mediasiatica (M), A1, B2, B3, and B5 of Francisella. Genetic group designations are found in Figure 1 and Table 1. The detection limit of the final assay was tested with serial logarithmic dilutions of F. tularensis subsp. holarctica Live Vaccine Strain (LVS) DNA, starting at one ng. The detection limit was set at the lowest amount of DNA with which PCR amplification of all 34 sequences at the National Centre for Biotechnology Information (NCBI). In addition, two INDELS and 12 SNPs previously shown to discriminate between isolates of Francisella were selected and tested for specificity [2,24,28–30].

For each SNP marker, two forward allele-specific primers with different 3′ bases, each matching one of the SNP allele states, and a reverse common primer, were designed using Primer3 [31] (Table 2). The primers were designed according to a SNP discrimination assay described by Germer and Higuchi [32,33], in which GC-rich tails of different lengths are added to each of the two allele-specific primers: a 14 bp (GGCGGCAGGGCGGC) tail was attached to the primer with G or C at the 3′-end, and a six bp (GGGGGC) tail was attached to the primer with A or T at the 3′-end. The GC-tails were in the original publication added primary to obtain a difference in the melting temperature, but a larger difference in the time of appearance between the two PCR-products was also obtained. For each INDEL marker, one common primer (CP) and two forward primers were designed: one inside (IN) and one outside (OUT) the deletion (Table 3). The CP-OUT primer pair was used as a positive control.

All primers were obtained from Eurofins MWG, Ebersberg, Germany and matched regions with an identical nucleic acid sequence in compared genomes and DNA sequences of the genus Francisella to minimize amplification failure of screened isolates.
| SNP | SCHU S4* SNP position | SCHU S4 locus ID | SCHU S4 gene | SNP state | Primer | Primer sequences
|-----|-----------------------|-----------------|--------------|-----------|--------|---------------------|
| F.1 | 1312210, SCHU S4a SNP position | FTTr04, FTTr10 | 16S T | D | gcgggcCTATGTGACTGAGCTTTGGT |
| F.2 | 1379332 | FTTr07 | G | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| T/N.1 | | TTT0080 | A | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| T.1 | 116590 | FTTr0901 | C | AGTTGGAAACGACTGTTAATACCGC |
| T.2 | 83976 | FTTr0901 | C | AGTTGGAAACGACTGTTAATACCGC |
| N.1 | 83943 | FTTr0901 | T | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| N.2 | 910194 | FTTr0901 | G | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| N.3 | 84027 | FTTr0901 | G | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| M.2 | 84027 | FTTr0901 | A | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| A.2 | 1199395 | FTTr0901 | T | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| A.3 | 62997 | FTTr0901 | G | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| A.4 | 830716 | FTTr0901 | A | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| A.5 | 350750 | FTTr0901 | C | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| A.6 | 1806912 | FTTr0901 | C | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| B.15 | 1113816 | FTTr0901 | G | D | gcgggcAGAAACACATCAATTTATCCTGTCa |
| B.16 | 608246 | FTTr0901 | G | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| B.17 | 1743251 | FTTr0901 | C | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
| B.18 | 1756146 | FTTr0901 | C | A | gcgggcAGAAACACATCAATTTATCCTGTCa |
markers occurred. The reproducibility of the assay was assessed using one ng DNA of LVS tested in three replicate runs.

MLVA
To assign MLVA clusters for isolates that had not been previously characterized in [26], MLVA was performed using a CEQ 8800 instrument (Beckman Coulters, Fullerton, CA), as previously described [26].

Accession Numbers
Completed genomic sequences (with GenBank accession numbers in parenthesis) used in this work were: U112 (CP000439), ATCC25017 (CP000937), WY96-3418 (CP000608), FSC147 (CP000915), FTNF002-001/FTA (CP000803), OSU18 (CP000437), LVS (AM233362) and SCHUS4 (AJ749949).

