The Genetic Diversity and Evolution of *Francisella tularensis* with Comments on Detection by PCR

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Abstract
Francisella tularensis has been the focus of much research over the last two decades mainly because of its potential use as an agent of bioterrorism. *F. tularensis* is the causative agent of zoonotic tularemia and has a worldwide distribution. The different subspecies of *F. tularensis* vary in their biogeography and virulence, making early detection and diagnosis important in both the biodefense and public health sectors. Recent genome sequencing efforts reveal aspects of genetic diversity, evolution and phylogeography previously unknown for this relatively small organism, and highlight a role for detection by various PCR assays. This review explores the advances made in understanding the evolution and genetic diversity of *F. tularensis* and how these advances have led to better PCR assays for detection and identification of the subspecies.

Introduction
Francisella tularensis is a small, non-motile, Gram-negative coccobacillus and is the causative agent of the zoonotic disease tularemia. This facultative intracellular pathogen was first discovered in Tulare County California in 1911 where it caused a plague-like illness in local rodents (McCoy and Chapin, 1912). *F. tularensis* is able to cause disease in rabbits, squirrels, and other mammals, including humans (Wherry and Lamb, 1914). The transmission of *F. tularensis* to humans is mediated through arthropod vectors such as ticks and deer flies, by the ingestion of contaminated food or water, or by inhalation of aerosolized bacteria (Akimana and Abu Kwaik, 2011). *F. tularensis* subsp. *tularensis* is highly infectious. It is estimated that an aerosol inoculation of as few as 10 organisms is sufficient to cause disease in humans (McCrumb, 1961). Because of its highly infectious nature, *F. tularensis* is considered a potential agent of bioterrorism and is categorized by the Centers for Disease Control and Prevention (CDC) as a Tier 1 select agent (Dennis et al., 2001).

Through the years, the taxonomy of *Francisella* has gone through many changes. Upon its discovery, McCoy and Chapin named their new discovery *Bacterium tularense* (McCoy and Chapin, 1912). Following the *Bacterium* genus, it was subsequently placed in *Pasteurella* and later *Brucella* (Salomonsson, 2008). Finally in 1959, it was placed in a new genus, *Francisella*, in honor of Edward Francis, in which genus it resides today (Olsufjev et al., 1959). There are currently four recognized subspecies of *Francisella tularensis*: *tularensis, holarctica, mediasiatica,* and *novicida*. While the inclusion of *novicida* as a subspecies of *F. tularensis* is still contested (Larsson et al., 2009; Kingry and Petersen, 2014), much of the recent scientific literature, including Bergey's Manual of Systematic Bacteriology, recognizes this classification (Garrity, 2005).

In 1950, the first *novicida* subspecies was isolated and characterized (Larson et al., 1955). This new isolate resembled *F.* tularensis morphologically, but differed in that it could ferment glucose, was not as virulent in humans, and did not cross-react with serum from rabbits inoculated with killed *F. tularensis.* Based on these differences, the authors proposed the name *Francisella novicida* (Larson et al., 1955). However, in the 1950s, researchers did not have the genetic tools which became available in later decades. In the 1980s, DNA-DNA hybridization experiments between *F. tularensis* and *F. novicida* demonstrated up to 92% homology (Hollis et al., 1989). Because of this high degree of genetic similarity, it was proposed that *F. novicida* be reclassified as a subspecies of *F. tularensis*. This reclassification was formally proposed in 2010 in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) (Huber et al., 2010). This proposal received a formal objection in IJSEM, contending that genetic similarity was not enough to reclassify *F. novicida* as *F. tularensis* subsp. *novicida*, but that the phenotypic differences were sufficient enough to justify separate species designation (Johansson et al., 2010).

Finally, in a rebuttal to the objection of Johansson et al., Busse et al. (2010), stood by their initial recommendation for reclassification, asserting that the genetic similarity meets the definition of a subspecies (Wayne et al., 1987). Furthermore, Busse et al. acknowledge the phenotypic differences between *F. tularensis* and *F. novicida*, but contend that the 11 phenotypic differences noted are not sufficient enough for a new species (Busse et al., 2010). There are many other examples of bacteria with a greater percentage of phenotypic differences which are classified as the same species (e.g. the various biovars of *Pseudomonas fluorescens*) (Busse et al., 2010). Despite this evidence, a formal reclassification has yet to occur. Based on the high genetic similarity, and taking into account the relatively few phenotypic differences, we also propose the reclassification of *F. novicida* as a subspecies of *F. tularensis*, and will refer to it as such throughout this work.

Each subspecies is predominantly associated with a specific geographic distribution and severity of disease. The subspecies *tularensis* is typically found in North America (Staples et al., 2006) while the subspecies *holarctica* is found across much of the Northern Hemisphere (Johansson et al., 2004). The subspecies *mediasiatica* has only been isolated from the central Asian republics of the former Soviet Union (Broekhuijsen et al., 2003) and the subspecies *novicida* has been isolated from North America and Australia (Hollis et al., 1989; Whipp et al., 2003). Phylogenetic relationships among these subspecies are inferred in Figure 1.

The two subspecies most associated with human disease are *tularensis* and *holarctica*. These are often abbreviated simply as Type A and Type B *tularensis*, respectively. Type A *tularensis* causes a more severe form of tularemia while the presentation of type B tularemia is somewhat milder (Owen et al., 1964; Weiss et al., 2007). The subspecies *mediasiatica* is fully virulent in mice, yet is believed to be of relatively mild virulence in humans (Broekhuijsen et al., 2003; Champion et al., 2009). Similar to the subspecies *mediasiatica*, the subspecies *novicida* is fully virulent in mice, yet rarely causes disease in humans (Hollis et al., 1989).

