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REVIEW ARTICLE

Review of processing and analytical methods for *Francisella tularensis* in soil and water

Erin E. Silvestri[1] · Sarah D. Perkins[2] · Eugene W. Rice[1] · Harry Stone[2] ·
Frank W. Schaefer III[1]

Abstract The etiological agent of tularemia, *Francisella tularensis*, is a resilient organism within the environment and can be acquired in many ways (infectious aerosols and dust, contaminated food and water, infected carcasses, and arthropod bites). However, isolating *F. tularensis* from environmental samples can be challenging due to its nutritionally fastidious and slow-growing nature. In order to determine the current state of the science regarding available processing and analytical methods for detection and recovery of *F. tularensis* from water and soil matrices, a review of the literature was conducted. During the review, analysis via culture, immunoassays, and genomic identification were the methods most commonly found for *F. tularensis* detection within environmental samples. Other methods included combined culture and genomic analysis for rapid quantification of viable microorganisms and use of one assay to identify multiple pathogens from a single sample. Gaps in the literature that were identified during this review suggest that further work to integrate culture and genomic identification would advance our ability to detect and to assess the viability of *Francisella* spp. The optimization of DNA extraction, whole genome amplification with inhibition-resistant polymerases, and multiagent microarray detection would also advance biothreat detection.

✉ Erin E. Silvestri
   Silvestri.Erin@epa.gov

[1] US Environmental Protection Agency, National Homeland Security Research Center, 26 W. Martin Luther King Drive, MS NG16, Cincinnati, OH 45268, USA

[2] Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201, USA

Introduction

The etiological agent of tularemia (rabbit fever), *Francisella tularensis*, is a Gram-negative bacterium that can be found in many vertebrate and invertebrate hosts (Johansson et al. 2000b; Oyston et al. 2004; Keim et al. 2007; Broman et al. 2011) and environmental matrices such as soils, aerosols, and water (Kuske et al. 2006). Human infections occur in several ways, including exposure to infectious aerosols and dust, contaminated food and water, contact with infected carcasses, contact with fluids or tissue from infected animals such as contaminated feces, and arthropod bites (example, ticks and deer flies), but human to human transmission has not been reported (Fujita et al. 2006; Keim et al. 2007; WHO 2007; Berrada and Telford 2010; Meric et al. 2010; CDC 2011). In the United States, there were 1208 cases of tularemia reported between 2000 and 2010 (CDC 2013) and the mortality rate is currently around 2 % (Dennis et al. 2001; WHO 2007). *F. tularensis* is transmitted easily and has the potential to cause a large number of cases of human morbidity and mortality in the population, hence its designation as a Category A select agent (Dennis et al. 2001; Cooper et al. 2011; DHHS 2012). Subspecies of *F. tularensis* include type A (*F. tularensis* subspecies *tularensis*), type B (*F. tularensis* subspecies *holarctica*, previously known as *F. tularensis* subspecies *palaearctica*), and *F. tularensis* subspecies *mediasiatica* (Turingan et al. 2013), with geographic distribution, occurrence, and pathogenicity varying by subspecies (Duncan et al. 2013). A majority of human infections are caused by *F. tularensis* types A and B (Euler et al. 2012) with type A being more virulent and highly infectious (Cooper et al. 2011).

Two distinct phylogenetic groups exist under type A and include A1 and A2 (Keim et al. 2007). Type A1 can be further split into clades A1a and A1b (Nakazawa et al. 2010), with type A1b causing the highest mortality rate of all *F. tularensis* strains (Nakazawa et al. 2010). Rarely, the closely related species, *F. tularensis* subspecies *novicida* (also known as *F. novicida*), is associated with human infections (Kingry and Petersen 2014).

Francisella tularensis is persistent within the environment; it has been found to persist from weeks to years in decaying animal carcasses, moist soil, straw, hay, and water at low temperatures (Mitscherlich and Marth 1984; Forsman et al. 2000; Dennis et al. 2001). Certain species or subspecies prefer one type of host or environment over another. Type A, for example, is found within blood-feeding ticks, deerflies, and wild rabbits, and prior to the 1950s, in sheep (Keim et al. 2007; WHO 2007; Whitehouse et al. 2012). Type B, on the other hand, can be found in blood-feeding ticks, hares, multiple rodent species, and tabanid flies (Keim et al. 2007). Type B, *F. tularensis* subspecies *novicida*, and *F. philomiragia* are often found in environmental waters; however, Type B is also associated with semi-aquatic animals such as muskrats and beavers (Keim et al. 2007; WHO 2007; Whitehouse et al. 2012; van Hoek 2013).

There are several hypotheses regarding the natural environmental life cycle or mechanisms supporting the persistence of *F. tularensis* in the environment, and these include: infection of *F. tularensis* within free-living protozoa such as amoeba in the environment (Kantardjiev and Velinov 1995; Abd et al. 2003; Sjostedt 2006; Svensson et al. 2009; Visvesvara 2010; Broman et al. 2011); survival of *F. tularensis* within biofilms or within and among amoeba in the biofilms (Durham-Colleran et al. 2010; van Hoek 2013); persistence and disease transmission due to ingestion of planktonic *F. tularensis* or *F. tularensis* associated with biofilms by mosquito larvae (Mahajan et al. 2011); the use of dog ticks by *F. tularensis* as sustaining microfoci (Goethert and Telford 2009); and the survival of *F. tularensis* in microcosms (Davis-Hoover et al. 2006).

