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Literature Review on Processing and Analytical Methods for *Francisella tularensis* in Soil and Water

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Executive Summary

Identifying pathogenic organisms within a soil sample can be a challenge, yet an understanding of the environmental distribution of bacterial pathogens and their fate over time is needed for multiple applications. *Francisella tularensis,* the etiological agent of tularemia in humans and animals, can be acquired by handling infected carcasses, ingesting contaminated food or water, from an infected arthropod bite, or inhaling infectious soil dust or aerosols[1]. *F. tularensis* is listed as a Category A agent by the Centers for Disease Control and Prevention (CDC) due to its extremely low infectious dose for humans[2] so identifying *F. tularensis* within an environmental soil or water matrix is a priority for protecting both human and animal lives. *F. tularensis* is widely distributed in the environment and has been isolated from nearly 250 wildlife species, ranging from mammals, invertebrates, birds, amphibians, and fish[3]. It is an environmentally hardy organism and can survive for weeks at low temperatures in water, moist soil, hay, straw, or decaying animal carcasses[4].

The purpose of this report was to survey the open literature to determine the current state of the science regarding the processing and analytical methods currently available for recovery of *F. tularensis* from water and soil matrices, and to determine what gaps remain in the collective knowledge concerning *F. tularensis* identification from environmental samples. Information for this review came from unclassified reports, peer-reviewed journal articles, published books, and government publications published in the last twenty years. The search was limited to articles published in the English language, but no restrictions were placed on the geographic focus of the documents.

The search identified three broad mechanisms of *F. tularensis* detection within environmental samples: culture analysis, immunoassays, and genomic identification. Isolating environmental cultures of *F. tularensis* is challenging as it is a slow-growing, nutritionally fastidious organism requiring 24 to 72 hours for growth[5] on supplemented media[6]. Even with antibiotic amended media, colonies are often out-competed by background organisms present in environmental samples. Antibiotic supplemented cysteine heart agar with blood (CHAB) was frequently cited in the literature to culture *F. tularensis* from environmental samples. While CHAB, or modified forms of CHAB, have been used to detect virulent *F. tularensis* from within environmental samples, the process is long, labor intensive, and rarely yields positive isolates. There were

[1] Fujita et al., 2006. Jpn J Infect Dis 59:46-51.
[2] Cooper et al., 2011. Sensors 11:3004-19.
[3] Broman et al., 2011. Int J Microbiol 2011.
[4] Dennis et al., 2001. JAMA 285:2763-73.
[5] Versage et al., 2003. J Clin Microbiol 41:5492-5499.
[6] van Hoek, M. L. 2013. Virulence 4:833-46.

multiple instances described in the literature where *F. tularensis* was identified by molecular assays, but culture identification was limited in the laboratory setting.

Immunoassay detection of *F. tularensis* can be amenable to hand-held devices or multiagent identification procedures; however, due to high limits of detection, the utility of these immunoassay detection applications might only be seen in highly concentrated samples[7]. The overall applicability of immunoassays are dependent upon the specificity of the selected antigens. Some antigens can have cross-reactivity to other microorganism, thus impeding the results.

Genomic identification of *F. tularensis* from environmental samples can rapidly yield detection results. However, it must be noted that molecular identification of *F. tularensis* does not necessarily indicate the presence of viable *F. tularensis* cultures. *F. tularensis* is a non-sporulating Gram-negative organism; therefore, its deoxyribonucleic acid (DNA) can be extracted for identification rather easily when compared to sporulated microorganisms. Yet, inhibitory chemical constituents within environmental samples are often coextracted and lead to confounding downstream polymerase chain reaction (PCR) responses. Therefore, special care must be taken to efficiently clean environmental DNA extracts prior to downstream analysis. A comparison of multiple commercial DNA recovery kits for isolating *F. tularensis* DNA from within various soil types highlighted the efficiency of the UltraClean® Microbial DNA Isolation kit and the PowerMax® Soil DNA Isolation kit, both products of MoBio Laboratories, Inc., Carlsbad, CA[8]. The UltraClean® Soil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) was the recovery kit most commonly used within this literature search. One study comparing two kits that used different amounts of the initial sample concluded that for samples of unknown biological agents it is preferable to extract DNA from as much of the original sample volume as possible[9].

Direct genomic DNA extraction was not the only method for sample preparation found within the literature. Sellek et al.[10] developed a filtration method for processing soil samples that allowed for both genomic analysis and immunologic analysis of the extracted sample with limited efficiency. Trombley Hall et al.[11] focused on finding PCR reagents with inhibitor-resistant capabilities. Use of inhibitor-resistant PCR reagents could eliminate the need for sample-specific sample preparation and increase the sensitivity of downstream real-time PCR.

Multiple studies within this review demonstrated the capability of one assay to identify multiple biothreat agents from a single sample. However, these studies also noted a trade-off between achieving multiple organism detection and producing a minimized limit of detection (LOD).

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[7] Huelseweh et al., 2006. Proteomics 6:2972-81.
[8] Whitehouse and Hottel. 2007. Mol Cell Probes 21:92-6.
[9] Offermans and Zegers. 2007. Test Results 7th NATO-SIBCRA BW Round Robin Trial 2006. TD2007-0043.
[10] Sellek et al., 2008. J Environ Monit 10:362-9.
[11] Trombley Hall et al., 2013. PLoS One 8:e73845.

While each of the multiagent technologies is promising, efficacy data from environmental assessments with complex matrices are lacking. Data from a broader range of complex environments are needed to enable evaluation of the usefulness of the approach.

Two studies included in this review combined culture and genomic analysis to rapidly quantify viable microorganisms. Using macrophage cell cultures to accelerate *F. tularensis* growth before DNA extraction and amplification, Day and Whiting[12] were able to detect viable *F. tularensis* in contaminated foods at a LOD of 10 colony forming units (CFU)/ milliliter (mL). Rapid viability-PCR (RV-PCR) is another promising technique that utilizes an enrichment step and the change in cycle threshold time between two PCR reactions to determine the presence or absence of viable cells[13]. While RV-PCR has not been optimized for *F. tularensis* detection, it has been shown to be effective for *Bacillus anthracis* spore detection in environmental samples. Future work incorporating a macrophage culture step with RV-PCR sample processes could significantly improve viable *F. tularensis* detection capabilities in environmental soil and waters.

Other areas for future work could include a combined comparison of multiple soils with various extraction kits and various inhibitor-resistant PCR reagents. Such an analysis would identify both an optimum extraction kit and optimum PCR reagents to yield real-time PCR reactions with increased sensitivity. Microarray detection technologies could be the future of high-throughput environmental detection of multiple biothreat agents of interest. The introduction of whole genome amplification prior to microarray detection might further improve sensitivity[14]. Future work combining the use of internal controls for each analytical step, optimized DNA extraction, whole genome amplification with inhibition-resistant polymerases, and multiagent microarray detection could significantly expand the detection capabilities of *F. tularensis* in soil and water.

[12]Day and Whiting, 2009. J Food Protect 72:1156-1164.
[13] Létant et al., 2011. Appl Environ Microbiol 77:6570-8.
[14]Brinkman et al., 2013. J Appl Microbiol 114:564-73.

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List of Acronyms and Abbreviations

°C	Degrees Celsius
µm	Micrometer
ABICAP	Antibody immuno columns for analytical process
AFLP	Amplified fragment length polymorphism
BAL	Bronchoalveolar lavage
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CHAB	Cysteine heart agar with blood
CHAB-A	CHAB agar supplemented with colistin, amphotericin, lincomycin, trimethoprim, and ampicillin
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ESI-MS	Electrospray ionization/time of flight mass spectrometry
fg	Femtogram
g	Gravitational force
g	Grams
GE	Genomic equivalents
kDa	Kilo Daltons
L	Liter
LOD	Limit of detection
LPS	Lipopolysaccharide
LVS	Live vaccine strain
Min	Minutes
mL	Milliliter
mPCR-EHA	multiplex PCR enzyme hybridization assay
ng	Nanogram
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pg	Picograms
qPCR	Quantitative PCR
R.A.P.I.D.®	Ruggedized Advanced Pathogen Identification Device
RI-test	Rapid immunochromatographic-test
RPA	Recombinase polymerase amplification
rRNA	Ribosomal RNA
RT-PCR-ESI-MS	Reverse transcription-PCR coupled to electrospray ionization mass spectrometry
RV-PCR	Rapid viability-PCR

S	Svedberg units
SETS	Swab extraction tube system
TRF	Time-resolved fluorescence

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1 Introduction

Francisella tularensis is the etiological agent of tularemia, “rabbit fever,” in humans and numerous wild animals. Humans can acquire the disease by handling infected carcasses, ingesting contaminated food or water, being infected by an infected arthropod bite, or inhaling infectious soil dust or aerosols [1]. *F. tularensis* subspecies *tularensis* (type A) is virulent and highly infectious, with an infective dose as little as 10-50 organisms for humans [2]. Therefore, due to its ease of transmission, potential for substantial morbidity and mortality to large numbers of people, and its capability to induce widespread panic, *F. tularensis* is listed as a Category A select agent by the U.S. Centers for Disease Control and Prevention (CDC) [2]. Identifying *F. tularensis* within a soil matrix and in surface, ground, and drinking water is a priority for protecting both human and animal lives. There have been multiple studies dealing with clinical samples. Due to the fastidious nature of the organism and the complexity of environmental isolation there has been little work on identifying the organism from within soil samples. This report is a compilation of soil and water sampling and processing information for microbial detection acquired from research conducted within the last two decades, and describes research gaps within the available literature.

Soil, in particular, is a complex matrix characterized by distinguishable layers, some of which are capable of supporting rooted plants [3]. The overall properties of a soil fluctuate with time due to changing weather patterns and plant growth cycles. For this reason, pH, soluble salts, organic mass, flora, fauna, temperature, moisture, and the number and types of microorganisms all change with the seasons and over extended periods of time [3]. Some naturally occurring organisms can be pathogenic to animals and humans. Appropriate sampling methods for soil are thus needed to help determine where, how, and to what extent soils might have been contaminated following a tularemia event.