Genetic analyses by multiple-locus variable-number tandem repeat analysis (MLVA) has identified further sub classifications and geographic structure of Type A and Type B *tularensis*. The major subdivisions of Type A *tularensis* include Type A.I and Type A.II, with the former generally isolated from the eastern United States and the latter generally isolated from the western United States (Johansson et al., 2004). This biogeographic separation is correlated with the geographic distribution of specific vectors, hosts, and other abiotic factors such as elevation and rainfall (Farlow et al., 2005; Oyston, 2008). The major divisions of Type B *tularensis* also display geographic structure, with Type B.I isolated from Eurasia, Type B.II isolated from North America and Scandinavia, Type B.III isolated from Eurasia and North America, Type B.IV isolated from North America and Sweden, and Type B.V isolated from Japan (Johansson et al., 2004). Unlike type A *tularensis*, the distribution of Type B *tularensis* has not been shown to correlate with the distribution of any specific vectors (Farlow et al., 2005).

The *F. tularensis* subsp. *holarctica* isolated from Japan was first differentiated from other *F. tularensis* subspecies based on its ability to ferment glucose (Olsufjev and Meshcheryakova, 1983). These isolates were further differentiated by demonstrating a reduced virulence from the subspecies *tularensis,* displaying a virulence similar to that of the subspecies *holarctica* (Sandstrom et al., 1992). As genomic tools became more widely available, this division was confirmed by microarray analysis (Broekhuijsen et al., 2003), restriction fragment length polymorphism (RFLP) analysis (Thomas et al., 2003), and multiple-locus variable number tandem repeat analysis (MLVA) (Johansson et al., 2004; Fujita et al., 2008).

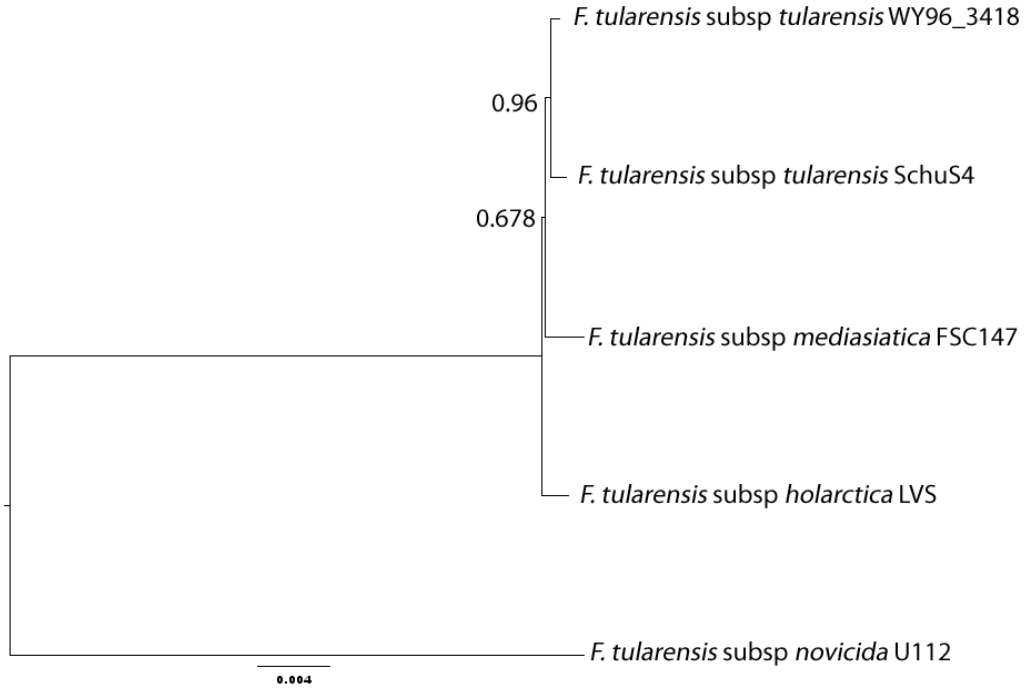

Figure 1. Maximum likelihood tree inferring the phylogenetic relationships of the *F. tularensis* subspecies. Tree was constructed by concatenating 10 housekeeping genes (*recA*, *gyrB*, *groEL*, *dnaK*, *rpoA1*, *rpoB*, *rpoD*, *rpoH*, *fopA*, and *sdhA*) followed by alignment with Clustal W and generation of the tree with MEGA 5.2. Bootstrap values are indicated at the nodes except where support was less than 0.65.

Genetic analyses have hinted that these isolates from Japan underwent a unique evolutionary process in a restricted area, separate from other *F. tularensis* subspecies (Fujita et al., 2008). Because of the phenotypic differences, the genetic differences, and the apparent isolated evolution, it has been proposed that these strains from Japan be classified as another subspecies of *F. tularensis* called *F. tularensis* subsp. *japonica* (Vivekananda and Kiel, 2006). However, since relatively few isolates from Japan have been analyzed, we recommend that this designation not be adopted at this time.

Genetic Diversity

The first complete genome of *Francisella tularensis* was sequenced in 2005 (Larsson et al., 2005). This first sequence was the classical type strain of *Francisella tularensis* subsp. *tularensis* representing the Type A.I sub classification. Since then, numerous other whole and partial genomes of *F. tularensis* have been sequenced: *F. tularensis* subsp. *holarctica* strain OSU18 (Type B) (Petrosino et al., 2006), a European isolate of Type A *tularensis* (Chaudhuri et al., 2007), *F. tularensis* subsp. *novicida* strain U112 (Rohmer et al., 2007), a Type A.II *tularensis* (WY96-3418) (Beckstrom-Sternberg et al., 2007), *F. tularensis* subsp. *mediasiatica* (Larsson et al., 2009) and at least 10 more comprising the 4 subspecies of *F. tularensis* (Barabote et al., 2009; Nalbantoglu et al., 2010; Modise et al., 2012; Svensson et al., 2012). With the advent of improved massively parallel sequencing technologies, more genomes continue to be sequenced at an ever-increasing rate (La Scola et al., 2008). In all, there are currently 16 complete genomes of *Francisella tularensis* deposited in GenBank and even more partial genomes. This collection of genomic information allows for the comparative analysis of these genomes and provides insight into the evolution of *F. tularensis* genome architecture.