Isolation and detection of pathogens from environmental matrices such as soil or water can be a difficult task due to humic acids, organics, chemical constituents or other microorganisms present in the matrices, which can impede detection methods (Zhou et al. 1996; Robe et al. 2003; Balestrazzi et al. 2009). Limited work has been done on isolation, processing, and identification of *F. tularensis* from soil samples due to the fastidious nature of the organism and the complexity of environmental isolation (Versage et al. 2003; Gilbert and Rose 2012). The purpose of this review was to conduct a survey of the open literature to determine the state of the science of currently available processing and analytical methods for detection of *F. tularensis* in water (drinking, ground, and surface) and soil matrices, and to identify remaining gaps concerning *F. tularensis* identification from environmental samples. The results of the review could be used to inform needed method development in the detection of *F. tularensis* in both water and soil matrices.

Unclassified reports, published books, peer-reviewed journal articles, and government publications written in English from primarily the last 20 years were used for this literature review. PubMed with Google Scholar and Science Direct were used as the primary search engines with the Homeland Defense and Security Information Analysis Center (US Department of the Air Force) used secondarily. Key search terms included the agent name plus one or more of the following: water, soil, environmental, methods, processing, extraction, detection, and recovery. Literature for processing protocols or analytical methods for similar pathogens or similar matrices were also included in the summary if found during the search and deemed to be applicable.

Current state of the science

Research articles for isolation, processing, and identification of *F. tularensis* in soil were limited. While the search for methods for water matrices was more fruitful, it was evident that there is a limited breadth of knowledge regarding these types of methods. During the review, analysis via culture, immunoassays, and genomic identification were the methods most commonly found for *F. tularensis* detection within environmental samples.

Sample processing

In order to eliminate inhibitors from environmental samples prior to detection, samples are often pre-processed or concentrated using methods such as filtration (Sellek et al. 2008; Berrada and Telford 2010; Meric et al. 2010; Simsek et al. 2012), ultrafiltration (Francy et al. 2009; EPA 2011), and centrifugation (Anda et al. 2001; Davis-Hoover et al. 2006; Petersen et al. 2009; Berrada and Telford 2010; Meric et al. 2010; Simsek et al. 2012; Whitehouse et al. 2012).

Filtration with a sterile deionized water wash and a 0.45 μm cellulose acetate filter [prior to DNA extraction and real-time polymerase chain reaction (PCR)] has been used to concentrate *F. tularensis* in reservoir water (Meric et al. 2010). A second study compared the efficiency of an 8 μm pore size glass fiber pre-filter and a 5 μm pore size polyvinylidene fluoride membrane filter (PVDF) for processing 0.5–1.0 g soil samples containing low concentrations of *F. tularensis* that would allow for simultaneous immunologic and molecular analysis of the extracted sample (Sellek et al. 2008). Recoveries for the filters were low; of the spiked *F. tularensis*, only 6–10 % were recovered in the filtrate of

the glass and only 20 % in the PVDF filters (Sellek et al. 2008). Development of more efficient filters for processing environmental soil samples might potentially help improve recovery of samples containing low concentrations of *F. tularensis*.

A more efficient method for concentrating contaminated water samples might be the use of hollow-fiber ultrafiltration (HFUF) techniques. Ultrafiltration can be used to concentrate large water samples (100 L) down to a much smaller sample size (e.g., 225 mL). Francy et al. (2009) were able to detect *F. tularensis* in all 14 water samples (raw ground water and finished surface and ground water) that were spiked and concentrated using HFUF followed by analysis via quantitative PCR (results recorded as detected or not detected). When using HFUF to recover multiple microbes from environmental waters, variable input seeding levels and the use of an overnight culture was needed for samples containing *F. tularensis*, which demonstrated lower recovery rates compared to the other microbes tested (EPA 2011). A study by the EPA (EPA 2011) found that, depending on the laboratory protocol used and the addition of 1 % ammonium chloride to treat ultrafiltration concentrates prior to culture, the average recovery efficiencies of *F. tularensis* from tap water samples using ultrafiltration can range from 13 % to 62 %.

Swab sampling is a common technique used for sampling particulates on solid interior surfaces. A disposable centrifugation system called the Swab Extraction Tube System (SETS) has been found to be a more efficient processing method for recovering pure cultures of *F. tularensis* cells spiked on swabs [10^3–10^5 colony forming units (CFU)/swab] compared to heating for 10 min at 65 °C, vortexing, and sonicating (followed by DNA extraction and real-time PCR; Walker et al. 2010). However, the application of SETS to processing water and soil samples is unknown (Walker et al. 2010).

Culturing *F. tularensis* from the environment

Culturing is considered the "gold standard" for identification and confirmation of microbial agents by the US Centers for Disease Control and Prevention; however, there is a potential risk to laboratory workers when working with highly virulent, infectious organisms. *F. tularensis* requires 24–72 h of growth on a rich medium supplemented with bio-available iron, cysteine, and up to 12 other nutrients before colonies can be visualized (Versage et al. 2003; van Hoek 2013). Complicating successful culturing of *F. tularensis* is the fact that background organisms, especially in environmental samples, often out-compete *F. tularensis*, even if selective agars are employed (Versage et al. 2003; Delmont et al. 2011; Humrighouse et al. 2011; EPA 2012).

Culture of *F. tularensis* from environmental samples is frequently accomplished using selective antibiotic-supplemented cysteine heart agar with blood (CHAB) (Anda et al. 2001; Versage et al. 2003; Petersen et al. 2004, 2009; Francy et al. 2009; Berrada and Telford 2010, 2011; Meric et al. 2010; Humrighouse et al. 2011; Simsek et al. 2012; Whitehouse et al. 2012). Various modifications using antibiotics have been made to CHAB to improve isolation of *F. tularensis* from environmental samples. CHAB containing amphotericin B, cefepime, cycloheximide, polymyxin B, and vancomycin has been used to isolate *Francisella* spp. from seaweed and seawater samples (Petersen et al. 2009). CHAB-A has been used to inhibit background organisms in prairie dog tissue cultures collected from the field and consists of a modified CHAB agar supplemented with amphotericin, ampicillin, colistin, lincomycin, trimethoprim (Petersen et al. 2004). Modified Thayer-Martin chocolate agar supplemented with IsoVitaleX^{TM} has also been used as a selective agar for *F. tularensis* recovery from water samples (Anda et al. 2001).