Water supplies are at risk of biological contamination through either natural or illicit means. A tainted water source creates a significant disruption to society [4]. Drinking water might be contaminated at the original source, during treatment, within distribution plumbing, or in distribution containers [5]. Analytical methods for early detection of waterborne pathogens within a variety of aqueous matrices (surface, ground, or drinking waters) are needed to help maintain water security.

Identifying pathogenic organisms within an environmental soil or water sample can be a challenging task. Direct culture of some bacteria can be difficult due to particular growth requirements, extensive growing times, and potential risk to laboratory workers when an organism is highly virulent. Identification can be impeded by chemical constituents within the soil or water that can interfere with the chemistry involved in downstream molecular detection methods [6-8]. An understanding of the environmental distribution of bacterial pathogens and their fate over time in nature is needed for multiple applications, including determining risk to

wildlife, livestock, and humans in a given area, and distinguishing between natural and anthropogenic sources during an epidemic. However, due to the number of organisms and impeding chemical constituents within soil and water, identifying a single virulent species within an environmental sample can be a difficult task.

1.1 Characteristics of *F. tularensis*

F. tularensis is a gram-negative intracellular pathogen. It was first isolated from diseased squirrels in Tulare county, California in 1912 [9], but was not officially named *Francisella tularensis* until 1947 [10, 11]. There are now three commonly recognized subspecies: *F. tularensis* subspecies *tularensis* (type A), *F. tularensis* subspecies *holarctica* (type B; previously known as F. tularensis subspecies *palaearctica* [12]), and *F. tularensis* subspecies *mediasiatica*. *F. tularensis* type A and *F. tularensis* type B cause a majority of human tularemia infections [13], while *F. tularensis* subspecies *mediasiatica* has only been isolated in Central Asia and exhibits virulence in rabbits similar to type B organisms [10]. Each subspecies differs in pathogenicity, prevalence, and geographic distribution [14]. *F. novicida* is a closely related species that is sometimes considered a subspecies of *F. tularensis*, but is only very rarely associated with human infections [9]. *F. tularensis* subspecies *holarctica*, *F. novicida*, and *F. philomiragia* are all associated with environmental waters [15].

Tularemia within the United States is most commonly associated with hunting activities or tick bites [16]. Tularemia incidents associated with the consumption of hunted animals typically occur in the summer/ early autumn months, while waterborne tularemia often occurs during the rainy season when swollen streams might extend onto contaminated animal carcasses in the surrounding area [17]. *F. tularensis* type A is primarily found in North America, however recently it was observed in Europe for the first time [13]. *F. tularensis* type A can be split further into two distinct phylogenetic groups, A1 and A2, based upon their geographic distribution and primary vector species [10]. Type A1 is found within the eastern United States and California correlating to the tick vectors *Dermacentor variabilis* and *Amblyomma americanum*, with the eastern cottontail rabbit as common tularemia host [10]. Type A2 is found at a significantly higher elevation within the Rocky Mountain regions of western United States matching the tularemia host mountain cottontail rabbit and the vectors *D. andersoni* (Rocky Mountain wood tick) and *Chrysops discalis* (deer fly). Type A1 can be further separated into two distinct clades, A1a and A1b, based upon phylogenic analysis [18]. *F. tularensis* type A1a and A1b are found primarily within the eastern United States, while type A2 strains are only found within the western United States [18]. *F. tularensis* type A1b exhibit the highest mortality rate for human mortality of all *F. tularensis* strains [18].

Tularemia manifests in a number of ways in humans depending upon the initial portal of infection [19]. Ulceroglandular and glandular forms occur after handling contaminated carcasses

or from an infected arthropod bite [20]. Oropharyngeal forms occur after ingesting contaminated food or water. Oculoglandular and pneumonic forms occur after direct contamination of the eye and inhalation of *F. tularensis*, respectively [20]. Grunow and Finke [21] established a list of criteria for assessing tularemia events to determine if they began from natural or illicit mechanisms. Two of the criteria included within the assessment are the natural geographic distribution of *F. tularensis* and the strain of *F. tularensis* within the affected area [21].

1.2 Persistence of *F. tularensis* in the environment

F. tularensis is widely distributed in the environment and has been isolated from nearly 250 wildlife species, ranging from mammals, invertebrates, birds, amphibians, and fish [10, 22, 23]. Whereas *F. tularensis* subspecies *tularensis* (type A) is found within wild mammals or blood-feeding ticks and deerflies, *F. tularensis* subspecies *holarctica* (type B) is primarily found within environmental surface waters [24].

F. tularensis is a hardy organism within the environment and can survive for weeks and potentially years at low temperatures in water, moist soil, hay, straw, or decaying animal carcasses [20, 25, 26]. Goethert and Telford [27] conducted a systematic analysis of dog ticks on the island of Martha's Vineyard during a sustained tularemia outbreak. Their results point toward dog ticks as a sustaining microfoci for *F. tularensis* for a minimum of four years. Davis-Hoover et al. [28] spiked *F. tularensis* into microcosms filled with sterilized municipal solid waste leachate. Replicate microcosms were stored either at 12 degrees Celsius (°C) or 37°C and cultured at specified intervals [28]. Results show that *F. tularensis* was culturable for up to six weeks within the microcosms, but were not culturable past six weeks at either incubation temperature [28].

While *F. tularensis* is known to persist in the environment, and has been found in soils and aerosols collected across the continental United States [29], the organism is extremely fastidious within the laboratory setting. One study showed that *F. tularensis* live vaccine strain (LVS) and *F. tularensis* NY98 were culturable in spiked tap water held at 8°C for 21 days [5]. However, if the temperature was decreased to 5°C or increased to 25°C neither strain was culturable after 24-hours. This exemplifies the specific nutrient conditions and high inoculum rates that are required in addition to the strict safety precautions needed to prevent laboratory-acquired infections [29, 30].

The natural lifecycle of *F. tularensis* within the environment is not fully understood. Many have hypothesized that protozoa have a significant role in the *Francisella* sp. lifecycle, but the actual activities have yet to be discerned [22, 31-33]. A parasitic interaction between *F. tularensis* and *Tetrahymena pyriformis*, a ciliate protozoa commonly found within fresh water, was first described by Kantardjiev and Velinov [34]. Their work indicates that *F. tularensis* can infect *T. pyriformis*, replicate, and remain viable within the protozoan host for over 30 days; thereby

providing a mode of transport for the bacterium within the environment [34]. More recently, Abd et al. [31] found that *F. tularensis* LVS cultured in the presence of the soil amoeba *Acanthamoeba castellanii* increased in concentration compared to *F. tularensis* LVS cultured alone. *F. tularensis* bacteria go through multiple stages of infection within *A. castellanii*. Bacterial growth was observed within intracellular vacuoles, released vesicles, and within amoeba cysts [31]. It has been hypothesized that *F. tularensis* utilizes carbon dioxide produced by live amoeba and the nutrients released from deceased amoeba to create an ideal setting for proliferation over an extended period [31]. Work by Svensson et al. [35] corroborated these findings by showing that identical *F. tularensis* genotypes overwinter at disease cluster sites; this, in combination with the ability of amoebae to form cysts during periods of famine adds to a potential *F. tularensis*-amoeba relationship that helps *F. tularensis* survive long-term in the environment [33, 35].

Another hypothesis is that long-term *F. tularensis* persistence is due to its survival within biofilms [15, 36]. Biofilms are naturally formed communities of organisms held within an extracellular polymeric matrix. Biofilm communities reduce the influence of shear stress from flowing waters and increase nutrient capture, while simultaneously protecting the inner bacteria from antibiotics and disinfecting chemicals [15]. A number of *Francisella* spp. have shown biofilm formation capabilities, including *F. novicida*, *F. tularensis* subspecies *holarctica* LVS, *F. tularensis* subspecies *tularensis* SchuS4, and *F. philomiragia* [15, 36]. While biofilm formation has been noted in the laboratory and from within environmental samples, the precise role that biofilm has in persistence is still uncertain. One hypothesis is that *F. tularensis* survive within and among amoeba in biofilms [15]. Another hypothesis is that mosquito larvae aid in long-term persistence of *F. tularensis* within the environment. Mahajan et al. [37] found that mosquito larvae can ingest planktonic *F. tularensis* or *F. tularensis* within biofilms. Once inside, the larvae could provide protection, nutrients, transportation, and a source of disease transmission for the *F. tularensis* [37]. Thus, biofilm formation within environmental waters and moist soils might be an additional mechanism by which *F. tularensis* could persist long-term in the environment [36].

1.3 Purpose

The purpose of this review was to survey the open literature on processing and analytical methods currently available for detection of *F. tularensis* in soil and water (drinking, ground, and surface), and to determine gaps in the collective knowledge concerning *F. tularensis* identification from environmental samples. The information presented here could be used to inform future development of standardizing methods used in detecting pathogens in environmental matrices.

1.4 Methods

Information about *F. tularensis* for this literature review was considered from unclassified reports, peer-reviewed journal articles, published books, and government publications focusing on the last twenty years. Published books were limited to the last ten years. The primary search engines used were Science Direct and PubMed with Google Scholar and the Homeland Defense and Security Information Analysis Center (U.S. Department of the Air Force) used secondarily. Search terms included the agent name plus one or more of the following key words: soil, water, environmental, methods, detection, extraction, recovery, and processing. The search was limited to articles published in the English language, but there was no restriction on geographic focus.

This report was generated using references (secondary data) that could not be evaluated for accuracy, precision, representativeness, completeness, or comparability and; therefore, no assurance can be made that the data extracted from these publications meet the stringent quality assurance requirements of the U.S. Environmental Protection Agency (EPA). However, the sources of secondary data were limited to peer-reviewed documents wherever possible. In the event that a pertinent study was found that had not been subject to review by fellow researchers, the scientific and technical information from these non-peer reviewed sources were evaluated, as outlined in the EPA General Assessment Factors for Evaluating the Quality of Scientific and Technical Information (EPA/100/B-03/001) using the assessment factors: focus, verity, integrity, rigor, soundness, applicability and utility, clarity and completeness, uncertainty and variability, and evaluation and review.

2 Current State of the Science

Overall, there is not a great depth of knowledge regarding methods for *F. tularensis* identification within a soil matrix. An initial PubMed search for "*Francisella tularensis,*" "English language" and "soil" returns 11 references; expanding the search to "water" yielded 112 references. While this review did not limit the findings to these articles, it is an indication of the limited breath of knowledge regarding *F. tularensis* in soil and water samples.