Even before the first *Francisella* genome was completed in 2005, studies analyzing the genomic diversity of *F. tularensis* were plentiful. Because of its potential use as a bioweapon and for public health reasons, rapid identification of *F. tularensis* became paramount (Dennis et al., 2001). Early DNA based techniques focused on 16S rDNA typing. This proved difficult since among the 4 subspecies, the 16S rDNA genes exhibit between 98.5 - 99.9% similarity, the result of only 6 nucleotide differences among the most divergent strains (Forsman et al., 1994). Other DNA based techniques for identification such as PCR, which is both rapid and accurate, helped spur further interest in the genetic diversity of the *F. tularensis* subspecies (Broekhuijsen et al., 2003; Pohanka et al., 2008). A genome wide microarray that analyzed 27 strains of all four subspecies confirmed the limited genetic variation within the subspecies, but identified 8 variable regions that were used to develop a subspecies-specific PCR assay (Broekhuijsen et al., 2003). Another microarray study analyzing the genetic diversity of 11 Type A isolates and 6 Type B isolates from various localities around the United States identified 13 regions of difference, including segments of several genes with implications for virulence (Samrakandi et al., 2004). While microarray and other studies revealed valuable information about the regional distribution and differences in virulence, complete genome sequences reveal a more complete picture (Broekhuijsen et al., 2003; Johansson et al., 2004; Samrakandi et al., 2004).

The first completed genome sequence of *F. tularensis* yielded insights to previously undiscovered features of its genetic makeup. Some of the genetic features discovered included previously uncharacterized virulence genes encoding type IV pili and iron acquisition systems (Larsson et al., 2005). The complete sequence also revealed a duplication of an approximately 30 kb region previously identified as a pathogenicity island containing 17 open reading frames (ORFs), perhaps shedding light on the enhanced virulence of Type A *tularensis* (Nano et al., 2004; Larsson et al., 2005; Nano and Schmerk, 2007). Finally, analysis of this genome indicated the loss of several biosynthetic pathways, which helps explain the fastidious nutritional requirements of *F. tularensis* and suggests the need to infect a host during its life cycle (Larsson et al., 2005).

The first comparative genomic study of *F. tularensis* was of the Type A (Schu S4) and Type B (OSU18) strains. This study revealed an extensive genomic similarity of 97.63%, indicating that the differences in virulence between the two strains are likely not due to large differences in gene content (Petrosino et al., 2006). This degree of sequence identity was confirmed among the remaining subspecies as well (Rohmer et al., 2007; Champion et al., 2009; Larsson et al., 2009). Perhaps the most striking difference between these two strains is the vast amount of genomic rearrangement. These rearrangements can mostly be attributed to homologous recombination using insertion (IS) elements (Petrosino et al., 2006).

After the genome sequence of *F. tularensis* subsp. *novicida* was complete, a 3-way comparison between three of the subspecies (*tularensis*, *holarctica*, and *novicida*) was possible. Again, a high degree of sequence identity among the subspecies was confirmed, as was the large amount of genomic rearrangement (Rohmer et al., 2007). Even though the length and the gene content of the *novicida* subspecies (1.91 Mb and 1,731 protein coding genes) are both greater than that of the *tularensis* subspecies (1.89 Mb and 1,445 protein coding genes) and the *holarctica* subspecies (1.89 Mb and 1,380 protein coding genes), these human pathogenic strains contain 41 genes which the non-human pathogenic strains (*novicida*) do not (Rohmer et al., 2007). Initial comparisons of these genomes revealed that the human pathogenic strains carry 2 copies of the *Francisella* Pathogenicity Island (FPI) while the non human pathogenic strains carry only 1 copy, shedding further light on the differences in virulence among the subspecies (Nano and Schmerk, 2007).

Many studies have been completed comparing the various subsets of available *F. tularensis* genomes. A comparison of the genomes of two *holarcitca* subspecies, the live vaccine strain (LVS) and strain FSC200, sought to uncover

the mode of attenuation for LVS (Rohmer et al., 2006), which was attenuated through the repeated passage of a *holarctica* strain between the 1930s and 1950s in the former Soviet Union (Green et al., 2005; Petrosino et al., 2006). The genomes of the LVS and FSC200 strains differ by only 0.08% but the LVS strain was able to confer immunity to infection with *F. tularensis* subsp. *tularensis* in BALB/c mice (Green et al., 2005; Rohmer et al., 2006). While the exact nature of genomic modifications leading to LVS attenuation were not found, comparison with other more virulent Type A strains revealed some candidate genes which could be targeted in the development of a future vaccine (Rohmer et al., 2006). When the sequence of *F. tularensis* subsp. *holarctica* FTNF002-00 was completed and compared to both LVS and the OSU18 strains, it was found to have greater than 99.9% sequence similarity (Barabote et al., 2009). Other studies have shown a stable genome architecture among Type B strains, but FTNF002-00 carries a 3.9 kb inversion compared to other Type B strains (Petrosino et al., 2006; Dempsey et al., 2007; Barabote et al., 2009).