Compared to PCR, use of culture to isolate *F. tularensis* from environmental waters has shown variable success. For example, the source of a tularemia outbreak in Turkey was sought through the collection and analysis of 154 surface water samples for *F. tularensis*. The results showed that only 4 samples were culture-positive using CHAB agar amended with antibiotics, while 17 were PCR positive (Simsek et al. 2012). Following a 2000 outbreak of pneumonic tularemia on Martha's Vineyard, Massachusetts, water, sediment, and soil samples were screened for *F. tularensis* by PCR utilizing 16S RNA, *fopA*, and other genetic primers (Berrada and Telford 2010). None of the samples collected from around a freshwater pond and a marsh were positive for *F. tularensis* subsp. *tularensis*. However, samples collected from the same freshwater pond were positive for *Francisella* spp., which was subsequently identified as *F. philomiragia* (Berrada and Telford 2010). Two other studies were able to identify *F. tularensis* only via PCR and not culture. For example, Meric et al. (2010) were able to identify *F. tularensis* from filter concentrated reservoir water samples only using PCR and not culture. In another study, *F. tularensis* subspecies *holarctica* was identified as the responsible agent only via PCR analysis and DNA sequencing and not culture via modified Thayer-Martin chocolate agar supplemented with IsoVitaleX during a tularemia outbreak in Spain connected to crayfish fishing in a contaminated freshwater stream (Anda et al. 2001).

Processing of samples prior to culture might help improve recovery. Use of ultrafiltration techniques for spiked water samples followed by culture on CHAB agar with antibiotics has reported a recovery range from 0.2 % to 40 % (Francy et al. 2009). One study comparing two similar ultrafiltration techniques found that when ultrafiltration filtrates were exposed to 1 % ammonium chloride for 2 h prior to culturing on antibiotics-amended CHAB, the recovery rates improved (recovery ranged from 17 % to 29 % without 1 % NH_4Cl, and

from 23 % to 62 % for samples with NH_4Cl) (EPA 2011). Use of a 15-min acid treatment on seeded water samples before culture on antibiotic-amended CHAB has been shown to aid in *F. tularensis* recovery by reducing native background organisms in the water samples (Humrighouse et al. 2011). Finally, *F. tularensis* recovery has been improved through use of acid shock prior to culture on modified Thayer-Martin chocolate agar (Anda et al. 2001).

Other factors could play a role in the culturability of *F. tularensis* from soil and water samples. For example, the effectiveness of the culture method might be dependent upon the sample collection techniques and the transport medium used (Johansson et al. 2000a). Another factor that might affect culturability is the temperature at which the samples are held at as well as the matrix in which *F. tularensis* is present. For example, *F. tularensis* spiked into tap water was not recovered after 24 h when held at 5 °C or 25 °C, but was recovered when held at 8 °C for 21 days and 28 days for *F. tularensis* LVS (live vaccine strain) and NY98 strains, respectively (Gilbert and Rose 2012). However, *F. tularensis* spiked into landfill leachates was culturable for 6 weeks when held at 12 or 37 °C (Davis-Hoover et al. 2006).

Immunoassay detection of *F. tularensis*

Infection source tracking has utilized testing for *F. tularensis* antigens within environmental samples for some time through the incorporation of immunoassays into hand-held field-deployable systems. However, some assay antigens can have cross-reactivity to other microorganisms (Quinn et al. 1984; Grunow et al. 2000; Fonseca et al. 2008; Pohanka and Skladal 2009). A summary of the immunoassay studies found through this literature review are summarized below.

The rapid immunochromatographic-test (RI-test) can be used to indicate the presence of the *F. tularensis* lipopolysaccharide (LPS) antigen in environmental waters (Berdal et al. 2000; Peruski et al. 2002). However, when compared to enzyme-linked immunosorbent assay (ELISA) and PCR, the RI-test and ELISA test were found to be better suited for detection of *F. tularensis* in tissue samples rather than water samples; PCR performed better with the environmental water samples. A limit of detection (LOD) for the three methods tested in the study was not given (Berdal et al. 2000).

Time-resolved fluorescence (TRF) is a technology based on lanthanide chelate labels with unique fluorescence properties. For one study, assay sensitivity was improved (2000-fold) and a wider dynamic range was noted for various matrices including sewage water, soil, urine, and sera when TRF was used compared to standard capture ELISA (Peruski et al. 2002). While the overall sensitivity was not impacted by sewage water and urine, capture efficiency was decreased by soil and serum. TRF for the study showed an overall lower LOD of approximately 48 CFU/mL and therefore might provide detection of low

concentrations of *F. tularensis* within environmental samples (Peruski et al. 2002).

The antibody immuno columns for analytical process (ABICAP) test is an immunoaffinity chromatographic column test that includes ELISA detection chemistry within a hand-held single use field-deployable device; it has been shown to have an LOD comparable to capture ELISA (Grunow et al. 2008). An LOD of 10^4 CFU/mL has been noted for spiked silt loam samples processed through glass fiber filters analyzed via capture ELISA (Sellek et al. 2008). In another study, the ABICAP classic test kit was able to detect *F. tularensis* subspecies *tularensis* and *F. tularensis* subspecies *holarctica* in the test kit buffer at a concentration of 10^4 cells/mL, although cross-reactivity was noted with other bacterial species (Zasada et al. 2015). When using the ABICAP to extract bacterial LPS from environmental waters (125 µL) containing various amounts of dissolved soil and from rabbit and mouse fecal matter during a Swedish tularemia outbreak, false positive test results and increasing background signal (with increased concentration of mud from the initial water samples) were noted (Grunow et al. 2008). Even with highly specific ELISA techniques, other challenges have been noted when testing environmental samples. For example, during two tularemia outbreaks in Kosovo involving *F. tularensis* LPS contamination in food storage areas contaminated with rodent feces and in water sources, only fecal samples yielded positive capture ELISA results (Grunow et al. 2012). Two lateral flow ELISA kits, the Smart II test and the BioThreat Alert test, were able to detect *F. tularensis* in phosphate buffered saline (PBS) only at concentrations of 10^7 cells/mL and 10^8 cells/mL respectively, thus having less sensitivity for detection of *F. tularensis* than the ABICAP test kit that was also evaluated (Zasada et al. 2015).