A review of these articles and others pointed to three broad mechanisms of *F. tularensis* detection within environmental samples: culture analysis, immunoassay, and genomic identification. While some sampling methods targeted only *F. tularensis* spp., other methods target multiple Category A and B agents within a single sample including, *Bacillus anthracis, Yersinia pestis, Brucella melitensis, Burkholderia mallei,* and/or *Burkholderia pseudomallei.*

2.1 Sample Processing

Environmental samples are often pre-processed before detection techniques are implemented to eliminate inhibiting constituents. While some procedures simply suspend a soil aliquot in buffered solution [38], others utilize filtration [16, 17, 39, 40], centrifugation [16, 17, 24, 28, 40-42], or ultrafiltration [4, 43] to process environmental water and/or soil samples. Johansson et al. [44] noted that the sampling method and transport medium (conditions) had a role in both the culturability and genomic analysis of the samples.

Meric et al. [17] found a 0.45µm cellulose acetate filter to be more efficient for concentrating *F. tularensis* from 1 liter (L) of reservoir water than centrifugation. The filters were washed with sterile deionized water prior to deoxyribonucleic acid (DNA) extraction and real-time polymerase chain reaction (PCR) analysis. In their analysis, only filtered water samples were PCR positive, whereas centrifuge concentrated water samples were not PCR positive [17]. Sellek et al. [39] developed a filtration method for processing soil samples that allowed for both genomic analysis and immunologic analysis of the extracted sample. Their study assessed the efficiency of two filters to capture *F. tularensis* and eliminate inhibiting constituents [39]. Briefly, 0.5 gram (g) of soil (sandy loam, silt loam, or clay) spiked with *F. tularensis* were mixed with 1.5 milliliter (mL) of phosphate buffer solution (PBS) and sufficiently mixed. The suspension was then collected into a sterile syringe and filtered through either an 8-micrometer (µm) pore size glass fiber pre-filter or a 5-µm pore size polyvinylidene fluoride membrane filter. *F. tularensis* cells within the flow-through were directly used for immunological analysis or the flow-through was concentrated by centrifugation and heat lysed prior to PCR analysis. While Sellek et al. [39] were able to show proof of concept for processing *F. tularensis* soils simply through filtration, the results were not efficient. The glass fiber filters only recovered 6%-10% of the *F. tularensis* cells while the polyvinylidene fluoride filters recovered approximately 20% of the spiked bacteria [39]. Therefore, until more efficient filtration procedures are developed, other soil sampling methods would appear to be more suitable for processing environmental soil samples with potentially low *F. tularensis* concentrations.

Ultrafiltration techniques offer a more efficient method for concentrating contaminated water samples. A study by the EPA [43] found ultrafiltration to be an effective sampling technique for simultaneous recovery of diverse microbes from environmental waters. *F. tularensis* was the most challenging microbe to recover during the experiments, yet the average recovery efficiencies ranged from 13 to 62% depending upon the laboratory protocol used and the use of ammonium chloride to treat ultrafiltration concentrates prior to culture. Francy et al. [4] demonstrated the utility of ultrafiltration to concentrate 100 L samples of raw ground water or of finished surface and ground waters into a 225 mL retentate for subsequent biological assessment. *F. tularensis* was detectable in each of the 14 spiked water samples [4].

Swab sampling is a common interior surface sampling method employed during bioterrorism investigations that yields solid particulates similar to some soils. Walker et al. [45] sought an

optimized swab processing method for maximum recovery of *F. tularensis* cells, followed by automated DNA extraction and real-time PCR detection. Four processing methods - heat, sonication, vortexing, and the swab extraction tube system (SETS) - were tested against three commonly used sampling swab materials: polyester, rayon, and foam. SETS is a disposable centrifugal system composed of an inner and outer collection tube. The inner tube contains an orifice to assist in separating collected bacteria from the swab tip. Rehydrated swab tips are aseptically placed within the inner tube of the SETS system. After a brief centrifugation, the rehydration fluids, along with recovered microorganisms, are collected in the outer SETS tube. The sample suspension can then be cultured or processed further for genomic identification. A careful statistical analysis determined that SETS was more efficient at recovering the spiked *F. tularensis* cells from the various swab materials [45]. However, it must be noted that this work utilized pure cultures of *F. tularensis* at high concentrations (10^3 – 10^5 colony forming units (CFU)/swab), and the correlation to field-collected environmental samples is still unknown [45].

2.2 Culturing *F. tularensis* from the Environment

While culturing an organism is considered the gold standard for identification, isolating environmental cultures of *F. tularensis* is challenging as it is a slow-growing, nutritionally fastidious organism that requires 24 to 72 hours for growth [46] on medium supplemented with bio-available iron, cysteine, and up to 12 other nutrients [15]. Even with selective agars, *F. tularensis* colonies are often out-competed by background organisms present in environmental samples [46, 47]. In a study where tap water samples were spiked with *F. tularensis* and held at various temperatures, the *F. tularensis* was not recovered after 24 hours when held at 5°C or 25°C, but was culturable for 21 days when held at 8°C [5]. Yet, when landfill leachates were spiked with *F. tularensis* cultures the organism could be cultured for six weeks when held at 12°C or 37°C [28]. Thus, temperature seems to have a profound effect for some matrices, but not all. Furthermore, due to the high risk of laboratory acquired infections, all *F. tularensis* culturing must be conducted under biosafety level 3 conditions [2, 46]. Yet, *F. tularensis* culturing remains a primary mechanism for confirming the presence of viable biothreat agents. Table 1 summarizes the methods and findings of environmental culturing studies included herein.

Antibiotic-supplemented cysteine heart agar with blood (CHAB) has been frequently used to culture *F. tularensis* from environmental samples [4, 16, 17, 24, 40-42, 46-49]. CHAB medium has been used to attempt to locate the environmental origin of *Francisella* strains isolated within the clinical setting. Two Utah hot springs were suspected as the original route of transmission to a patient; therefore, Whitehouse et al. [24] collected soil, water, vegetation, sediment, and pond scum samples from two suspect springs. Aliquots of the collected samples were centrifuged before culturing on CHAB agar. Suspected *Francisella* spp. colonies were picked and further processed to determine phylogeny and biochemical analysis [24]. While the study was unable to

discern the origins of the human isolated strain, the authors were able to characterize multiple presumptive *Francisella* isolates and identify them as either *F. philomiragia* or *F. novicida* [24].

CHAB agar has been modified with various antibiotics to aid environmental *F. tularensis* isolation. Petersen et al. [49] developed a modified CHAB agar supplemented with colistin, amphotericin, lincomycin, trimethoprim, and ampicillin (CHAB-A) to inhibit background organisms when culturing environmental tissue samples in the field. CHAB has been further modified for isolating *Francisella* spp. from environmental water and seaweed samples [42]. Utilizing CHAB containing polymyxin B, amphotericin B, cyclohexamide, cefepime and vancomycin , Petersen et al. [42] were able to isolate three new *Francisella* spp. from seawater and seaweed collected near Houston, Texas [42]. Their findings were significant, as BioWatch (a federal bio-agent release detection technology program) filters stationed nearby had detected the presence of *Francisella* spp. in the past [42, 50].

Following an outbreak of pneumonic tularemia on Martha's Vineyard, Massachusetts in 2000, significant work was conducted to determine the natural foci for *F. tularensis* type A on the island [16]. Water and soil samples collected from the island were initially screened for *F. tularensis* by PCR detection of the *fopA* gene [46]. Samples PCR positive for *fopA* were cultured on CHAB-A agar to investigate the culturability of the organism. *F. philomiragia* was cultured from only one of five *fopA*-positive water samples [16]. The isolate came from a brackish-water sample and led Berrada and Telford [48] to hypothesize that brackish-water is a more suitable environment for the persistence of *F. tularensis* Type A than freshwater. By culturing fresh and brackish-water microcosms spiked with *F. tularensis* Type A, another study confirmed that brackish-water is a superior environment for *F. tularensis* Type A persistence [48].

Other studies have attempted to culture *F. tularensis* from environmental waters with limited success. Şimşek et al. [40] cultured multiple surface water samples on CHAB agar amended with antibiotics in an effort to identify the source of a tularemia outbreak in Turkey. Of the 154 water samples collected, four were culture positive for *F. tularensis* while 17 were PCR positive. Meric et al. [17] attempted to identify *F. tularensis* by both PCR and culture techniques using filter concentrated reservoir water samples; only PCR yielded positive results. Anda et al. [41] sought the environmental source of a tularemia outbreak associated with crayfish fishing in a contaminated freshwater stream in Spain. *F. tularensis* subspecies *holarctica* was identified as the responsible agent; however, identification was accomplished through PCR analysis and DNA sequencing and not culture, as no *F. tularensis* were isolated on the modified Thayer-Martin chocolate agar utilized within the study. For each of these studies a maximum of 1.5 L of water was concentrated before culture analysis.

Ultrafiltration has been used to concentrate large volumes of water prior to biological assessment. Using ultrafiltration techniques Francy et al. [4] concentrated 100 L samples of 14 spiked waters. They observed that *F. tularensis* was an extremely fastidious organism with a maximum culture recovery rate of 40% (minimum 0.2% recovery) on CHAB agar with

antibiotics. A study comparing two similar ultrafiltration techniques found *F. tularensis* to be the most challenging organism to recover of those tested, which included viruses, bacteria, and protozoa [43]. However, when ultrafiltration filtrates were exposed to 1% ammonium chloride for two-hours prior to culturing on antibiotics amended CHAB the recovery rates dramatically improved [43]. Humrighouse et al. [47] saw a similar effect when seeded water samples were acid treated for 15 minutes before culture on antibiotic amended CHAB. Acid treatment reduced the indigenous background organisms present in the environmental water samples, allowing better *F. tularensis* recovery. Anda et al. [41] also demonstrated the use of acid shock to enhance *F. tularensis* recovery on modified Thayer-Martin chocolate agar.