Other whole genome comparisons focused on comparing different strains of Type A *tularensis*. A comparison between *F. tularensis* subsp. *tularensis* Schu S4 (Type A.I) and WY96-3481 (Type A.II) revealed only one whole gene difference, a hypothetical protein with an unknown function (Beckstrom-Sternberg et al., 2007). Despite the fact that these two strains are very closely related, there were still many other differences, including numerous single nucleotide polymorphisms (SNPs), small indels, differences in IS elements, and even 31 large chromosomal rearrangements (Beckstrom-Sternberg et al., 2007). Many of the chromosomal rearrangements are frequently bordered by IS elements, providing a mechanism for the translocations (Beckstrom-Sternberg et al., 2007; Larsson et al., 2009; Nalbantoglu et al., 2010). Another genome comparison of a Type A.I clinical isolate to the Schu S4 genome showed that except for some minor changes, the genomes were virtually identical, suggesting a high degree of sequence conservation within the Type A.I subgroup (Nalbantoglu et al., 2010). The genome of another Type A.I strain (TI0902) isolated from a cat in Virginia, United States, is also highly similar to Schu S4 as it only differs by 103 SNPs (Modise et al., 2012). Other researchers compared a European isolate of Type A.I *tularensis* (which is typically restricted to North America) to Schu S4 and found that the two were virtually identical, with only 8 SNP and 3 variable number tandem repeat (VNTR) differences (Chaudhuri et al., 2007). The fact that these two strains are so alike suggests that the European isolates are descended from the Schu S4 strain and did not evolve independently in Europe (Chaudhuri et al., 2007).

The completion of a fourth subspecies genome of *F. tularensis*, the *mediasiatica* subspecies, enabled full genome comparisons of the four subspecies of *F. tularensis*. It was demonstrated that the subspecies *mediasiatica* and *tularensis* are highly similar, which raises more questions about their differences in virulence (Olsufjev and Meshcheryakova, 1982; Broekhuijsen et al., 2003; Larsson et al., 2009). Phylogenetic analysis of the complete genomes of the subspecies *mediasiatica* also demonstrated that it is a monophyletic taxon of *F. tularensis*, contradicting previous evidence suggesting that the subspecies *mediasiatica* was not a member of the *F. tularensis* clade (Nübel et al., 2006). However, since isolates of the *mediasiatica* subspecies are rare, it is difficult to know the true genetic diversity within the subspecies. Figure 2 shows the overall genome architecture of representative strains of *F. tularensis*,

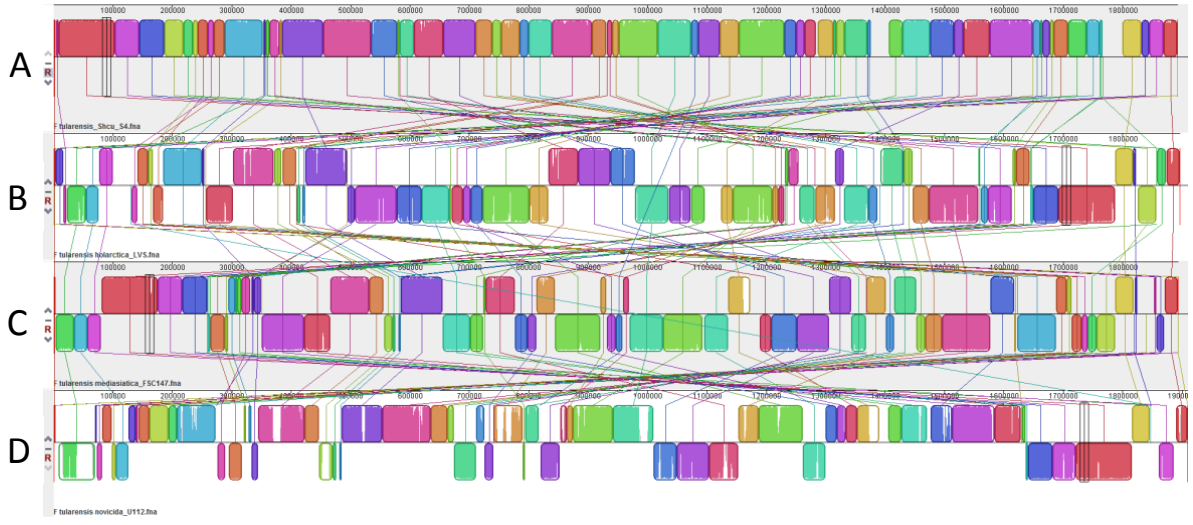

Figure 2. Whole genome alignment of representative strains from each of the four subspecies of *Francisella tularensis* using Mauve (Darling et al., 2004) highlighting differences in the macro genome architecture relative to the reference strain (A). Colored blocks represent homologous sections of each genome. A) *F. tularensis* subsp. *tularensis* Schu S4. B) *F. tularensis* subsp. *holarctica* LVS. C) *F. tularensis* subsp. *mediasiatica* FSC147. D) *F. tularensis* subsp. *novicida* U112.

highlighting the large-scale genomic rearrangements between the subspecies.

The evolution of the *Francisellacaea* is complicated by the discovery of *Francisella*-like endosymbionts (FLEs) of ticks, which have an unknown pathogenicity in humans (Niebylski et al., 1997; Scoles, 2004; Goethert and Telford, 2005; Dergousoff and Chilton, 2012). While these endosymbionts lack sufficient evidence to be classified as *F. tularensis*, they are similar enough to cross react with many molecular-based methods of detection (Szigeti et al., 2014). Because of the potential to misidentify FLEs as *F. tularensis*, which could impact the diagnosis of tularemia in public heath settings, many have cautioned about the use of PCR assays for the detection of *F. tularensis* (Kugeler et al., 2005; Sreter-Lancz et al., 2009). Despite this caution, PCR remains the standard of practice for the detection and identification of *F. tularensis* subspecies (Pohanka et al., 2008).

Detection

The ability to accurately detect and diagnose *F. tularensis* infection carries significant implications in public health and bioterror (Dennis et al., 2001; Gunnell et al., 2012). Because of the different pathogenic profiles and biogeography of the various subspecies of *F. tularensis*, it is important to be able to accurately discriminate among them (Gunnell et al., 2012). Polymerase chain reaction (PCR) has become the method of choice for the identification of various pathogens because it is rapid, sensitive and highly specific (Foroshani et al., 2013; Sting et al., 2013; Celebi et al., 2014). Detection and differentiation of the subspecies of *F. tularensis* by PCR is complicated by the lack of significant variability in their genomes (Forsman et al., 1994; Petrosino et al., 2006). Various methods for the detection of *F. tularensis* have been reviewed in the last decade, however much more work has since been completed on the detection of *F. tularensis* using PCR (Splettstoesser et al., 2005; Pohanka et al., 2008).