Draft genome sequences (with GenBank accession numbers in parenthesis) used in this work were: ATCC25015 (ABYY00000000), FSC200 (AASP00000000), FTE (ABSS00000000) and FTG (ABXZ00000000).

Preliminary sequence data were obtained from the MIT Broad Institute website at www.broad.mit.edu for the following Francisella strains: GA99-5349, GA99-5348, FSC033, FSC022, and FSC257/RC503.

The following Francisella genomes from Baylor College of Medicine Human Genome Sequencing Center website at www.hgs.bcm.tmc.edu were not available at the time of the study, but are mentioned here: ATCC6223, KO97-1026, MI00-1730 and OR96-0246/BSA; The OR96-0463 genome was sequenced by the Joint Genome Institute and Lawrence Livermore National Laboratory, and is available from http://genome.ornl.gov.

The following previously published genes found to discriminate between isolates of Francisella, were used: dnaA (AM261088 to AM261101) [29]; tpiA (AM261102 to AM261115, AY794514 to AY794528 and AY794495) [2]; atpA (AY794498 to AY794513) [2]; vacJ (DQ451123 to DQ451126) [28]; fabH (DQ863407 to DQ863420) [30]; FTT0086 (DQ863472 to DQ863483) [30]; asd/FTT0425 (Ftind18) and lpyR/FTT0492 (Ftind33) [24]; appC/FTT0092 (Ftind38) [24], and aceF, RD17 (AY794422) [2].

Results
Selection of Genetic Markers
We identified 49 SNPs and 15 INDELs with potential canonical properties by analyzing various available DNA sequences. Strain
polymorphism was verified using a pair of isolates showing the two possible allelic states. Twenty-four SNPs and three INDELs were not used in further analyses because of amplification failure, or (in SNP analysis) because there was a less than one cycle difference in the time of appearance of different PCR products. In evaluation of the remaining SNPs and INDELs in a panel of 62 Francisella isolates of diverse genetic and geographical origins, two SNPs and one INDEL were found to be incongruent with the phylogenetic structure of F. tularensis determined by Vogler et al [1], and were therefore also discarded. The final set of markers comprised 23 SNPs and 11 INDELs, which were arrayed in a hierarchical assay structure in 68 wells of a 96-well plate (one primer pair per well was used) (Figure 2).

Detection Level and Typing Resolution

The limit of detection of our assay was found to be 100 pg DNA. Three replicate runs using F. tularensis strain LVS showed identical results. An indefinite typing result occurred on average in 0.3 to one marker per plate. However, unambiguous strain classification was still possible using the information obtained from the other markers. The assay successfully detected and discriminated among the three species of Francisella, the five major genetic clades of F. tularensis, and the subclades of F. tularensis subsp. holarctica. A comparison with a set of recently published canonical SNPs [1] showed perfect correlation with the results obtained in our assay (as shown in the Francisella phylogeny depicted in Figure 3, which indicates names of markers and subclades from both research groups). Our markers B.20 to B.23, B.16 and A.4 added typing resolution to the genetic branches B.Br.013/014, B.Br.002/003, and A.I.001/002 previously defined by Vogler et al [1] (Figure 3, Table 5). In addition, the use of INDELs Ftind44, Ftind48 and Ftind49 provided resolution at phylogenetic nodes where no corresponding SNP was identified by Vogler et al.