Johansson et al. [44] concluded that the successful culture of wound specimens (and therefore other environmentally collected samples) was dependent upon the transport medium and sampling techniques employed during collection. Consistent growth curves of *F. tularensis* subspecies *holarctica* and *tularensis* Schu S4 have also been noted as difficult to achieve for verification purposes in the laboratory setting [30]. Therefore, to circumvent the laboratory challenges of directly culturing environmental isolates of *F. tularensis*, other methods of identification, including immunoassays and genomic methods, have been developed to target *F. tularensis*.

Table 1. Comparison of *Francisella tularensis* Culturing Studies

Reference	Sample Matrix and Tested Organism	Sample Preparation Method	Culture Media	Summary
Anda et al., 2001 [41]	River and sewage water naturally contaminated with *Francisella tularensis* subspecies *holarctica*	10 mL water samples were concentrated by centrifugation, the pellet was resuspended in 1 mL of the original sample water and subject to an acid shock to reduce contaminants before plating	Modified Thayer-Martin chocolate agar, supplemented with IsoVitalex™ and 1% L-cysteine	No *F. tularensis* isolates were detected in the sewage or river water samples. One sewage water sample was polymerase chain reaction (PCR) positive for *F. tularensis.*
Berrada and Telford, 2010 [16]	Surface soil, sand, sediment, water naturally contaminated with *F. philomiragia*	Large particles were removed from 100-300 mL samples by centrifugation before filtering through a 0.22 µm cellulose nitrate filter. Filters were washed and resulting particles were collected for culture or DNA extraction	Cysteine heart agar supplemented with 9% sheep blood (CHAB) and antibiotics colistin, lincomycin, trimethoprim, and ampicillin	Only environmental samples that were PCR positive for *fopA* were cultured. From these, *F. philomiragia* was isolated from a single brackish-water sample.
Berrada and Telford, 2011 [48]	Microcosms of fresh or brackish water spiked with *F. tularensis* Type A, Type B live vaccine strain (LVS), or *F. novicida*	10 µL from each microcosm was directly cultured	Cysteine heart agar supplemented with 8% rabbit blood and antibiotics as supplied by Remel (Lenexa, Kansas)	*F. tularensis* Types A and B persist in brackish water longer than freshwater.
Davis-Hoover et al., 2006 [28]	Landfill leachate microcosms spiked with pure cultures of *F. tularensis, Bacillus anthracis, Clostridium botulinum,* or *Yersinia pestis*	5 mL microcosm samples were centrifuged, and resuspended in phosphate buffered saline (PBS) before dilution and triplicate plating.	Cysteine heart agar, chocolate agar, Thayer-Martin agar, Buffered Charcoal yeast extract	*F. tularensis* was not viable after 7 weeks in the landfill leachate held at 12°C or 37°C.
EPA, 2011 [43]	Drinking water spiked with *F. tularensis* LVS, *Bacillus anthracis, B. atrophaeus, Yersinia pestis, Brevundimonas diminuta, Clostridium perfringens, Enterococcus faecalis*	100L of drinking water was spiked before concentration by ultrafiltration. Ultrafiltration concentrates were assayed immediately for *F. tularensis* by membrane filter plates.	Cysteine heart agar with chocolatized 9% sheep blood and antibiotics colistin, amphotericin, lincomycin, trimethoprim, and ampicillin	It was found that average recovery efficiencies for *F. tularensis* were higher when water sample ultrafiltration concentrates were exposed to 1% ammonium chloride for 2 hour prior to culturing.
EPA, 2012 [30]	Pure cultures of *F. tularensis* Schu4 and LVS	None	Trypticase soy broth with Isovitalex	Study attempted to determine recovery methods from swabs, however *F. tularensis* could not be reliably grown within broth cultures and was thus cut from the study.
Francy et al., 2009 [4]	Raw water and drinking water spiked with *F. tularensis* LVS, *B. anthracis* Sterne, *Salmonella typhi, Vibrio cholerae, Cryptosporidium parvum*	100 L samples were concentrated to 225 mL by ultrafiltration before culture.	Cysteine heart agar supplemented with hemoglobin, penicillin, and polymyxin B and blood.	*F. tularensis* recoveries by culture were the lowest of the six tested organisms. Whereas *F. tularensis* was detectable within all 14 ultrafiltration water samples by quantitative (or real time) PCR (qPCR).
Gilbert and Rose, 2012 [5]	Autoclaved tap water spiked with *F. tularensis* LVS and NY98, *Y. pestis, Burkholderia pseudomallei, Brucella melitensis, Bacillus suis*	1 mL aliquots of spiked water were diluted in Butterfield's Buffer and plated at each time point.	Chocolate II agar	*F. tularensis* LVS survived 8 days and NY98 survived 28 days in natural waters at 8°C.

Reference	Sample Matrix and Tested Organism	Sample Preparation Method	Culture Media	Summary
Humrighouse et al., 2011 [47]	Creek water spiked with *F. tularensis* subspecies *holarctica* and *tularensis*	Spiked waters were subject to a 15-min acid treatment (potassium chloride- hydrogen chloride) before neutralization (potassium hydroxide) and streak plating on selective agar.	Cysteine heart agar with rabbit blood and antibiotics as supplied by Remel (Lenexa, Kansas)	The combination of acid treatment and selective agar allowed the recovery of *F. tularensis* from water and effectively reduced indigenous background organisms. Differences in acid resistance were observed among the 7 *F. tularensis* strains assessed.
Johansson et al., 2000 [44]	Clinical isolates collected from infected wounds and *F. tularensis* LVS	Wounds were directly cultured using rayon-tipped applicators.	Thayer-Martin agar	Study compared PCR to culture and found PCR to more sensitive; however, sampling methods can cause PCR difficulties. Differentiating between a tularemic wound and a non-infected wound can be difficult.
Meric et al., 2010 [17]	Reservoirs of spring water naturally contaminated with *F. tularensis*	1 L water samples were filtered with 0.45 µm cellulose acetate filters. Filters were washed with distilled water before filtrate was cultured and DNA extracted.	Glucose cysteine heart agar with 2.5% blood	No cultures were recovered. PCR was attempted following filter concentration, however no samples were PCR positive. Filtration is a better concentration method than centrifugation. Sera, throat swabs, lymph node aspirates, filter concentrated reservoir waters were all culture negative.
Petersen et al., 2004 [49]	Environmental tissues (prairie dog spleen and liver) naturally contaminated with *F. tularensis* subspecies *holarctica*	Necropsied tissue samples were plated and sealed on-site	Cysteine heart agar with chocolatized 9% sheep blood supplemented with colistin, amphotericin, lincomycin, trimethoprim, and ampicillin	Antibiotic supplementation of CHAB media controlled the growth of contaminating bacteria and significantly improved the ability to recover *F. tularensis* and culture sensitivity.
Petersen et al., 2009 [42]	Naturally contaminated seawater and seaweed from Houston, TX. *F. tularensis, F. novicida* and *F. philomiragia*	100 µL was directly plated and an additional 10 mL was centrifuged. The resulting pellet was resuspended in PBS and plated. Seaweed was homogenized in 500 µL of PBS before plating. DNA from selected colonies were boil-lysed before PCR.	Cysteine heart agar with 9% chocolatized sheep blood supplemented with antimicrobials	*F. tularensis* can be directly cultured from environmental seawater and seaweed samples. Presumptive *F. tularensis* colonies were confirmed through PCR analysis.
Şimşek et al., 2012 [40]	Environmental water naturally contaminated with *F. tularensis* subspecies *holarctica* strain LVS	0.3 - 1.5 L water samples were filtered through cellulose acetate membranes (pore size 22 µm). The membranes were placed directly on plates.	Antibiotic (Oxoid SR147)-added cysteine heart agar base with blood	Real-time PCR was more sensitive as 17 of 154 samples were PCR positive and only 4 were culture positive. 16S rRNA sequencing identified the cultured strains as *F. tularensis* subspecies *holarctica* strain LVS.
Versage et al., 2003 [46]	55 wild-type *F. tularensis* isolates collected from naturally infected tissues and laboratory infected animals including *F. tularensis* subspecies *tularensis* and *holarctica*, *F. novicida*, and *F. philomiragia*	Tissue samples were directly cultured while DNA from mouse and prairie dog tissues were extracted.	Cysteine heart agar with 9% chocolatized blood	Comparison of TaqMan® PCR assays to culturing determined that PCR was significantly more sensitive than culturing.

Reference	Sample Matrix and Tested Organism	Sample Preparation Method	Culture Media	Summary
Whitehouse et al., 2012 [24]	Water, vegetation, soil, sediment, and pond scum naturally contaminated with *F. philomiragia* and *F. novicida*	50 mL samples were centrifuged at 8,000 gravitational force (*g*), supernatant was decanted and resuspended in sterile saline before culturing. DNA from presumptive isolates was extracted.	Cysteine heart blood chocolate agar	Samples were centrifuged and the pellet was cultured. DNA from the isolates were further processed to identify *F. philomiragia* and *F. novicida* within the Utah natural warm springs.

2.3 Immunoassay Detection of *F. tularensis*

Testing for *F. tularensis* antigens within environmental samples has been used as a means of infection source tracking for some time due to the ability to incorporate immunoassays into hand-held field-deployable systems. A summary of the immunoassay studies found within this review are presented in Table 2. Care must be taken when developing assay antigens, as some can have cross-reactivity to other microorganisms [51].

Berdal et al. [52] developed a rapid immunochromatographic-test (RI-test) where upon direct addition of environmental waters the presence of *F. tularensis* lipopolysaccharide (LPS) antigen is indicated by a red line within the test window. A comparison of the RI-test to enzyme-linked immunosorbent assay (ELISA) and PCR analyses demonstrated that PCR performs best with environmental water samples, while the RI-test and ELISA were better suited for detecting *F. tularensis* within tissue samples. However, no specifics were given regarding limit of detection (LOD) for any of the three tested methods in the study [52].

Peruski et al. [53] demonstrated the effectiveness of time-resolved fluorescence (TRF), a technology based on lanthanide chelate labels with unique fluorescence properties, within various matrices including soil, serum, urine, and sewage water. The authors determined that TRF improved assay sensitivity by 2000-fold when compared to standard capture ELISA. Sewage water and urine did not impact the overall sensitivity, but soil and serum decreased the capture efficiency. An overall lower LOD of the TRF assay was determined to be approximately 48 CFU/mL [53]. The authors concluded that the TRF assay is more sensitive, has a wider dynamic range than standard ELISA, and could prove to be an invaluable tool for detecting low levels of *F. tularensis* within environmental samples.