Development of new technologies to incorporate immunoassay detection chemistry are underway. A protein chip has been developed by Huelseweh et al. (2006) to simultaneously and rapidly detect two to five bioagents at similar LODs as ELISA, but faster than ELISA. Individual affinities to the antibodies, however, affect the overall quality of the immunoarray. A prototype biosensor was developed recently by Cooper et al. (2011b) that includes detection of *F. tularensis* via label-free, specific antibody and single-stranded oligonucleotides. A piezoelectric immunosensor was developed by Pohanka and Skládal (2007) to allow direct detection of *F. tularensis* in drinking water and milk samples with a LOD of 10^5 CFU/mL for both matrices. In an effort to develop automated biodefense systems, the utility of a bidiffractive grating biosensor has been explored as a potential field deployable system (O'Brien et al. 2000). A novel competitive ELISA for clinical identification of *F. tularensis* might have the potential to be applicable to environmental samples (Sharma et al. 2013).

Genomic identification of *F. tularensis*

Multiple studies identified by this review have shown that PCR identification is faster and more sensitive than culture or immunoassay (Anda et al. 2001; Versage et al. 2003; Sellek et al. 2008; Berrada and Telford 2010; Meric et al. 2010; Simsek et al. 2012). These assays also have limitations. A summary of studies that utilized genomic analysis to identify *F. tularensis* within environmental samples including use of DNA extraction kits and PCR identification is summarized below.

Extraction of *F. tularensis* DNA

Soil and water samples contain humic acids and other inhibitory compounds that might be coextracted with bacterial DNA and could confound downstream PCR reactions, thus requiring cleanup of extracts prior to analysis (Robe et al. 2003). In order for DNA extraction to be efficient, an unbiased yield of quality DNA that can be used for downstream analysis is needed. From a single sample, long-DNA segments from diverse species are needed in sufficient concentrations (Gillings 2014). Table 1 illustrates the DNA extraction kits that were found through this literature review for *F. tularensis* DNA extraction.

Isolation of *F. tularensis* DNA from silt loam, clay, and commercial potting soil was conducted using a comparison of five commercial DNA recovery kits (Whitehouse and Hottel 2007). The lowest and most consistent LOD of the kits tested were reported by the UltraClean® Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) and the PowerMax® Soil DNA Isolation kit (MoBio Laboratories), which had LODs of 20 CFU/g soil and 100 CFU/g soil, respectively (Whitehouse and Hottel 2007). The pure culture *F. tularensis* extraction (positive control) for the same study had an LOD of 10 CFU/mL (Whitehouse and Hottel 2007). Another study compared isolation of *Salmonella enterica* (non-sporulating Gram-negative bacteria) DNA from soil, manure, and compost samples using five commercial DNA recovery kits (Klerks et al. 2006). The kits that yielded the highest quality and quantity of DNA from the tested samples were the UltraClean® Soil DNA Isolation kit (MoBio Laboratories), the UltraClean® Fecal DNA Isolation kits (MoBio Laboratories), and the Bio101 extraction kit (Q-Biogene, Carlsbad, CA) (Klerks et al. 2006). The remaining kits tested, the QIAGEN Plant DNeasy™ DNA (QIAGEN, Westburg, The Netherlands) extraction kit and the Soilmaster™ DNA extraction kit (Epicentere, Madison, WI), were not found to be optimal for *S. enterica* from soil samples during that study (Klerks et al. 2006). However, the Soilmaster™ kit was used successfully by Broman et al. (2011) during two reoccurring tularemia outbreaks in Sweden to identify the presence of *F. tularensis* within 32 %

of the surface water samples and 20 % of the sediment samples collected.

Utilizing the UltraClean® Soil DNA Isolation kit and a modified kit protocol (the bead-beating time was reduced from 10 min to 5 min to reduce DNA shearing) (Berrada and Telford 2010), mud, soil, and sediment samples collected on Martha's Vineyard were analyzed for *Francisella* spp. by Berrada and Telford (2010). After DNA extraction, they were able to identify PCR positives for specific primers (*Francisella* spp. 16 svedberg units [S] ribosomal ribonucleic acid [rRNA] primers [16S rRNA]) in four brackish-water soil/sediment samples and three samples positive for *F. tularensis* specific sequences (Berrada and Telford 2010). In October 2003, 364 water and soil samples were collected around the Houston area of Texas and were analyzed for *Francisella* species and relatives following BioWatch (a federal bioagent release detection technology program) aerosol samples being positive for *F. tularensis* (Barns et al. 2005). DNA was extracted using the UltraClean® Soil DNA Isolation kit and samples were analyzed by 16S rRNA sequencing (Barns et al. 2005). The results indicated the presence of *F. philomiragia* in one water sample and the presence of new subspecies of *F. tularensis* with unknown pathogenicity in seven soil samples (Barns et al. 2005).