Conventional PCR

Since 2008, research on the use of conventional PCR for the detection of *F. tularensis* has dropped off considerably, with only a handful of publications on the subject. In alignment with an earlier review (Splettstoesser et al., 2005), the gene *tul4* was a popular choice to detect all subspecies of *F. tularensis* (He et al., 2009; Kormilitsyna et al., 2013). Since *F. tularensis* is a potential agent of bioterrorism, some assays included the multiplex detection of other biothreat agents. One such study developed two multiplex assays to detect “Tier 1” select agents; one assay for DNA based organisms (Variola Major, *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and Varicella zoster virus) and another assay with a reverse transcriptase for RNA based viruses (Ebola virus, Lassa fever virus, Rift Valley fever, Hantavirus Sin Nombre and the four serotypes of Dengue virus) (He et al., 2009). A major drawback to these multiplex assays however, is the use of a reporter dye and a colormetric detection system, because a positive result is unable to distinguish between the agents. The assay is intended only as a broad screening tool and further testing is required to differentiate between the organisms comprising the assay. Furthermore, since the genome of Variola Major (the causative agent of Smallpox) is so highly regulated, testing was completed with a plasmid control containing a small segment of the Variola Major genome (He et al., 2009).

Real-time PCR is known for being efficient and sensitive, but is not ideal for multiplexing beyond a 4- or 6-plex reaction because of the limited number of fluorescent channels available on most instrument platforms (Varma-Basil et al., 2004; Skottman et al., 2007). Researchers have overcome this limitation by using modified primers to bind the PCR products of a 15-plex reaction to fluorescent beads that can then be analyzed by a flow cytometer for the simultaneous detection of 11 pathogens with similar sensitivities to real-time reactions (Hsu et al., 2013). While effective, flow cytometers can be large, difficult to use, and costly. The Luminex Corporation (Austin, TX) has developed a similar, yet easier to use technology in their MAGPIX® system. Rather than a flow cell, the MAGPIX® uses a magnet to capture fluorescently labeled magnetic beads and a CCD camera to capture images of up to 50 different analytes (Bergval et al., 2012; Munro et al., 2013). Because of its relatively low cost and ease of use, the MAGPIX® may be more ideally suited for integration in clinical labs for the simultaneous detection of multiple pathogens (Bergval et al., 2012).

While it may be useful to detect broad categories of pathogens, because of the virulence status of various subspecies of *F. tularensis*, it is also important to be able to differentiate among them as well. Using the *tul4* gene and variations in the *pilA* gene, researchers were able to differentiate the four subspecies of *F. tularensis* (Kormilitsyna et al., 2013). Another study used suppression subtractive hybridization (SSH) to identify regions of difference between the genomes of Type A.I and Type A.II *tularensis.* This information was used to create a conventional PCR assay to differentiate between Type A.I, Type A.II, Type B, and *F. tularensis* subsp. *novicida* isolates (Molins-Schneekloth et al., 2008). Later, this same assay was adapted to a real-time PCR platform (Molins et al., 2009).

Real-time PCR

Real-time PCR is a popular choice for the detection of *F. tularensis* because it is sensitive, reliable, cost-effective, and eliminates the need for time consuming gels, though this time commitment has been significantly reduced with the introduction of rapid dry gels (Zasada et al., 2013). A popular method of real-time PCR incorporates the use of SYBR Green which will fluoresce upon binding double stranded DNA. Thus, the fluorescent signal will increase as PCR progresses and more amplicons are synthesized. SYBR green is a popular alternative to other real-time technologies because of its relatively low cost (Sellek et al., 2008). However, it is not ideal for multiplex reactions since the dye will bind to all double stranded DNA in the reaction and produce a fluorescent signal. Sellek *et al*. (2008) developed an assay to detect *F. tularensis* from soil using the *tul4* gene, previously used in conventional PCR assays

(He et al., 2009; Kormilitsyna et al., 2013). However, the assay was only validated with *F. tularensis* subsp. *holarctica* and subsp. *novicida*. Lacking were representatives from the subsp. *tularensis* and *mediasiatica*. Furthermore, positive fluorescent signals were obtained from other non-related bacteria. These were later ruled out as true positives after analyzing the PCR products on a gel and finding only primer dimers (Sellek et al., 2008).

Genome comparisons aided the development of SYBR green assays (Pandya et al., 2009; Svensson et al., 2009; Woubit et al., 2012). Woubit *et al*. (2012) compared several genomes from the *Escherichia, Francisella, Salmonella, Shigella, Vibrio, and Yersinia* genera to develop a series of 27 assays to detect and differentiate these common food and biothreat pathogens. With respect to *Francisella*, the assays were so specific that assays intended to detect all subspecies of *Francisella* were only able to detect the *tularensis* and *novicida* subspecies (Woubit et al., 2012).

The propensity of PCR assays to cross-react with environmental, non-pathogenic *Francisella* or other closely related organisms (Kugeler et al., 2005) requires the development of more specific assays to avoid false positives or incorrect diagnoses. To solve this problem, results from resequencing microarrays were compared to identify SNPs along the phylogeny of *F. tularensis* and build real-time PCR assays capable of differentiating Type A.I, A.II, A.Ia, A.Ib, Type B.I, and B.II *tularensis* (Pandya et al., 2009). Similarly, another group analyzed publically available whole genome sequences to identify defining SNPs and small insertion/deletion elements (INDELs) to design a series of 35 assays capable of distinguishing the four subspecies of *F. tularensis* and the major subtypes of Type A and Type B *tularensis,* including Type A.I, A.II, and B.I, B.II, B.III, B.IV, and B.V (Svensson et al., 2009). Both assays were able to accurately assign isolates to the correct subspecies and clade while avoiding any cross-reactivity to near neighbors (although the former includes only one *novicida* strain in the analysis).