Grunow et al. [54] validated antibody immuno columns for analytical process (ABICAP) which are an immunoaffinity chromatographic column test that includes ELISA detection chemistry within a hand-held single use device. The ABICAP system uses small disposable plastic columns within which all assay components are added in flow-through, and thus could allow larger sample volumes to increase sensitivity. Bacterial LPS was directly extracted from environmental waters (125 µL) collected from a Swedish reservoir during a tularemia outbreak and from rabbit and mouse fecal matter. The water samples contained various amounts of dissolved soil. The concentration of mud within the initial water samples directly correlated with increasing background signal during analysis and false positive test results [54]. Yet, the system offers a field-deployable assay with a LOD comparable to capture ELISA [54]. Capture ELISA for spiked silt loam samples processed through glass fiber filters have shown a LOD of 10^4 CFU/mL [39].

Even with highly specific ELISA techniques, testing environmental samples can be challenging. Capture ELISA tests were utilized to track *F. tularensis* contamination within water and fecal samples in Kosovo during two tularemia outbreaks [55]. Unsanitary conditions were targeted as the source of infection in Kosovo as rodent feces collected from food storage areas were found to

contain *F. tularensis* LPS. Water sources were also tested as a probable contamination source, however no water samples yielded positive capture ELISA results [55]. During a biodefense training exercise Offermans and Zegers [38] determined that a 20% suspension of soil in PBS was not suitable for their sandwich ELISA assay using monoclonal antibodies as neither the soil samples nor the control positive sample yielded positive results.

New technologies are being developed that incorporate immunoassay detection chemistry. Huelseweh et al. [56] have developed a protein chip for rapid detection of multiple biowarfare agents. Their method was capable of simultaneously detecting two to five bioagents at similar limits of detection as ELISA, but in less time. However, the overall quality of the immunoarray is still dependent upon the individual affinities to the antibodies. Cooper et al. [2] recently published details of their prototype biosensor for label-free, specific antibody and single-stranded oligonucleotide detection of *F. tularensis.* Pohanka and Skládal [57] have developed a piezoelectric immunosensor for direct detection of *F. tularensis*. While the detection limit is still high, their method has been tested on drinking water and milk samples (LOD of 10^5 CFU/mL for both). The utility of bidiffractive grating biosensor has been explored as a field deployable biosensor for automated biodefense systems [58]. Sharma et al. [59] have developed a novel competitive ELISA for clinical identification of *F. tularensis*, but there is potential that the method could be useful for environmental samples in the future.

Table 2. Comparison of *Francisella tularensis* Immunoassay Studies

Reference	Sample Matrix and Tested Organism	**Detection method** (Sample Amount)	Antibody	Summary
Berdal et al., 2000 [52]	Environmental water naturally containing *F. tularensis*	Rapid immunochromatographic-test (RI- test) (200 µL) Enzyme-linked immunosorbent assay (ELISA) (50 µL)	Lyophilized mouse monoclonal IgG antibody specific for *F. tularensis* lipopolysaccharide (LPS) antigen Mouse monoclonal *F. tularensis* LPS antibody Ft-27 and antibody Ft-11.	Three wells were tested. One gave a weakly positive signal for 2 of 4 collected samples. Liver, spleen and kidney supernatants from a lemming carcass tested strongly positive.
Cooper et al., 2011 [2]	Pure cultures of *F. tularensis* subspecies *tularensis* and *holarctica*	Antibody and DNA photonic biosensors	IgG LPS directed antibody	At least 10^5 colony forming units (CFU) of *F. tularensis* were detectable to present a proof-of-principle.
Grunow et al., 2008 [54]	Environmental water and feces collected during a tularemia outbreak or spiked with *F. tularensis* live vaccine strain (LVS).	Antibody immuno columns for analytical process (ABICAP) (125 µL)	*F. tularensis* LPS monoclonal IgG1antibody FF/11/6	The ABICAP system is useful for identifying reservoirs of *F. tularensis* from within environmental waters and feces. Limit of detection (LOD) 10^3 bacteria.
Grunow et al., 2012 [55]	Environmental water and feces collected during a tularemia outbreak	Capture ELISA	*F. tularensis* LPS-specific monoclonal IgG1antibody FF/11/6 as capture antibody bound to the solid phase	Kosovo outbreak due to poor conditions following war activity. No water samples yielded positive capture ELISA results.
Huelseweh et al., 2006 [56]	Pure cultures of *F. tularensis* WIS 140, *Yersinia pestis, Burkholderia pseudomallei, B. mallei, Brucella melitensis, Escherichia coli*	Protein chip	LPS-specific capture and detector monoclonal antibody: FT140/11/1/06	Microarray detection limits were comparable to ELISA, but require less time even when detecting multiple bioagents. LOD 10^6 CFU/mL.
O'Brien et al., 2000 [58]	Pure cultures of *F. tularensis* LVS	Bidiffractive grating biosensors	Goat capture antibody	Proof of principle to assess if bidiffractive grating biosensors could be used for biosurveillance monitoring. LOD 10^5 CFU/mL.
Offermans and Zegers, 2007 [38]	Soils spiked with unknown quantities of *F. tularensis, B. anthracis, Brucella pseudomallei* or Vaccinia	ELISA (2g)	Capture antibody: Monoclonal antibody FF27/1/7 anti *F. tularensis*; Detector antibody: Monoclonal antibody FF1 1/1/6-biotin anti *F. tularensis*	No *F. tularensis* was detected with ELISA techniques in either the spiked samples or the positive control.
Peruski et al., 2002 [53]	Serum, urine, dirt, sewage water spiked with *F. tularensis* LVS	Time-resolved fluorescence (TRF) and ELISA (100 µL or 10mg dirt/mL phosphate buffered saline (PBS))	Biotinylated capture antibodies: monoclonal antibody Ft-03 or polyclonal antibodies to *F. tularensis* and detected by TRF Europium-labeled antibodies	Capture biotinylated antibodies to *F. tularensis* were used to compare ELISA to TRF. TRF was 2000 times more sensitive for *F. tularensis* than standard ELISA. Additionally when tested within sera, urine, sewage water, and dirt (10 mg/mL PBS) the sensitivity decreased for sera and dirt, but not for sewage water or urine.

Reference	Sample Matrix and Tested Organism	**Detection method** (Sample Amount)	Antibody	Summary
Pohanka and Skládal, 2007 [57]	Pure cultures of *F. tularensis* LVS	Piezoelectric immunosensor (500 µL)	Mouse polyclonal antibody	The novel device was capable of detecting *F. tularensis* within tap water and milk at a LOD of 10^5 CFU/mL.
Sellek et al., 2008 [39]	Sandy loam, silt loam, or clay soil spiked with FMA-inactivated *F. tularensis* subspecies *holarctica* LVS	Capture ELISA, quantitative-polymerase chain reaction (qPCR)	Anti-*F. tularensis* LVS monoclonal antibody T14	Comparisons showed that qPCR had a lower LOD than capture ELISA (10^2 and 10^4 CFU/mL, respectively)
Sharma et al., 2013 [59]	Human and animal serum and *F. tularensis* subspecies *holarctica*	Competitive ELISA	LPS monoclonal antibody M14B1	Currently this method is limited to clinical identification of tularemia.

2.4 Genomic Identification of *F. tularensis*

While culture is considered the gold standard for identifying viable pathogenic microorganisms from environmental or clinical samples, multiple reports have shown that PCR identification is faster and more sensitive than culture or immunoassay [17, 39]. However, these assays also have limitations. Versage et al. [46] compared the capabilities of culture versus real-time PCR to identify *F. tularensis* from contaminated animal tissues. Their analysis determined that real-time PCR assays were significantly more sensitive than culture. Anda et al. [41] and Meric et al. [17] each attempted to identify *F. tularensis* within environmental water samples. Neither study was able to recover *F. tularensis* isolates, but both detected *F. tularensis* through genomic analysis. Work at Martha's Vineyard following a pneumonic tularemia outbreak screened water and soil samples for *F. tularensis* [16]. Of the 156 samples assessed, 23 were PCR positive for *F. tularensis* genes, yet only one sample yielded a *F. philomiragia* culture. Şimşek et al. [40] also attempted to identify *F. tularensis* by culture and real-time PCR following a tularemia outbreak in Turkey. Here again, culture was less sensitive than PCR as only four water samples were culture positive, but 17 of the 154 samples were PCR positive for IS*Ftu2*. This review identified a number of studies that utilized genomic analysis to identify *F. tularensis* within environmental samples. Table 3 outlines the studies discussed in sections 2.4.1 and 2.4.2, their processing methods, and a brief summary of their conclusions.

2.4.1 Extraction of *F. tularensis* DNA

F. tularensis is a non-sporulating Gram-negative organism; therefore, its DNA can be extracted for identification rather easily when compared to sporulated microorganisms. However, humic acids and other inhibitory compounds within environmental soil and water samples are often coextracted and lead to confounding downstream PCR responses [7]. Therefore, special care must be taken to efficiently clean environmental DNA extracts prior to analysis. An efficient method of DNA extraction ought to produce an unbiased yield of quality DNA suitable for downstream analysis, meaning that a high concentration of long-DNA segments from diverse species present within a single sample is needed [60].