Rather than identifying an optimum extraction kit, Trombley Hall et al. (2013) investigated the use of recognized inhibitor-resistant PCR reagents to purify nucleic acids and to remove inhibiting constituents from environmental samples. The need for sample-specific preparation was eliminated and the sensitivity of real-time PCR increased through the use of inhibitor-resistant PCR reagents (Trombley Hall et al. 2013). The KAPA Blood PCR Kit (KAPA Biosystems, Wilmington, MA) gave the most consistent LOD among the five PCR chemistries and matrices (buffer, soil, sand, swab, sputum, whole blood, and stool) investigated (Trombley Hall et al. 2013). It was determined that no single chemistry performed well across all the matrices tested. When the PCR reaction was composed of 0.05 % soil, a LOD of 0.2 picograms (pg; ~103 genomic equivalents) *F. tularensis* DNA, was yielded for the KAPA Blood PCR Kit, Ampdirect® buffer (Rockland Immunochemicals, Gilbertsville, PA) with Phire® Hot Start DNA Polymerase (Finnzymes/New England Biolabs, Ipswich, MA), and STRboost™ buffers (Clontech Laboratories, Mountain View, CA) with Phire® Hot Start DNA Polymerase (Trombley Hall et al. 2013).

PCR amplification for genomic identification of *F. tularensis*

In recent years, progress has been made in PCR identification of *F. tularensis*. Initial identification methods for *F. tularensis* within environmental waters by PCR amplification were conducted by either manual DNA extraction followed by genus-specific *Francisella* PCR amplification (Forsman et al. 1995),

Table 1 Studies that investigated commercial DNA kits for *Francisella tularensis* DNA extraction from soil and water sample found in the literature. *LOD* Limit of detection, *CFU* colony forming units

Reference	Kit	Organism	Matrix	Sample size and prep method prior to DNA extraction	Detection method	LOD
Barns et al. 2005	UltraClean® Soil DNA Isolation Kit	*Francisella tularensis*	Surface soil / Grab water sample	0.25 g soil directly processed / 50 mL water samples were centrifuged to pellet cells before DNA extraction	PCR detection: *F. tualrensis* specific 16 s rRNA, IS*Ftu2*, *tul14*, *fopA*, *23 kDa*	ND[a] / ND
Berrada and Telford 2010	UltraClean® Soil DNA Isolation Kit	Targeted *F. tularensis*, but samples naturally contained *F. philomiragia*	Surface soil, sand, and sediments / Water from marsh, brackish water pond, and freshwater pond	0.25 to 0.5 g processed directly / 100–300 mL samples centrifuged before filtering. Filter wash collected for culture or DNA extraction	PCR: *shdA*, IS*Ftu2*, *tul14*, *fopA*,	ND
Broman et al. 2011	SoilMaster® DNA Extraction Kit	*F. tularensis* subspecies *holarctica*	Sediment / Surface water	2.0 mL of soil centrifuged / 2.0 mL of water centrifuged	Real-time PCR detecting *lpnA* and FtM19 internal deletion region	LOD of PCR *lpnA* assay is 10³ bacteria/mL in water, but no LOD reported for DNA kit
Escudero et al. 2008	QIAamp DNA Blood Extraction Kit	*F. tularensis* subspecies *tularensis*, *holarctica*, or *F. novicida*	Clinical tissue samples	Direct extraction	PCR detecting *lpnA* followed by hybridization to various probes for subspecies differentiation	ND
Francy et al. 2009	Powersoil® DNA I solation kit	*F. tularensis* LVS, *Bacillus anthracis* Sterne, *Salmonella typhi*, *Vibrio cholerae*, *Cryptosporidium parvum*	Raw water and drinking water	Ultrafiltration retentate filtered through 0.4 μm polycarbonate filters which were placed into extraction tubes	Real-time PCR targeting *fopA* and *tul4* and culture	ND
Fujita et al. 2006	SepaGene DNA Extraction Kit	*F. tularensis* subspecies *tularensis*, *holarctica*, *philomiragia*, *F. novicida*	Pure cultures	Genomic DNA from pure cultures was manually extracted or extracted using the SepaGene DNA extraction Kit	Real-time PCR detecting *fopA* gene	1.2 CFU or 10 copies of the *fopA* gene
Matero et al. 2011	MagNA Pure Nucleic Acid Isolation Kit 1	*F. tularensis*, *Bacillus thuringiensis*, *B. anthracis*, *Yersinia pestis*, *Brucella* spp.	Pure culture	Direct extraction	PCR targeting *23 kDa* gene	10 fg for both the RAZOR to ABI instrumentation
Meric et al. 2010	QIAamp DNA mini Kit	*F. tularensis*	Reservoir spring water	1 L samples filtered with cellulose acetate filters. Filters washed with sterile distilled water before filtrate was cultured or DNA extracted	Culture and real-time PCR targeting: IS*Ftu2* element, *23 kDa* gene, and the *tul4* gene	ND
O'Connell et al. 2004	DNeasy mini spin columns	*F. tularensis* subspecies *holarctica* LVS	Creamer, cornstarch, baking powder, flour	DNA from pure cultures of *F. tularensis* extracted with columns	Direct PCR in Bio-Seeq® handheld system	10³ cells/ reaction or less when the consumable

Table 1 (continued)