Another method for the real-time detection of *F. tularensis* is the 5' nuclease or TaqMan® assay. These assays incorporate fluorescently labeled DNA probes specific to the template DNA resulting in even more specific identification than the SYBR Green assays, eliminating the need to perform a melt curve analysis. Strategies for single-plex real-time assays for the detection of *F. tularensis* with TaqMan® assays are varied. Gene targets include a gene for an outer membrane protein, FopA, a single-copy gene for detection and quantification of all subspecies of *F. tularensis* (Abril et al., 2008), the 16S rRNA gene to detect all subspecies of *F. tularensis* (Yang et al., 2008; Angelakis et al., 2009), the insertion element IS*Ftu2,* which is unique to *Francisella* species (Simsek et al., 2012), intergenic regions of differentiation to distinguish Type A.I from Type A.II *tularensis* (Molins et al., 2009), and SNP-based assays to differentiate the species and subspecies of *Francisella* isolates (Birdsell et al., 2014b). Some assays can be used in concert with others to detect a wide variety of agents. These include biothreat agents (Yang et al., 2008) or other organisms with similar disease presentations (Angelakis et al., 2009), while others were used solely for the differentiation of subspecies and subpopulations of *F. tularensis* (Molins et al., 2009; Birdsell et al., 2014b). The advantage of using a single-copy gene for detection is the ability to quantify the amount of the agent, which can be useful in clinical and diagnostic settings (Abril et al., 2008). Conversely, multicopy-genes such as the 16S rRNA gene and the IS*Ftu2* gene should achieve lower detection limits, which is ideal given the low infectious dose of *F. tularensis* (McCrumb, 1961; Yang et al., 2008; Simsek et al., 2012). A significant drawback of using the 16S rRNA gene for detection is that since it is so conserved, there is some cross reactivity with near neighbors and other *Francisella*-like species, requiring further confirmatory analyses (Forsman et al., 1994; Yang et al., 2008).

Multiplex real-time TaqMan® assays incorporate the added convenience of running multiple reactions in a single tube using probes labeled with various fluorophores. However, as mentioned previously, multiplexing with TaqMan® assays is generally limited to a 4- or 6 plex reaction because of the limited number of fluorescent channels on the instruments (Varma-Basil et al., 2004; Skottman et al., 2007). One multiplex assay is a 2-plex assay designed from genome comparisons to detect the four subspecies of *F. tularensis* but does not differentiate among them. Another multiplex assay is capable of differentiating the four *F. tularensis* subspecies with only a 3-plex assay. This assay was developed using both unique and shared genome regions among the subspecies with the addition of a scoring matrix (Gunnell et al., 2012).

Since *F. tularensis* has the potential to be used as a bioweapon, a commercial market has arisen for field-ready detection of biothreat agents, including *Bacillus anthracis, Francisella tularensis, Yersinia pestsis, Brucella* species, and others. A comparison of one such commercial instrument, the RAZOR®, (BioFire Defense; previously Idaho Technologies, Salt Lake City, UT) and another instrument designed for laboratory use, the Applied Biosystems 7300/7500 system (Thermo Fisher Scientific, Grand Island, NY) used assays developed for *B. anthracis, Brucella* species, *F. tularensis,* and *Y. pestis*, comparing sensitivities and specificities of the two platforms. Results showed that for all agents, the sensitivities were between 10-100 fg of target DNA per reaction, and no cross reactivity was observed with other closely related bacteria (Matero et al., 2011). Run time on the RAZOR® was notably shorter than that of the 7300/7500 instrument.

Another diagnostic tool, the FilmArray® system (BioFire Defense, Salt Lake City, UT), uses a lab-in-a-pouch approach to process raw samples and detect 17 biothreat pathogens with an array of single-plex real-time PCR assays in about an hour (Seiner et al., 2013). An evaluation of the Biothreat Panel using DNA samples from *B. anthracis, F. tularensis,* and *Y. pestis* indicated sensitivities of 250 genome equivalents or lower and the authors conclude that the system is both sensitive and selective (Seiner et al., 2013). However, since the FilmArray®

system is designed to be a complete sample to answer system, sensitivities may vary when tested with whole organisms in different matrices like blood or serum rather than purified DNA.

Another evaluation compared the FilmArray® system with TaqMan® Array Cards developed for the detection of biothreat agents (Rachwal et al., 2012; Weller et al., 2012). Here, researchers tested for *B. anthracis, F. tularensis,* and *Y. pestis* in the blood of murine infection models. Results showed that blood culture was the most sensitive means of detection followed by the FilmArray and Array Cards for *B. anthracis,* and *F. tularensis.* All three methods demonstrated similar detection levels for *Y. pestis* (Weller et al., 2012). While blood culture was the most sensitive means of detection for two of the three agents tested, it requires much more time for detection compared to the PCR assays. Each of these methods for detection carries drawbacks and benefits and must be weighed appropriately to ensure the best possible outcome.

Other PCR assays

Recently, other PCR-based assays have been developed for the detection of *F. tularensis* and other bacteria. One such assay involves analyzing PCR products with electrospray ionization-mass spectrometry (ESI-MS). In this technique, the actual base composition of the PCR products are identified and compared to a library of sequences for identification rather than relying on the fluorescent signal obtained from real-time PCR (Jacob et al., 2012). This PCR/ESI-MS technique has been applied to the wide-spread identification of biothreat agents, respiratory pathogens, and other pathogenic bacteria and viruses (Jacob et al., 2012; Jeng et al., 2013). Others have used this technology specifically for identifying *F. tularensis* from natural sources (Whitehouse et al., 2012) and even for typing the subspecies of *F. tularensis* (Duncan et al., 2013).