Table 3. INDEL markers, genes affected by the INDELs, and primers.

| INDEL | SCHU S4 INDEL position | SCHU S4 locus ID | SCHU S4 gene | Primer | Primer sequences |
|-------|------------------------|------------------|--------------|--------|------------------|
| Ftind43 | 1541234..1541239 | FTTt30, FTTt31 | Arg-tRNA, Gly-tRNA | IN | GTTCCACAAATTTGCGGGAA (intergenic) OUT AATCCCTTTGGGTGTGCCAT |
| Ftind44 | 895956..896021 | FTT0886, FTT0887 | recN, FTT0887 | IN | TCGCAAGCATTGCTGACCCTA (intergenic) OUT TAAACTCTAGTGGCTGATTAAAT |
| Ftind45 | 725227..725228 | FTT0706 | glk2 | IN | ACCTAATAGCACCATTAGGAT (pseudogene) OUT TCACCAATAGCTCCTACAA |
| Ftind46 | 1830698..1830699 | FTT1739 | asd | IN | AGTTTCGATCGCAAGGGA (pseudogene) OUT AGTTTTCTGATCGCCTGCT |
| Ftind47 | 271674..271683 | FTT0255 | recA | IN | AGTACATATCCATGCTCAATCAG (pseudogene) OUT ATCTTTCAGATCGATCAGT |
| Ftind48 | 960987..961011 | FTT0948 | attC | IN | ATCTCATTACATTCATATTCCAGT (pseudogene) OUT ATCTTTCAGATCGATCAGT |
| Ftind49 | 834341..834349 | FTT0816 | attC | IN | AGTACATATCCATGCTCAATCAG (pseudogene) OUT ATCTTTCAGATCGATCAGT |
| Ftind50 | 88484..88576 | FTT0086 | lysR | IN | CATCAGCGCCACCAAGGATAT |
Our markers T.1 and FInd44 also conveniently discriminated all *F. tularensis* strains from *F. novicida*, *F. philomiragia* and *F. noatunensis* isolates (Figure 3, Table 5).

Concordance to MLVA

The categorization of *F. tularensis* isolates based on 23 SNPs and 11 INDELs was consistent with the MLVA-groupings presented by Johansson et al in 2004 [26] (Table 1, Figure 1) with one exception. In our SNP/INDEL analysis, strain FSC186 was classified as belonging to B1, while it was classified as B3 by MLVA [26]. An analysis of MLVA data showed that the inconsistency was likely caused by homoplasy (characters shared by a set of strains but not present in their common ancestor) at the highly variable MLVA markers Ft-M3 and Ft-M6 (Table 6).

Categorization of *Francisella* Strains by the Real-time PCR array

Twenty-one genotypes were detected by the hierarchical array (Table 5). The typing accuracy of the final one-plate assay was assessed in a blind test, in which we correctly categorized six isolates previously tested individually for each marker. We further used the assay to categorize 14 isolates obtained from patients with tularemia in Sweden in 2008 (Figure 4, Table 4), and five isolates that were not included in the development of the assay (Table 1). We characterized six human tularemia ulcer specimens that were positive by the standard PCR for diagnosis of ulceroglandular tularemia [27] by amplifying the four selected markers B.20 to B.23 (Figure 4, Table 4), since we could not apply the new assay with all 34 markers due to scarcity of DNA.

Discussion

In the present study we combined analysis of INDELs and SNPs in a real-time PCR array for robust, rapid and flexible hierarchical identification of *F. novicida* and *F. philomiragia* and typing of human pathogenic members of the genus *Francisella*. In contrast to previously published real-time PCR assays, our assay was designed to cover the full currently known phylogenetic range of *Francisella*. The assay was also tailored to provide high typing resolution for *F. tularensis* subsp. holarctica isolates originating from Scandinavia, where our laboratory is located. Hierarchical typing based on cultivation and bacterial phenotypes has long been a fundamental element of the characterization of bacteria in diagnostic microbiology laboratories. Hierarchical typing based on genetic characters has only recently been applied, for classification of

### Table 4: Fourteen isolates and six ulcer specimens from tularemia patients in Sweden 2008 characterized by the developed hierarchical real-time PCR array.