Reference	Kit	Organism	Matrix	Sample size and prep method prior to DNA extraction	Detection method	LOD
						sampling assembly is utilized
Simsek et al. 2012	QIAamp DNA mini Kit	F. tularensis subspecies holarctica LVS	Environmental water	0.3–1.5 L samples filtered through cellulose acetate membranes. Membranes placed directly on CHAB or washed with sterile water before DNA extraction	Culture and real-time PCR targeting ISFtu2 gene	ND
Versage et al. 2003	MasterPure™ Purification Kit	F. tularensis subspecies tularensis, holarctica, philomiragia, F. novicida	Pure cultures isolated from tissued of infected animals	Tissue samples directly cultured or DNA extracted using kit	Multitarget PCR targeting tul4, fopA, ISFtu2, 23 kDa compared to culture	ND
Whitehouse and Hottel 2007	Gentra Puregene® Yeast/Bacteria Kit	F. tularensis	Silt loam, clay, potting soil	1 g soil processed through kit	PCR targeting fopA gene	200 CFU/g for potting soil, 2000 CFU/g for silt loam, and 20,000 CFU/g for clay
	PowerMax® Soil DNA Isolation Kit		Silt loam, clay, potting soil	10 g soil processed through kit		100 CFU/g for all matrices tested
	QIAamp DNA Stool Mini Kit		Silt loam, clay, potting soil	0.5 g soil processed through kit		500 CFU/g for all matrices tested
	SoilMaster® DNA Extraction Kit		Silt loam, clay, potting soil, sand, garden soil	0.1 g soil processed through kit		Silt loam: 100 CFU/g, Clay and potting soil: 1000 CFU/g, Sand: 10^6 CFU/g
	UltraClean® Soil DNA Isolation Kit		Silt loam, clay, potting soil	1 g soil processed through kit		20 CFU/g for all matrices tested

[a] Not detected

or restriction enzyme analysis (Berdal et al. 2000) and visual gel electrophoresis detection. In more recent years, commercial sample extraction kits (Whitehouse and Hottel 2007) and rapid real-time PCR analysis allow for sensitive detection at low concentrations (Fujita et al. 2006). Genomic identification studies have commonly targeted the genes *fopA*, *tul4*, IS*Ftu2*, and *23 kDa*. Outer membrane proteins include the *fopA* and *tul4* genes (Versage et al. 2003), which encode a 43-kDa protein (Berrada and Telford 2010) and a 17-kDa protein (Francy et al. 2009), respectively. IS*Ftu2* targets an insertion element-like sequence in *F. tularensis* (Barns et al. 2005). The *23 kDa* gene encodes a protein that is expressed during macrophage infection (Versage et al. 2003).

PCR analysis (using 16S rRNA primers) was used to determine the natural presence of *F. tularensis* among 15,000 aerosol samples and 89 soil samples collected from 15 major US cities (Kuske et al. 2006). Results indicated that *F. tularensis* or its near relatives are naturally present in urban aerosols but the study did not find the organism within the studied soils (Kuske et al. 2006). Diverse *Francisella* spp. have been identified via PCR analysis of environmental samples from Martha's Vineyard following a natural tularemia outbreak (Berrada and Telford 2010). Out of 156 samples analyzed, 23 were positive for *F. tularensis* 16S rRNA, 19 positive for IS*Ftu2*, 15 were positive for *fopA*, 14 were positive for *tul4* and one *fopA* PCR positive sample yielded a culture of *F. philomiragia*. Meric et al. (2010) targeted IS*Ftu2*, *23 kDa*, and *tul4* genes during PCR analysis of reservoir spring water samples linked to a tularemia outbreak in Turkey. Fujita et al. (2006) established a sensitive and specific real-time PCR assay for rapid detection of *F. tularensis* within a prepared DNA sample that targeted *fopA* and has an LOD equivalent to 1.2 CFU bacterial cells/reaction.

Molecular methods have been developed to discriminate between *Francisella*-like organisms and *F. tularensis*. One study was able to develop a genomic method for differentiating between *F. tularensis* and *Francisella*-like organisms by recognition of a 36-bp deletion in *lpnA* sequences within *F. tularensis* subspecies (Escudero et al. 2008). During a comparison of three molecular methods for separating *F. tularensis* strains [amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), and 16S rRNA gene sequencing], PFGE and AFLP were able to distinguish *F. tularensis* subspecies, which could be useful for epidemiological tracking during a tularemia event (Garcia Del Blanco et al. 2002). PCR assays for hierarchical identification of *Francisella* isolates have been developed by Duncan et al. (2013) and Svensson et al. (2009). In order to differentiate various *F. tularensis* subspecies, Duncan et al. (2013) utilized 24 multilocus PCR reactions followed by electrospray ionization/time of flight mass spectrometry (ESI-MS) detection. Svensson et al. (2009) generated a hierarchical identification system using 68 individual real-time PCR reactions by

utilizing specific deletions and insertions within the *F. tularensis* genome. The utility of both of these studies would be most useful for tracking analysis in a tularemia outbreak situation.

Many primers previously developed for *F. tularensis* yield false positives due to the extremely low specificity (Ahlinder et al. 2012). Identification of specific species or subspecies can be challenging. In an outbreak study, it was reported that real-time PCR assays incorrectly identified *F. tularensis* subspecies *novicida* and *F. tularensis* (Brett et al. 2014). While this finding is not surprising owing to the very similar genetic make-up of these two species, it does point to the need for thorough characterization of isolates that share close sequence identity. Primer sequences need to be continually evaluated and redesigned using up-to-date genomic databases in order to mitigate false positive PCR results. Furthermore, to improve *Francisella* strain resolution, an optimized combination of markers could be used (Ahlinder et al. 2012). When PCR was used to target the *tul4* gene for identification of *F. tularensis* Schu S4A during a study by Bader et al. (2003), a higher number of false positive and false negative identifications were reported for soil sample unknowns than for liquid sample unknowns.

PCR master mixes and PCR thermocycler instruments do not all function equally. The LOD for the *F. tularensis* *23 kDa* gene was found to be the same [10 f. genomic DNA (or 5 genomic equivalents) per reaction] for the RAZOR (Idaho Technology, Salt Lake City, UT) and ABI 7300/7500 (Applied Biosystems, Foster City, CA) real-time PCR thermocyclers during a comparison study (Matero et al. 2011). However when Buzard et al. (2012) compared three real-time PCR instruments and ten commercially available PCR master mixes, all ten master mixes tested yielded positive results for *F. tularensis* on the 7500 Fast Dx (Applied Biosystems) and SmartCycler (Cepheid, Sunnyvale, CA) instruments, but only seven were positive on the LightCycler (Roche, Indianapolis, IN) instrument.