Whitehouse and Hottel [61] conducted a comparison of five commercial DNA recovery kits for isolating *F. tularensis* DNA from three types of soil: silt loam, clay, and commercial potting soil. They determined that the UltraClean® Microbial DNA Isolation kit (MoBio laboratories, Inc., Carlsbad, CA) and the PowerMax® Soil DNA Isolation kit (MoBio laboratories, Inc.) yielded the most consistent and lowest limits of detection (LOD) of the tested kits. The UltraClean® Microbial DNA Isolation kit yielded an LOD of 20 colony forming units (CFU)/g soil, and the PowerMax® Soil DNA Isolation kit yielded an LOD of 100 CFU/g soil. These limits of detection were similar to the positive control LOD of 10 CFU/mL achieved for pure culture *F. tularensis* extraction and real-time PCR [61]. Klerks et al. [62] conducted a similar study comparing five commercial DNA recovery kits for isolating *Salmonella enterica* DNA, another non-sporulating Gram-negative organism, from within various environmental matrices. They determined that the UltraClean® Soil DNA Isolation kit (MoBio laboratories, Inc.), Bio101 extraction kit (Q-Biogene

Inc. Carlsbad, CA), and the UltraClean® Fecal DNA Isolation kits (MoBio laboratories, Inc.), yielded superior quality and quantity of DNA from the tested soil, manure, and compost samples [62]. The Soilmaster™ DNA extraction kit (Epicentere, Madison, WI) and QIAGEN plant DNeasy™ DNA (QIAGEN, Westburg, The Netherlands) extraction kit were not found to be optimal for *S. enterica* from soil samples [62]. Yet, Broman et al. [22] successfully utilized the Soilmaster™ DNA extraction kit to identify the presence of *F. tularensis* in 20% of the sediment samples and 32% of the surface water samples collected within two regions of reoccurring tularemia outbreaks in Sweden.

Berrada and Telford [16] utilized the UltraClean® Soil DNA Isolation kit in their analysis of soil, mud, and sediment samples collected on Martha's Vineyard. They followed the manufacturer's protocol with one exception; in an effort to reduce DNA shearing, the bead-beating time was reduced from ten minutes (min) to five min [16, 63]. Utilizing the modified UltraClean® Soil DNA Isolation kit protocol, the study identified four brackish-water soil/sediment samples that were PCR positive for specific primers (i.e., *Francisella* spp. 16 svedberg units [S] ribosomal ribonucleic acid [rRNA] primers [16S rRNA]) and three samples positive for *F. tularensis* specific sequences [16]. Barns et al. [50] also utilized the UltraClean® Soil DNA Isolation kit to broadly survey the Houston, Texas area for *Francisella* species and relatives. Following a *F. tularensis* positive sample by the BioWatch aerosol monitors in October 2003, 364 soil and water samples were collected around the Houston area. The 16S rRNA sequencing results from one water sample showed the presence of *F. philomiragia* while the 16S rRNA sequencing results from seven soils pointed to the presence of new subspecies of *F. tularensis* with unknown pathogenicity [50].

Defense Research and Development Canada included the UltraClean® Soil DNA Isolation kit as part of two biothreat response readiness exercises [64, 65]. During the 2001 exercise [64], *F. tularensis* was spiked into a single liquid sample. No processing was conducted for liquid samples before PCR analysis, but DNA was extracted from the unknown soil samples with UltraClean® Soil DNA Isolation kits prior to PCR analysis. All 13 laboratories involved in the exercise accurately identified *F. tularensis* within the aqueous sample [64]. In 2002, a single soil sample spiked with *Brucella suis* and a chemical nerve agent stimulant was assessed as an unknown sample by Defense Research and Development Canada, Suffield. Through the course of the exercise *Brucella* spp. was accurately identified through SYBR® Green PCR, culture, and BIOLOG™ within the unknown soil sample [65]. Another biodefense training exercise conducted in 2006 sought to identify the presence of biological warfare agents within supplied unknown soil samples through either real-time PCR or immunochemical assays [38]. Fourteen soils were screened for the presence of *B. anthracis, F. tularensis, B. pseudomallei,* or vaccinia individually and as a mixture. Prior to real-time PCR analysis, soil samples were extracted with either the UltraClean® Soil DNA isolation kit (200 µL) or the PowerMax® DNA isolation kit (6.0 mL). The significant difference in loading size of the two extraction kits was clearly seen in the real-time PCR analyses results. Only soil samples spiked with the high concentrations of

biological agents were detectable within the UltraClean® Soil extracts. Therefore, it was concluded that for samples of unknown biological agents it is preferable to extract DNA from as much of the original sample volume as possible [38].

Trombley Hall et al. [66] recognized the need for purified nucleic acids from environmental samples; however, rather than seeking an optimum extraction kit that removes inhibiting constituents, they sought inhibitor-resistant PCR reagents. Use of inhibitor-resistant PCR reagents eliminates the need for sample-specific preparation and increases the sensitivity of real-time PCR [66]. Among the five PCR chemistries tested, KAPA Blood PCR Kit (KAPA Biosystems, Wilmington, MA) yielded the most consistent estimated LOD results across the range of tested matrices (buffer, whole blood, sputum, stool, soil, sand, and swab) [66]. When looking at soil results alone, 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 Inc., Mountain View, CA) with Phire® Hot Start DNA Polymerase all yielded the same LOD, 0.2 picograms (pg) *F. tularensis* DNA, when the PCR reaction was composed of 0.05% soil [66].

Table 3. Comparison of *Francisella tularensis* Genomic Studies.

Reference	Sample Matrix and Tested Organism(s)	Sample Preparation Method	Detection method	Summary
Ahlinder et al., 2012 [67]	*Francisella tularensis* subspecies *holarctica, mediasiatica, tularensis, F. novicida, F. hispaniensis, F. philomiragia*	None given	*in silico* polymerase chain reaction (PCR) analyses were conducted for a large dataset of primers and *Francisella* genomes.	No single marker topology of the entire genus is currently available to classify all *Francisella* spp. to their proper subspecies. This indicates that several markers utilized for detection are unspecific resulting in false positives. No environmental samples were assessed.
Anda et al., 2001 [41]	River and sewage water naturally contaminated with *Francisella tularensis* subspecies *holarctica*	1mL of water was centrifuged and the pellet was resuspended in 100 µL of sample water. After a low speed centrifugation to eliminate solids, cells within the supernatant were chemically lysed and DNA precipitated.	PCR targeting the *F. tularensis* specific 16S rRNA	No *F. tularensis* isolates were detected in the sewage or river water samples- one sewage water sample was PCR positive for *F. tularensis.*
Bader et al., 2003 [64]	Phosphate buffered saline (PBS) spiked with *F. tularensis* Schu S4	1 mL liquid was directly processed	PCR targeting *tul4* gene	A higher number of false positive and false negative identifications were reported for soil sample unknowns than for liquid sample unknowns. *F. tularensis* was properly identified within a phosphate buffered solution (PBS) by 10 of 13 reporting laboratories.
Bader et al., 2004 [65]	Soil spiked with *Brucella suis* and simulant nerve agent	0.25 g of soil was processed in a MoBio UltraClean Soil DNA isolation kit	PCR targeting *tul4* and *fopA* genes	Soil sample was correctly identified to not be spiked with *F. tularensis* but rather with *B. suis* and a G nerve agent simulant.
Barns et al., 2005 [50]	Houston, TX surface soil, grab water naturally containing *F. tularensis*	0.25 g soil directly processed while cells within the 50 mL water samples were pelleted by centrifugation before DNA extraction with a MoBio UltraClean Soil DNA isolation kit	PCR detecting *F. tularensis* specific 16S rRNA, IS*Ftu2, tul4, fopA, 23kDa*	DNA from soil and water samples collected from Houston, TX showed the presence of new *F. tularensis* subspecies.
Berdal et al., 2000 [52]	Well water naturally containing *F. tularensis*	100 µl incubated with lysis buffer before using 2 µl directly in PCR reactions.	PCR followed by restriction analysis with endonuclease DraI	Three wells were tested. One gave a positive PCR signal in 4 of 4 collected samples. Liver, spleen and kidney supernatants from a tested lemming carcass were all PCR negative, but ELISA and RI-tests were positive.
Berrada and Telford, 2010 [16]	Environmental surface soil, sand, sediment, water naturally found with *F. philomiragia*	DNA within 0.25 to 0.5 g of sediments, mud, or soil were directly extracted using a MoBio UltraClean Soil DNA isolation kit. Large particulates from 100-300 mL of water were removed by centrifugation before filtering through a 0.22 µm cellulose nitrate filter. Filter wash collected for culture or DNA extraction with MoBio UltraClean Soil DNA isolation kits.	PCR: *sdhA,* IS*Ftu2, tul4, fopA*	All samples collected near the freshwater pond and the marsh were PCR negative for *F. tularensis* 16S rRNA, but only one brackish water sample was culture positive for *F. philomiragia.*

Reference	Sample Matrix and Tested Organism(s)	Sample Preparation Method	Detection method	Summary
Broman et al., 2011 [22]	Environmental soil and water naturally contaminated with *F. tularensis* subspecies *holarctica*	2.0 mL of soil or water centrifuged. Cell pellet processed through Soil Master™ DNA Extraction (Epicenter Biotechnologies) to yield sample DNA.	Real-time PCR detecting *lpnA* and FtM19 internal deletion region	Clinically relevant subspecies *F. tularensis* subspecies *holarctica* found in water and sediment samples during three consecutive years.
Buzard et al., 2012 [68]	Pure cultures of *F. tularensis* LVS, *Bacillus anthracis* Ames, *Brucella melitensis, B. mallei*	DNA extracts from pure cultures were obtained for this study.	real-time PCR for *tul4*	Ten commercial PCR master mixes and three real-time PCR instruments were compared: all ten yielded positive results for *F. tularensis* on the 7500 Fast Dx and Smart Cycler® instruments, but only seven were positive on the Light Cycler® instrument.
Duncan et al., 2013 [14]	*F. tularensis* subspecies *tularensis, holarctica, mediasiatica, F. novicida*	Pure DNA was procured for the study.	hierarchical PCR analysis using electrospray ionization/time of flight mass spectrometry (ESI-MS) detection	This method can differentiate between pathogenic and nonpathogenic *F. tularensis* strains for epidemiological or investigation studies.
Escudero et al., 2008 [69]	Clinical and environmental tissues contaminated with *F. tularensis* subspecies *tularensis, holarctica*, or *F. novicida*	QIAamp DNA blood extraction kit (Qiagen) used to extract DNA from human and tick tissue samples.	PCR detecting *lpnA* followed by hybridization to various probes for subspecies differentiation	Method able to differentiate pathogenic *F. tularensis* from non-pathogenic subspecies within tissue samples. Limit of detection (LOD) 1 plasmid copy OR 10 genomic equivalents (GE).
Forsman et al., 1995 [70]	Environmental water spiked with *F. tularensis* LVS	A) 1 mL filtered and freeze thaw DNA lysis; B) 1 mL centrifuged, pellet treated with a commercial ion exchange suspension to purify DNA; C) Treated by alkaline method to prep DNA; D) Chromosomal DNA from water samples were prepared; E) 1 ml filtered, filtered bacteria chemically lysed, DNA purification by phenol chloroform isoamyl alcohol, DNA filter purified by microspin column	PCR with genus specific *F. tularensis* primers	An early study that looked at various methods for processing environmental water samples for *F. tularensis* detection. LOD 10 bacteria/mL.
Francy et al., 2009 [4]	Raw water and drinking water spiked with *F. tularensis* LVS, *B. anthracis* Sterne, *Salmonella typhi, Vibrio cholerae, Cryptosporidium parvum*	Ultrafiltration retentate filtered through 0.4 µm polycarbonate filters. DNA from the organisms collected on the filters was extracted using MoBio PowerSoil® DNA extraction kit (filters directly placed into the extraction tubes.)	Real-time PCR targeting *fopA* and *tul4*	Determined qPCR of ultrafiltration retentate is an effective method to sample large-scale drinking water samples.