| Category         | FSC no. | Sample ID | Location of the receiving hospital | Table 5 genotype | Figure 3 subclade |
|------------------|---------|-----------|------------------------------------|-------------------|-------------------|
| Isolates         | 792     | 32–92     | Säffle                             | 13                | B4.FInd49/18      |
|                  | 844     | 32–280    | Uddevalla                          | 13                | B4.FInd49/18      |
|                  | 780     | 32–51     | Luleå                              | 16                | B3.23/24,25       |
|                  | 785     | 32–75     | Falun                              | 16                | B3.23/24,25       |
|                  | 812     | 32–123    | Sunderbyn                          | 16                | B3.23/24,25       |
|                  | 816     | 32–142    | Boden                              | 16                | B3.23/24,25       |
|                  | 823     | 32–155    | Lövånger                           | 16                | B3.23/24,25       |
|                  | 831     | 32–173    | Skellefteå                          | 16                | B3.23/24,25       |
|                  | 794     | 24–95     | Östersund                           | 19                | B1.20/21          |
|                  | 777     | 32–38′    | Örebro                             | 19                | B1.21/22          |
|                  | 787     | 32–79     | Umeå                                | 20^f              | B1.21/22          |
|                  | 778     | 32–47^    | Ljusdal                            | 20^f              | B1.21/22          |
|                  | 783     | 32–69     | Färla                              | 21                | B1.FSC200         |
|                  | 817     | 32–145    | Bollnäs                            | 21                | B1.FSC200         |
| Ulcer specimens  | –       | 32–151^   | Jönköping                          | 16, 17 or 18^     | B3.23/24,25       |
|                  | –       | 32–300^   | Gavle                              | 16, 17 or 18^     | B3.23/24,25       |
|                  | –       | 32–87^    | Umeå                                | 16, 17 or 18^     | B3.23/24,25       |
|                  | –       | 32–215^   | Uddevalla                          | 19^               | B1.20/21          |
|                  | –       | 32–38^    | Örebro                             | 20^f              | B1.21/22          |
|                  | –       | 32–47^    | Ljusdal                            | 20^f              | B1.21/22          |

^aStrain ID in the Francisella Strain Collection, Swedish Defense Research Agency, Umeå, Sweden. ^bSample ID at the Department of Clinical Bacteriology, Umeå University, Umeå, Sweden. ^cIsolate FSC777 and ulcer specimens 32–38 are from the same patient. Isolate FSC778 and ulcer specimens 32–47 are from the same patient. ^dThe exact genotype could not be determined due to detection failure of marker B.22 (the difference in time of appearance between the two PCR products was less than one cycle). ^e*F. tularensis* cultures were negative. ^fThe patient reported probable acquisition of tularemia when visiting the county of Jämtland, where the regional center is Östersund.

gThe genotype and subclade were assigned based on marker B.20, which exhibited an A for all three specimens, and on marker B.23, which exhibited a T. No other markers were screened due to scarcity of DNA. ^hThe genotype and subclade were assigned based on: marker B.20, which exhibited a G for all three specimens; on marker B.21, which exhibited a G for specimens 32–215, and an A for specimens 32–38 and 32–47; and on marker B.22, which exhibited a G for specimens 32–38 and 32–47. No other markers were screened due to scarcity of DNA.
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Bacillus anthracis and Francisella tularensis strains [1,24,34,35]. This work demonstrates that a genetic hierarchical approach, based on carefully selected markers with canonical properties, can be used across an extensive phylogenetic typing range in the genus Francisella.

We have identified 34 genomic markers serving as phylogenetic guides, which can be added to or excluded from an assay depending on the testing objectives, i.e. according to the taxonomic and geographical resolution required. For example, in diagnostics, where the purpose is to verify the presence or absence of F. tularensis specimens, including canonical markers for species and subspecies levels in the assay may be sufficient. In contrast, in epidemiological investigations, where the aim is to track disease-transmission paths and/or sources, higher typing resolution might be desired, and thus markers that characterize the complete phylogeny, or alternatively only a selected subset with high resolution, should be included in the assay. In forensic investigations, complete characterization of isolates is needed to provide statistical and unambiguous evidence to infer relationships between isolates, and thus all canonical markers may be included in the assay. Geographical aspects could also be taken into consideration when selecting markers to be included. For example, in clinical laboratories located in Scandinavia it is not expected to find F. tularensis subsp. tularensis isolates in clinical samples tested,