Methods for environmental sampling and detection of multiple biothreat organisms

Genomic techniques to detect pathogenic organisms alone or with other organisms are constantly being developed. Rugged, sensitive, specific, and easily manipulated field-deployable detection systems are needed for first responders. Three technologies discussed in the literature that could potentially be utilized by first responders included the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.), Bio-Seeq®, and FilmArray® systems. R.A.P.I.D. is a real-time PCR platform that is field-deployable and for which *F. tularensis*-specific primers have been established but not evaluated with an LOD of 10 fg DNA (McAvin et al. 2004). The Bio-Seeq® instrument is a novel, portable, handheld, and

self-contained real-time PCR system that includes a consumable sampling and reaction tube assembly (sampling swab, buffer, and assay reagents) and has an LOD for *F. tularensis* detection of 10^3 cells per reaction (O'Connell et al. 2004). However, although *F. tularensis* was detectable when spiked into cornstarch, wheat flour, coffee creamer, and baking soda, inhibition was noted and could also be an issue for environmental soils or waters (O'Connell et al. 2004). The FilmArray® is a newly developed system which utilizes a "Lab-in-a-Pouch" approach for conducting liquid sample-to-answer detection of 17 biothreat agents, but has only been demonstrated as proof of concept for *F. tularensis* genomic DNA, *Bacillus anthracis* cells and spores, and *Yersinia pestis* cells (Seiner et al. 2013). Additional research on detection systems for environmental waters and soils that are field deployable is needed.

Multiplex qPCR detection methods allow simultaneous amplification of several DNA targets and could save both time and resources during a remediation event. A multiplex qPCR for simultaneous detection of three genes of *F. tularensis* (*fopA*, IS*Ftu2*, *pdpD*) and use of an internal positive control (*Bacillus thuringiensis* spores) for both nucleic acid extraction and amplification was developed by Janse et al. (2010) to reduce false positive and false negative results. The authors have utilized the method for hundreds of solid and liquid samples, but the method has not been verified specifically with soils (Janse et al. 2010). Janse et al. (2012) have also developed protocol for simultaneous detection of four biothreat agents. The multiplex asymmetric PCR protocol amplifies 16 DNA signatures and targets 4 gene signatures from *F. tularensis*, *Y. pestis*, and *Coxiella burnetii*; three signatures from *B. anthracis,* and a single signature for the internal positive control (*B. thuringiensis*; Janse et al. 2012). Standard multiplex platforms are unable to differentiate the PCR products due to the number of amplified signatures. Therefore, Janse et al. (2012) also compared two labeling chemistries for microarray detection: (1) target-specific primer extension followed by universal hybridization, which incorporates a unique capture tag sequence during strand extension by DNA polymerase; and (2) direct hybridization in which labeled PCR products are generated using in-house labeled primers in the multiplex PCR. Multiple pathogens could be detected simultaneously with high sensitivity and specificity using both microarray formats, and both formats had an LOD of 12 copies/reaction when targeting the internal spacer region, IS*Ftu2* for *F. tularensis* at 4.1 amplicons (Janse et al. 2012).

There is a trade-off with being able to minimize LOD and being able to detect multiple organisms simultaneously. While the TaqMan® Array Card, which incorporates ten PCR reactions for five agents (*Bacilllus anthracis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *F. tularensis* and *Y. pestis*) is capable of detecting all five target organisms, its LOD has been shown to be one order of magnitude greater than singleplex qPCR using pure genomic DNA (Rachwal et al. 2012). Brinkman et al. (2013) developed a microarray-based method for simultaneously detecting *B. anthracis*, *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Enterococcus faecium,* and *F. tularensis* in concentrated tap water samples in an attempt to minimize LODs while still achieving identification of multiple pathogens. While the assay developed by Brinkman et al. (2013) was capable of detecting *F. tularensis* genomic DNA at 20 genomic copies without PCR preamplification, the method has not been verified with soil samples.

Other research has focused on the detection of multiple pathogens in a single assay. A multiplexed PCR and sequencing assay for simultaneous detection of three pathogens (ten loci per pathogen) has been developed using a microfluidic biochip (Turingan et al. 2013). The Luminex® liquid array platform system can achieve LODs of 0.1 to 10 ng DNA and uses genetically marked beads to simultaneously identify multiple pathogenic microorganisms (Schweighardt et al. 2014). A protocol has been developed by Schweighardt et al. (2014) using the Luminex® system to detect *B. anthracis*, *Clostridium botulinum*, *Y. pestis*, and *F. tularensis.* A multi-targeted liquid array method for simultaneously detecting *Bacillus anthracis*, *Burkholderia pseudomallei*, *Brucella* spp., *F. tularensis*, and *Y. pestis* within a simulated white-powder sample has been assessed by Yang et al. (2012). In the latter study, universal 16S rRNA primers were used for amplification and pathogen-specific hybridization probes were used for identification (Yang et al. 2012).

Based on results of spiking *B. anthracis* and *Y. pestis* into various household white powders (milk powder, corn starch, wheat flour, instant drink mix) the Bio-Plex assay is another liquid array method that could have the potential to detect pathogens of interest from environmental samples (Yang et al. 2012). Methods for simultaneous detection of multiple biothreat agents in clinical samples have included a multiplex PCR enzyme hybridization assay (mPCR-EHA) (He et al. 2009) and high-throughput reverse transcription-PCR coupled to electrospray ionization mass spectrometry analysis (RT-PCR-ESI-MS) (Jeng et al. 2013). Another potential field deployable method promising quick results (~10 min) is the qualitative real-time isothermal recombinase polymerase amplification assay for *F. tularensis* alone (Euler et al. 2012) or in combination with *B. anthracis*, *Y. pestis*, and variola virus (Euler et al. 2013).