Reference	Sample Matrix and Tested Organism(s)	Sample Preparation Method	Detection method	Summary
Fujita et al., 2006 [1]	Pure cultures of *F. tularensis* subspecies *tularensis, holarctica, philomiragia, F. novicida*	Genomic DNA from pure cultures was manually extracted or with the SepaGene DNA Extraction Kit (Sanko Junyaku Co., Tokyo, Japan)	Real-time PCR detecting *fopA* gene	Development of real-time PCR primers for identifying *F. tularensis* in 1hour. LOD 1.2 colony forming units (CFU) or 10 copies of the *fopA* gene.
Garcia Del Blanco et al., 2002 [71]	Clinical and environmental isolates of *F. tularensis* subspecies *tularensis, holarctica,* and *F. novicida*	DNA from pure cultures grown on Thayer-Martin agar was obtained through manual extraction.	Pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and 16S rRNA amplification for subspecies identification of *F. tularensis.*	PFGE and AFLP can discriminate between *Francisella* species, but 16S rRNA amplification cannot.
Johansson et al., 2000 [44]	Clinical isolates collected from wounds and *F. tularensis* subspecies *holarctica* LVS	Heat-killed whole cell extraction	Cotton-tipped applicator used to collect material from *F. tularensis* suspected ulcers. Cotton applicators transported in guanidine isothiocyanate buffer before 450µL manually extracted for DNA and assessed for *tul4* gene presence.	Study compared PCR to culture and found PCR to more sensitive; however, sampling methods can cause PCR difficulties. Differentiating between tularemic wounds and a non-infected wounds can be difficult.
Klerks et al., 2006 [62]	Soil, manure, and compost spiked with *Salmonella enterica*	DNA from 100mg of spiked sample was extracted by 1 of 5 commercial kits: Ultraclean soil DNA isolation kit (MoBio); Ultraclean fecal DNA kit (MoBio); Bio101 extraction kit (Q-Biogene); Soilmaster DNA extraction kit (Epicenter); plant DNeasy DNA extraction kit (QIAGEN); or a combination of the microbial DNA extraction kit (MoBio) with bacterial isolation using Optiprep™	Real-time PCR using *S. enterica*-specific detection probe	MoBio soil, Bio101, and MoBio fecal were found to be most efficient for DNA extraction from, respectively, soil (eight different substrates), manure (six substrates), and compost (two substrates).
Kuske et al., 2006 [29]	USA soil and aerosol samples targeting *F. tularensis* subspecies *holarctica, B. anthracis, Y. pestis, Clostridium perfringens*	DNA from 0.5 g of soil was manually extracted with bead beating, ethanol DNA precipitation and spin Sephadex® G-200 column cleanup. Aerosol filters washed in PBS before DNA extracted with same process.	PCR targeting *F. tularensis* specific 16S rRNA and *tul4* gene	*F. tularensis* 16S rRNA found in aerosol samples from two US cites: Denver and San Diego. No *tul4* genes were detected. No soil samples were positive. LOD 0.1 pg or 17-46 GE

Reference	Sample Matrix and Tested Organism(s)	Sample Preparation Method	Detection method	Summary
Matero et al., 2011 [72]	B. *thuringiensis, F. tularensis, B. anthracis, Yersinia pestis, Brucella* spp.	DNA from pure cultures were extracted using MagNA Pure Nucleic Acid Isolation Kit I	PCR targeting 23kDa gene	Study compared RAZOR to ABI instrumentation and assessed a *B. thuringiensis* protocol with environmental samples to show proof-of-principle for *F. tularensis*.
Meric et al., 2010 [17]	Reservoir spring water naturally contaminated with *F. tularensis*	1 L water samples filtered with 0.45 µm cellulose acetate filters. Filters washed with sterile distilled water before filtrate was cultured or DNA extracted with QIAamp DNA mini kits.	Culture and real-time PCR targeting: *ISFtu2* element, *23 kDa* gene, and the *tul4* gene.	No cultures were recovered. PCR was also attempted following centrifugation concentration, however no samples were PCR positive. Filtration is a better concentration method than centrifugation. Sera, throat swabs, lymph node aspirates, centrifuge concentrated reservoir water, and filter concentrated waters were all culture negative.
O'Connell et al., 2004 [73]	Creamer, cornstarch, baking powder, flour spiked with *F. tularensis* subspecies *holarctica* LVS	DNA from pure cultures of *F. tularensis* extracted with QIAGEN DNeasy mini spin columns.	Direct PCR in BioSeeq® handheld system	Bio-Seeq® technology is a novel system for use in areas with high concentrations of bacteria. LOD of *F. tularensis* determined to be 10^3 cells/reaction or less when the consumable sampling assembly is utilized with household powders.
Offermans and Zegers, 2007 [38]	Soils spiked with unknown quantities of *F. tularensis, B. anthracis*, *Brucella pseudomallei* or Vaccinia	UltraClean Soil DNA Isolation Kit (200 µL); PowerMax Soil DNA Isolation Kit (6 mL)	Real-time PCR targeting *tul4* gene and ELISA	PowerMax DNA isolation kit extracts yielded much stronger reactions than the UltraClean extracts. No *F. tularensis* was detected by ELISA techniques.
Sellek et al., 2008 [39]	Sandy loam, silt loam, or clay soil spiked with FMA-inactivated *F. tularensis* subspecies *holarctica* LVS	0.5 g soil in PBS processed through Glass fiber pre-filter (pore size 8 µm) to separate cells followed by heat lysis OR Millex®-SV filter unit (pore size 5.0 µm) followed by heat lysis	qPCR with SYBR Green I targeting *tul4* gene	Millex filter was more efficient for filtering soils samples; however, it allowed more PCR inhibiting compounds through to the final sample than the glass fiber filter. Comparisons between qPCR and capture ELISA show that qPCR has a lower LOD (10^2 and 10^4 CFU/mL, respectively)
Şimşek et al., 2012 [40]	Environmental water naturally contaminated with *F. tularensis* subspecies *holarctica* LVS	0.3 - 1.5 L water samples filtered through cellulose acetate membranes (pore size 22 µm). Membranes place directly on cysteine heart agar with blood (CHAB) plates. For PCR detection, filters were washed with sterile water before DNA extracted using a QIAamp DNA Mini Kit.	Culture and real-time PCR targeting IS*Ftu2* gene	Real-time PCR was more sensitive than culture as 17 of 154 samples were PCR positive and only 4 were culture positive. 16S rRNA sequencing identified the cultured strains as *F. tularensis* subspecies *holarctica* strain LVS.
Svensson et al., 2009 [35]	62 *Francisella* isolates of diverse genetic and geographical origins	DNA from pure cultures was manually extracted for this study.	68 real-time PCRs for hierarchical identification of *F. tularensis*	Study established a 68-well assay for differentiating between *F. tularensis* strains.

Reference	Sample Matrix and Tested Organism(s)	Sample Preparation Method	Detection method	Summary
Trombley Hall et al., 2013 [66]	Pure DNA from *F. tularensis* SCHU S4 spiked into sand and soil	No extraction; direct PCR with 5 µl sample slurry and added pure DNA	PCR targeting *tul4* gene	Study assessed various PCR chemistries for inhibitor resistant capacity for use with environmental samples. Phire Hot Start DNA polymerase with SRT Boost reagents was the best combination found for detecting spiked DNA in soil samples.
Versage et al., 2003 [46]	Pure cultures isolated from tissues of laboratory infected animals including *F. tularensis* subspecies *tularensis, holarctica, philomiragia, F. novicida*	Tissue samples were directly cultured while DNA from mouse and prairie dog tissues were extracted with MasterPure™ Purification Kit (epiCenter).	Multitarget PCR targeting *tul4*, *fopA*, IS*Ftu2*, *23kDa* compared to culture	Comparison of TaqMan PCR assays to culturing determined that PCR was significantly more sensitive than culturing.
Whitehouse et al., 2007 [61]	Silt loam, clay, and potting soil spiked with *F. tularensis* Shu-4	0.1 to 10 g of soil processed through Puregene® DNA purification Kit OR QIAmp DNA Stool Mini Kit OR Epicentre SoilMaster DNA Extraction Kit OR MoBio UltraClean Soil DNA Isolation Kit OR MoBio PowerMax soil DNA isolation Kit	PCR targeting *fopA* gene	UltraClean and PowerMax soil DNA isolation kits were the most consistent and sensitive methods for extracting *F. tularensis* from soil.

2.4.2 PCR amplification for genomic identification of *F. tularensis*

PCR identification has progressed significantly in recent years. *F. tularensis* identification within environmental waters by PCR amplification was initially conducted by manual DNA extraction followed by genus specific *Francisella* PCR amplification [70] or restriction enzyme analysis [52] and visual gel electrophoresis detection. Now, commercial sample extraction kits [61] and rapid real-time PCR analysis allow for sensitive detection at low concentrations [1]. Genes commonly targeted in genomic identification studies were *tul4, fopA,* IS*Ftu2,* and *23kDa* genes. The *tul4* and *fopA* genes are outer membrane proteins [46] encoding for a17-kiloDalton (kDa) protein [4] and a 43-kDa protein [16], respectively. IS*Ftu2* targets an insertion element-like sequence in *F. tularensis* [50]. The *23kDA* gene encodes a protein that is expressed during macrophage infection [46].