Figure 2. Example of plate design and interpretation of results for the genetic classification of F. tularensis strain LVS. A) The allelic state of each marker in the LVS strain is indicated in boldface. A colored well corresponds to a phylogenetically determining (canonical) marker for a specific genetic subclade. B) A phylogenetic tree is generated from hierarchical analysis of the typing results. Thick lines indicate the inferred evolutionary history of strain LVS. D = derived state, A = ancestral state. doi:10.1371/journal.pone.0008360.g002
Figure 3. Schematic SNP and INDEL phylogeny, indicating genetic markers and Francisella subclades. Markers presented in this study are indicated in black and, for comparison, SNP markers developed in a recent study by Vogler et al. 2009 [1] are indicated in gray. The branch names of Vogler et al. have been abbreviated to simplify the nomenclature. Stars indicate terminal subclades defined by Francisella genomes and circles represent collapsed branch points along the genetic lineages that contain isolates of a particular genotype (a subclade). The subclades are named for the flanking SNPs and INDELS. The branch lengths do not represent true phylogenetic distances. The position of B.15/Ftind47 (marked by the asterisk in the figure) could not be definitively determined; it could be either where shown, or be descendant from B.1/2.

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Table 5. Francisella genotypes in this study.

| Genotype | Ftind1 | Ftind18 | Ftind | Ftind18 | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind | Ftind |
|----------|--------|---------|-------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. P.ATCC25017 | T       | DEL     | G     | T       | ND     | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    | ND    |
| 2. N.U112     | IN     | A       | T     | DEL     | T     | G     | C     | IN    | A     | IN    | C     | IN    | T     | C     | T     | C     | IN    | A     | IN    | G     | IN    | G     | IN    |
| 3. N.FSC156   | T       | IN      | A     | T      | DEL    | G     | A     | C     | IN    | A     | IN    | C     | IN    | T     | C     | T     | C     | IN    | A     | IN    | G     | IN    | C     |
| 4. N.FSC454   | T       | IN      | A     | T     | ND     | G     | T     | IN    | A     | ND    | C     | ND    | T     | C     | T     | C     | IN    | A     | IN    | ND    | G     | ND    | C     |
| 5. N.Ftind44/[1,2,3] | T   | IN      | A     | T     | DEL    | G     | G     | C     | IN    | A     | IN    | C     | IN    | T     | C     | T     | C     | IN    | A     | IN    | G     | IN    | C     |
| 6. M.FSC147   | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | G     | IN    | C     | IN    | T     | C     | T     | C     | IN    | A     | IN    | G     |
| 7. A1.3/[4,5] | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | C     | C     | T     | T     | C     | IN    | A     | IN    | G     | IN    |
| 8. A1.FSC033  | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | C     | T     | T     | C     | IN    | A     | IN    | G     | IN    | C     |
| 9. A1.SCHUS4  | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | C     | C     | C     | C     | C     | IN    | A     | IN    | G     | IN    |
| 10. A2.1/2    | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | IN    | T     | C     | T     | T     | C     | IN    | A     | IN    | G     | IN    |
| 11. B5.FSC022 | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | IN    | T     | C     | T     | T     | C     | IN    | A     | IN    | G     | IN    |
| 12. B2.OSU18  | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | IN    | T     | C     | T     | T     | C     | IN    | A     | IN    | G     | IN    |
| 13. B4.Ftind18 | T     | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | G     | DEL   | G     | DEL   | T     | IN    | C     | IN    | C     |
| 14. B4.FTNF002-00 | T | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | G     | DEL   | G     | DEL   | T     | IN    | C     | IN    | C     |
| 15. B3.19/[20,23] | T  | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | G     | DEL   | G     | DEL   | T     | IN    | C     | IN    | C     |
| 16. B1.21/22  | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | G     | DEL   | G     | DEL   | T     | IN    | C     | IN    | C     |
| 17. B1.FSC200 | T       | IN      | A     | C     | IN     | G     | G     | C     | IN    | A     | DEL   | A     | DEL   | G     | DEL   | G     | DEL   | T     | IN    | C     | IN    | C     |

*Ftind18, Ftind33 and Ftind38 were previously used in [24].