A prototype photonic biosensor which utilizes label-free single-stranded oligonucleotides without PCR amplification has been used to develop an assay for detection of *F. tularensis* in low concentrations (tested 1.7 ng) from aqueous samples (Cooper et al. 2011). Optimization is needed for field use, but the method shows promise as a tool that can rapidly detect *F. tularensis* in the field or laboratory facilities (Cooper et al. 2011). While each of these technologies are

promising, efficacy needs to be assessed using complex environmental matrices.

Combining culture with PCR to detect live *F. tularensis*

While PCR techniques are not able to discriminate between viable and non-viable target microorganisms, the combination of culture with PCR has been used to improve rapid detection of viable cells from various matrices. Use of culture prior to PCR can help increase the concentration of DNA in the sample due to growth of viable organisms present in the sample, and therefore improve recovery. For example, Day and Whiting (2009) have established a procedure to detect *F. tularensis* from contaminated foods (liquid baby formula, liquid egg whites, and iceberg lettuce mixed 1:1 with PBS) using mammalian macrophage cell cultures. The macrophage cell cultures engulf *F. tularensis*, are washed with PBS, reconstituted with macrophage growth medium, and incubated to allow for propagation of the engulfed *F. tularensis* within the macrophages. A supernatant is created by scraping macrophage monolayers from the plates, and cleaning and boiling them to lyse the cells; the supernatant is then used directly for real-time PCR analysis (Day and Whiting 2009). The method has an LOD of 10 CFU/mL for formula or egg whites and 10 CFU/g for lettuce (Day and Whiting 2009).

Rapid viability PCR (RV-PCR) is a technique that has been used to detect the presence or absence of viable *B. anthracis* spores from water, dust, and dirty air filters. RV-PCR combines a growth medium enrichment step (broth culture) and the calculation of the change in cycle threshold time of two real-time PCR reactions measured on aliquots taken at time zero and after a 9 h incubation (Kane et al. 2009; Letant et al. 2011). A change in cycle threshold that is greater than nine indicates that the spore concentration in the sample has increased in the 9 h aliquot compared to the time zero aliquot. Recently, Lamont et al. (2014) developed a combined enrichment protocol for detection of *F. tularensis* subsp. *holarctica* LVS NR14 in soil and lettuce matrices. The protocol included adding spent culture filtrate to standard medium (TSA containing 0.1 % L-cysteine) to increase growth during overnight incubation as well as using a DNA aptamer cocktail (including M-280 streptavidin beads) to capture and separate *F. tularensis* from other bacteria in the matrices (Lamont et al. 2014). Real time-PCR was used on spiked samples targeting the *fopA* gene. The method proposed that detection for all spike inoculums evaluated (1–10^6 CFU/mL) are possible using this combined enrichment method.

Culturing prior to PCR might show promise for detection of low concentrations of viable *F. tularensis*, however additional work is needed to determine the capabilities for environmental samples.

Summary and identified data gaps

This review found limited research pertaining to *F. tularensis* detection in soil. More research has been conducted on the detection of *F. tularensis* in environmental waters compared to soil matrices. However, additional information is needed pertaining to the complete lifecycle of *F. tularensis* in the environment. For example, the role that protozoa and biofilms play in *F. tularensis* persistence must be elucidated in order to determine which detection technologies would be most appropriate for targeting the specific *F. tularensis* in environmental samples and microenvironments. While isolation of viable *F. tularensis* from environmental samples would be ideal, the slow-growing, nutritionally fastidious nature of *F. tularensis* makes culturing the bacteria from environmental samples challenging. Background organisms in environmental samples often out-compete *F. tularensis* colonies, even if selective agars are used.

Studies have utilized culture analyses with varying success. Other methods that have been developed for identification of *F. tularensis* from environmental samples include genomic methods and immunoassays. Immunoassay techniques in the literature included either single reaction immunoassays or immunoassays as part of an immunoarray chip; however, both have high LODs. Immunoassay quality is dependent upon the selected antigen specificity and potential cross-reactivity with other microorganisms. New immunosensor assays being developed might provide alternative methods for environmental samples once optimized.

The most common identification method found in the literature was genomic identification. The literature listed the *tul4*, *fopA*, IS*Ftu2*, and *23 kDa* repeatedly as genes used to identify *F. tularensis*. With genomic analysis, the methods used to collect and purify samples, and the PCR primers used can influence the results. DNA extraction kits have been used to remove inhibiting constituents within soil and environmental waters prior to PCR analysis to increase processing efficiency. UltraClean® DNA extraction kits were the extraction kits most commonly mentioned in the literature. DNA extraction from environmental soils could be complicated by aggregation of cells with other constituents in the soil. Use of inhibitor-resistant PCR reagents is a new technique to prevent inhibition in PCR reactions; however, more research is needed to compare various extraction kits, inhibitor-resistant PCR reagents, and soil types to identify an optimum extraction procedure and increase sensitivity of qPCR reactions.

High-throughput detection of multiple biothreat agents of interest from environmental samples might be improved by microarray detection technologies. Sensitivity could be further improved by the use of whole genome amplification prior to microarray detection. Biothreat detection capabilities for environmental soil and water samples could be improved. The optimization of DNA extraction, whole genome amplification

with inhibition-resistant polymerases, and multiagent micro-array detection would advance biothreat detection. In addition, further work to integrate culture and genomic identification would advance our ability to detect *Francisella* spp. and to assess its viability.

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