Recombinase Polymerase Amplification (RPA) is a PCR-like assay in which amplification is carried out at one temperature (isothermal) instead of cycling temperatures as in PCR. Recently, RPA assays have been applied to the detection of *F. tularensis* and other biothreat agents (Euler et al., 2012; Euler et al., 2013; del Rio et al., 2014). Two of these assays showed comparable sensitivities to real-time PCR assays with an instrument run time of about 10 minutes (Euler et al., 2012; Euler et al., 2013). A third assay using electrochemical detection rather than fluorescent probes seemed less sensitive than other assays, with detection levels on the order of 10^4 copies/µL (del Rio et al., 2014).

Finally, as the cost of sequencing continues to fall, more sequencing-based detection assays are being used to detect biological agents such as *F. tularensis*. One such assay used a pyrosequencing method to sequence the variable region of 16S rDNA to identify and group *F. tularensis* isolates by subspecies (Jacob et al., 2011). The results from analyzing the SNPs in 16S rDNA are more distinctive than SNP analysis from real-time PCR. Another sequencing assay was multiplexed for the detection and strain typing of *B. anthracis, F. tularensis,* and *Y. pestis* by interrogating 10 loci per pathogen (Turingan et al., 2013). While sequencing assays provide some promise for the rapid detection and classification of *F. tularensis*, there is a noticeable lack of information on the sensitivity or detection limits of these assays. In the world of clinical diagnostics and biodefense, the ability to detect low quantities of *F. tularensis* and other agents is paramount.

Evolution

Numerous studies have been conducted on the evolution of the subspecies of *F. tularensis* to define specific clades and to reveal their evolutionary history. Before next generation whole genome sequencing was widely available, various techniques were used to recover the phylogenetic relationships among strains of *F. tularensis,* such as microarrays (Broekhuijsen et al., 2003; Samrakandi et al., 2004), MLVA (Johansson et al., 2004), and sequencing specific genes or other genetic loci (Svensson et al., 2005; Nübel et al., 2006). One of the earliest of these studies produced a phylogenetic tree in which the subspecies *tularensis* and *mediasiatica* shared a major clade along with the Japanese isolates of the *holarctica* subspecies (Broekhuijsen et al., 2003). A later analysis provided better resolution, differentiating the *tularensis* and *mediasiatica* subspecies, and grouping the Japanese isolates of the *holarctica* subspecies with the other *holarctia* subspecies (Johansson et al., 2004). These authors also determined that *F. tularensis* subsp. *holarctia* appears to have recently spread globally from a single geographic origin, while *F. tularensis* subsp *tularensis* appears to have experienced most of its evolutionary history in North America, and may even have originated in the central United States (Birdsell et al., 2014a). However, *F. tularensis* subsp. *tularensis* is now clearly distributed beyond North America into parts of Europe (Chaudhuri et al., 2007).

The finding that the subspecies *holarctica* recently spread from a single origin seems likely because of the small amount of genetic diversity within the subspecies, that has been identified by a variety of molecular methods (Farlow et al., 2005; Dempsey et al., 2006; Rohmer et al., 2006; Keim et al., 2007; Larsson et al., 2007). However, the precise area of origin of the subspecies *holarctica* is unknown. Based on phylogenetic analyses, there are two competing hypothesis as to its origin: 1) the subspecies *holarctica* originated in Asia or 2) the subspecies *holarctica* originated in North America before spreading around the Northern Hemisphere (Vogler et al., 2009). There appears to be more evidence for the origination of the subspecies *holarctica* in North America, though this may be due to the lack of Asian isolates for analysis. Regardless, it appears that the *holarctica* subspecies is a highly fit clone that originated from a single source and spread throughout the Northern Hemisphere (Keim et al., 2007; Vogler et al., 2009). However, if *F. tularensis* subsp. *tularensis* originated in North America (Johansson et al., 2004; Birdsell et al., 2014a) and the subspecies *holarctica* is descended from the *tularensis* subspecies (Svensson et al., 2005), then it seems likely that the subspecies *holarctica* may have originated in North America as well. This hypothesis is

supported by the fact that sequences of various housekeeping genes and some outer membrane proteins from the subspecies *tularensis* and *holarctica* align well, while those from the subspecies *novicida* and *mediasiatica* do not (Nübel et al., 2006).

It is generally accepted that *F. tularensis* subsp. *novicida* is the oldest of the *F. tularensis* subspecies and evidence suggests that *F. tularensis* subsp. *novicida* and *Francisella philomiragia* share a common, aquatic ancestor (Svensson et al., 2005; Sjödin et al., 2012; Zeytun et al., 2012). These two species are generally considered non-pathogenic to humans. However, their association with aquatic sources is further substantiated in that documented human infections by these two species have occurred in near-drowning victims (Hollis et al., 1989; Wenger et al., 1989). Furthermore, *F. philomiragia* contains one copy of the FPI, similar to *F. tularensis* subsp. *novicida* while the remaining subspecies of *F. tularensis* contain 2 copies (Nano and Schmerk, 2007; Zeytun et al., 2012).

Molecular evidence suggests that the four subspecies of *F. tularensis* have evolved by vertical descent (Svensson et al., 2005). A common method of acquiring genetic variation in bacteria is through horizontal gene transfer. This is well documented in many species of bacteria, and especially in the conference of antibiotic resistance (Bliven and Maurelli, 2012; Turner et al., 2014; Dunlop et al., 2015; Ying et al., 2015). However, in the subspecies of *F. tularensis*, genetic variation, including antibiotic resistance seems to have arisen by mutation rather than the acquisition of new genes through horizontal gene transfer (Gestin et al., 2010; Siddaramappa et al., 2012; Sutera et al., 2014).