**A boldfaced marker corresponds to a phylogenetically determining (canonical) marker for a specific genetic subclade.

*DEL = derived deletion.

*ND = not detected.

*IN = ancestral state.

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since this subspecies is confined to North America. Thus, only one canonical marker specific for the subspecies *tularensis* may be included and not all markers characterizing subclades of the subspecies. Instead, a very high discriminatory power for all the *F. tularensis* subsp. *holarctica* genetic groups that are known to be present in Sweden would be desired, i.e., groups B1 to B4 in Figure 1. Therefore, all canonical markers defining these subclades may be included. Finally, since we have included genomic markers for discriminating human pathogenic *F. tularensis* isolates from *F. philomiragia* and *F. novicida* which are of less clinical relevance and often present in environmental sources, the assay could potentially be used to monitor environmental *Francisella*.

A comparison of results obtained from SNP and INDEL markers shows good agreement. Both marker types apparently provide similar and stable phylogenetic information. Further, INDELS and SNPs are slowly mutating markers that provide very similar typing resolution. The lower typing resolution of INDELs in our assay was probably due to marker discovery bias: INDELs were easier to identify in the relatively few and genetically diverse available genome sequences than in the many available short sequence stretches from closely related isolates. In contrast, SNPs could be readily identified in both kinds of DNA sequences. We note that INDEL markers in the real-time PCR assay strengthen the SNP marker information at the main phylogenetic nodes (Figure 3). Deletion events should be evolutionarily unidirectional [2], while SNPs may revert. Thus, SNPs may (at least theoretically) display homoplastic patterns, while INDELs should not do so in a clonally structured bacterial population. We found that use of INDELS made the assay more robust and provided additional resolution at nodes where no corresponding SNP was identified.

The limit of detection of our assay was 100 pg of DNA, based on the lowest amount of DNA from which all 34 markers included in a single plate were amplified; a higher quantity than minimum amounts reported for other real-time PCR assays with fewer targets. This is a limitation that should be addressed in future work.Possibly, adaptation to a real-time PCR system including probes such as TaqMan SNP Genotyping Assay or the SNaPshot (Single Nucleotide Primer Extension) Assay (Applied Biosystems), could provide higher sensitivity. However, the reproducibility of the results was good and the failure of classification low, indicating that the assay was technically robust. The applicability of our assay to clinical isolates was also demonstrated, since we were able to characterize *F. tularensis* isolates including Ft-M3 and Ft-M6 should be

![Figure 4. Example of use.](image-url)
complemented with analysis of more robust markers, such as SNPs and/or INDELs to ensure correct phylogenetic classification.

In summary, real-time PCR assays based on a hierarchical classification concept, as exemplified in this work, are flexible typing tools for phylogenetic and geographical resolution of Francisella. The level of discrimination can be easily adjusted by adding or removing genetic markers, a property which is not generally provided by conventional PCR methods or by previously developed real-time PCR assays. The presented hierarchical real-time PCR array could be used in public health laboratories as well as in research laboratories for a wide range of Francisella identification and typing purposes.

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Author Contributions

Conceived and designed the experiments: KS MF AJ. Performed the experiments: KS MG LK VN. Analyzed the data: KS MG LK VN AJ. Contributed reagents/materials/analysis tools: KS MG LK AJ. Wrote the paper: KS MF AJ.