An *in silico* analysis has recently shown that the non human-pathogenic *F. tularensis* subsp. *novicida* possesses a CRISPER/Cas system to defend against invading genetic elements. This finding further supports the hypothesis that mutation is responsible for much of the evolution of *F. tularensis* (Gallagher et al., 2008; Schunder et al., 2013). Analyses of the other three virulent subspecies of *F. tularensis* (*tularensis*, *holarctica*, and *mediasiatica*), reveal that the genes responsible for the CRISPER/Cas system are non-functional (Schunder et al., 2013). This is somewhat puzzling since deletion of the CRISPER/Cas system in other pathogens such as *Neisseria meningitidis*, *Camphylobacter jejuni*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* result in decreased virulence. It is hypothesized that in the case of *F. tularensis*, other mutations in the genome have compensated for the degeneration of the CRISPER/Cas system in the virulent subspecies of *F. tularensis* (Sampson and Weiss, 2013).

Concluding Remarks

The genetic diversity of the subspecies of *F. tularensis* appears to be quite limited. Genome comparisons among the subspecies reveal similarities greater than 95% (Champion et al., 2009; Larsson et al., 2009). Many of the differences in the genomes of *F. tularensis* are large-scale genomic rearrangements and a duplication of the pathogenicity island in the *tularensis*, *holarctica*, and *mediasiatica* subspecies (Petrosino et al., 2006; Nano and Schmerk, 2007). However, because the *mediasiatica* subspecies is so rare, assessments of its true genetic diversity must be considered preliminary.

There are many pros and cons to the various PCR detection methods and the individual user's needs should dictate which method to use. Conventional PCR is easy and inexpensive but is known for being time consuming because of the need to run gels. However, since the introduction of rapid dry gels, the time commitment usually associated with gels has been shortened considerably. Utilizing fast PCR technology in combination with rapid dry gels, it is possible to get a result in approximately 50 minutes (Zasada et al., 2013). In general, conventional PCR has fallen out of favor with many researchers. However, this approach allows for large multiplex reactions for the detection of many organisms at once, especially when coupled with another detection system such as the MAGPIX® (Bergval et al., 2012; Munro et al., 2013).

Real-time PCR is one of the most popular methods for detection because it is simple, cost effective, and sensitive. SYBR Green assays are inexpensive and accurate and can even be multiplexed with the incorporation of a melting curve analysis. TaqMan® assays are more expensive than SYBR Green assays, but carry an additional layer of specificity with the sequence of the probe. Multiplexing with TaqMan® assays is possible, but usually only up to a 4- or 6-plex because of the limited number of available fluorescent channels on most instruments (Varma-Basil et al., 2004; Skottman et al., 2007). The limited amount of multiplexing with TaqMan® assays can be overcome by setting up an array of single-plex reactions similar to the FilmArray® system (Seiner et al., 2013).

Many current PCR assays lack the specificity to differentiate between environmental, non-pathogenic *Francisella* and other closely related organisms such as FLEs (Kugeler et al., 2005; Szigeti et al., 2014). Perhaps in these situations, it would be wise to use whole genome sequencing assays for the detection of *Francisella* subspecies (Jacob et al., 2011; Turingan et al., 2013).

As whole genome sequencing has become more widely available, genome comparisons between the subspecies of *F. tularensis* are possible and shed further light on the genetic diversity and evolution of this pathogen. It is apparent that the more virulent subspecies of *F. tularensis* have evolved from *F. tularensis* subsp. *novicida* primarily by genomic decay, genomic rearrangements, and the duplication of the FPI (Rohmer et al., 2007). Many of the interrupted genes (pseudogenes) in the virulent subspecies of *F. tularensis* are metabolic genes, further supporting an intracellular life cycle, while other interrupted genes include secreted effector proteins that may have led to excessive virulence, furthering the patho-adaption of *F. tularensis* as an intracellular pathogen (Hager et al., 2006; Larsson et al., 2009; Siddaramappa et al., 2011; Bliven and Maurelli, 2012).

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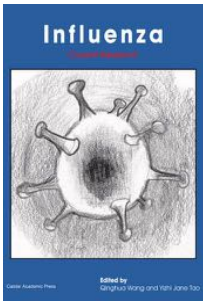

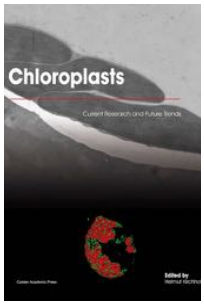

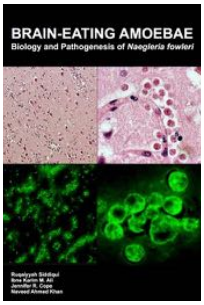

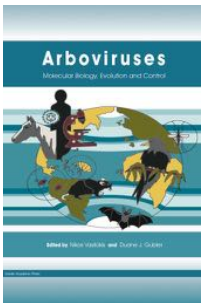

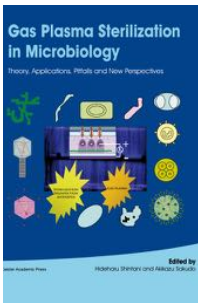

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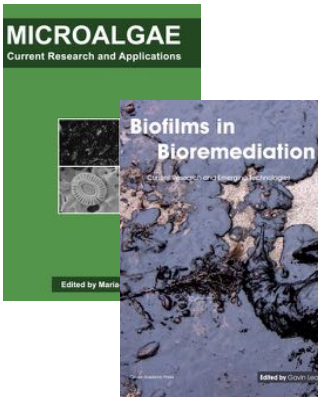

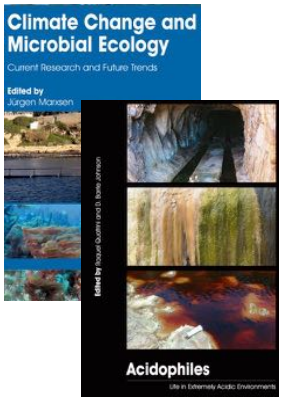

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