PCR analysis was used to determine the natural presence of *F. tularensis* among soil and aerosol samples collected across the United States. In total, 89 soils from across the US and over 15,000 aerosol samples from 15 major US cities were evaluated [29]. Utilizing 16S rRNA primers, the study found that *F. tularensis* or its near relatives are naturally present in urban aerosols; however, no 16S rRNA sequences for *F. tularensis* were found within the studied soils [29]. Following a natural tularemia outbreak at Martha's Vineyard, Berrada and Telford [16] were able to identify diverse *Francisella* spp. within the environment through PCR analysis. Of the 156 samples assessed, 23 were positive for *F. tularensis* 16S rRNA, 15 were positive for *fopA,* 19 positive for IS*FTu2,* and 14 were positive for *tul4*. Of the PCR positive samples, only one *fopA* PCR positive sample yielded a culture of *F. philomiragia.* Meric et al. [17] linked a tularemia outbreak in Turkey to consuming reservoired spring water by targeting IS*Ftu2, 23kDa*, and *tul4* genes in their PCR analyses. Targeting *fopA,* Fujita et al. [1] established a specific and sensitive real-time PCR assay for rapid detection of *F. tularensis* within a prepared DNA sample. This method can achieve detection equivalent to 1.2 CFU of bacterial cells/reaction.

Molecular methods have been developed to discriminate between *F. tularensis* and *Francisella*-like organisms. Differentiation between pathogenic and nonpathogenic strains of *F. tularensis* is critical to epidemiological and outbreak investigation studies. Recognition of a 36 base pair deletion in *lpnA* sequences within *F. tularensis* subspecies allowed Escudero et al. [39] to develop a genomic method for differentiating between *F. tularensis* and *Francisella*-like organisms. One study compared three molecular methods for separating *F. tularensis* strains: pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and 16S rRNA gene sequencing [71]. PFGE and AFLP were able to distinguish *F. tularensis* subspecies that could be useful for epidemiological tracking during a tularemia event. Duncan et al. [14] and Svensson et al. [35] used PCR assays for hierarchical identification of *Francisella* isolates. Duncan et al. [14] utilized 24 multilocus PCR reactions followed by electrospray ionization/time of flight mass spectrometry (ESI-MS) detection to differentiate *F. tularensis* subspecies. Svensson et al. [35] utilized specific deletions and insertions within the *F. tularensis* genome to generate a hierarchical identification system using 68 individual real-time PCR

reactions. While both of these studies have the ability to differentiate between *F. tularensis* subspecies, their utility would be most useful for tracking analysis in a tularemia outbreak situation.

Results of a study focusing on published PCR primers and their specificity among whole-genome sequences now available showed that many primers previously developed for *F. tularensis* contain extremely low specificity, and therefore yield false positives [67]. Identification of specific species or subspecies can be challenging. Real-time PCR assays incorrectly identified *F. tularensis* and *F. novicida* during an outbreak [74]. This finding points to the need for thorough characterization of isolates that share close sequence identities. To mitigate false positive PCR results, primer sequences need to be continually evaluated and redesigned using up-to-date genomic databases. Furthermore, as no single-marker was capable of distinguishing all the *Francisella* strains within the Ahlinder et al. [67] study, an optimized combination of markers could be used to improve *Francisella* strain resolution.

It has been recognized that PCR master mixes and PCR thermocycler instruments do not all function equally. In a comparison of the ABI 7300/7500 (Applied Biosystems, Foster City, CA) to the RAZOR (Idaho Technology Inc., Salt Lake City, UT) real-time PCR thermocyclers the LOD for *F. tularensis 23kDa* gene was found to be the same at 10 fentagram (fg) genomic DNA per reaction [72]. However when Buzard et al. [68] compared ten commercially available PCR master mixes and three real-time PCR instruments, 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.

2.4.3 Methods for Environmental Sampling and Detection of Multiple Biothreat Organisms

New technologies utilizing genomic techniques to detect pathogenic organisms alone or in concert with other organisms are constantly being developed. Table 4 gives details on developing assays for *F. tularensis* identification and multiagent identification methods discussed in this review.

Table 4. Comparison of Developing Methods for Genomic Identification of *Francisella tularensis* Alone and Simultaneously with Other Organisms.

Reference	Organism(s)	Sample Matrix	Sample Preparation Method	Detection Method	Summary
Brinkman et al., 2013 [75]	*Francisella tularensis* LVS, *Bacillus anthracis, Cryptosporidium. parvum, C. hominis, Enterococcus faecium*	Tap water	DNA extracted from pure cultures with Gentra PureGene Genomic Prep kit (Qiagen) added to a background of concentrated tap water. 1000 L of tap water was repeatedly filtered before ultracentrifugation and solvent extraction to remove PCR inhibitors.	Microarray	Designed to identify *F. tularensis* and other human pathogens under periods of high concentration from within tap water samples.
Cooper et al., 2011 [2]	*F. tularensis* subspecies *tularensis, holarctica*	Phosphate buffered saline (PBS)	Pure cultures were boiled to lyse cells before ethanol precipitation to concentrate DNA.	Antibody and DNA photonic biosensors targeting *yhhW* gene for type A strains, *lpnA* gene for both type A and B strains	Photonic biosensor only requires nanogram quantities of target DNA to differentiate *F. tularensis* subspecies without polymerase chain reaction (PCR) amplification.
Euler et al., 2012 [13]	*F. tularensis* subspecies *tularensis, holarctica, F. hispaniensis , F. novicida F. philomiragia*	Rabbit tissue	DNA extracted from pure cultures with QIAamp DNA blood extraction kit (Qiagen) small pieces of tissue was homogenized before DNA extracted by same kit.	Real-time recombinase polymerase amplification (RPA) assay on an isothermal amplification methods ESEQuant tube scanner device detecting *tul4*	RPA is comparable to real-time PCR with ~10 min run times and limit of detection (LOD) of 10-100 molecules.
Euler et al., 2013 [76]	*F. tularensis* (Ft 12), *B. anthracis, Yersinia pestis,* variola virus	Spiked human plasma	DNA from spiked plasma was extracted with an innuPREP MP basic kit A (Jena Analytik)	Real-time RPA assay on an isothermal amplification methods ESEQuant tube scanner device detecting *tul4*	RPA performed equally as PCR and showed not cross-detection among targets. RPA run time is ~10 min with a LOD of 10 molecules.
He et al., 2009 [77]	*F. tularensis, B. anthracis, Y. pests, variola major*	Spiked clinical samples	Manual DNA extraction	Multiplex PCR-enzyme hybridization assay targeting *tul4*	Only spiked clinical samples were assessed for method development of multiplex PCR enzyme hybridization assay (mPCR-EHA). LOD established at 10 copies/mL.
Janse et al., 2010 [78]	*F. tularensis* subspecies *holarctica* and *tularensis, and F. novicida, B. anthracis, Y. pestis*	Spiked milk powder, soy powder, silica, and maize powder	NucliSens Magnetic Extraction Reagents (bioMérieux) were used to extract DNA from pure cultures. DNA was added to interfering agents before analysis.	Multiplex quantitative PCR targeting *fopA, ISFtu2,* and *pdpD*	This multiplex reaction incorporates an internal positive control (*B. thuringiensis* spores) for both nucleic acid extraction and amplification. It allows rapid detection of three pathogen-specific targets simultaneously without compromising sensitivity. *F. tularensis* LOD 0.6-11.8 fg DNA/reaction.

Reference	Organism(s)	Sample Matrix	Sample Preparation Method	Detection Method	Summary
Janse et al., 2012 [79]	*F. tularensis* subspecies *holarctica, tularensis, and novicida, B. anthracis, Y. pestis, Coxiella burnetii*	Spiked blood, water, surface swab	200 L surface water was filter concentrated. DNA in filtrate extracted with NucliSens Magnetic Extraction Reagents (bioMérieux). Cotton swab samples collected at a goat farm were added to 10 mL of NucliSens lysis buffer and vortexed before DNA extraction. *B. thuringiensis* spore suspension (10^5 spores) added as an internal control to each sample before DNA extraction.	qPCR detected through direct hybridization to microarray probes OR target-specific primer extension followed by universal hybridization targeting: *fopA, wbtK, ISFtu2, pdpD*	The microarrays were capable of detecting multiple signature sequences with an internal control, making it possible to identify targeted pathogens and assess virulence potential.
Jeng et al., 2013 [80]	*F. tularensis, B. anthracis, Y. pestis, Brucella spp., Burkholderia spp, Rickettsia prowazekii*	Clinical bronchoalveolar lavage (BAL) fluids spiked with purified DNA	DNA from 300 µL of BAL fluid was extracted with the automated Roche Magna Pure LC robot with DNA isolation kit III protocol	Reverse transcription-PCR-electrospray ionization mass spectrometry	High-throughput reverse transcription-PCR coupled to electrospray ionization mass spectrometry analysis (RT-PCR-ESI-MS) can be used to detect biothreat agents in clinical samples.
McAvin et al., 2004 [81]	*F. tularensis* LVS	Purified DNA	DNA from pure cultures was extracted with MagNA Pure Nucleic Acid Isolation Kits.	R.A.P.I.D® platform for real-time PCR in the field	Study established a real-time PCR protocol for sputum and blood samples in the field using the R.A.P.I.D. system. LOD of 10 fg of DNA or 5 genomic equivalents (GE).
O'Connell et al., 2004 [82]	*F. tularensis* LVS	Spiked creamer, cornstarch, baking powder, flour	DNA from pure cultures of *F. tularensis* extracted with QIAGEN Dneasy mini spin columns.	Direct PCR in BioSeeq® handheld system	Bio-Seeq® technology is a novel system for use in areas with high concentrations of bacteria. LOD of *F. tularensis* determined to be 10^3 cells/reaction or less when the consumable sampling assembly is utilized with household powders.
Rachwal et al., 2012 [83]	*F. tularensis* Schu4, *B. anthracis, B. mallei, B. pseudomallei, Y. pestis*	Purified DNA	None- purified DNA was acquired for the study	TaqMan Array Cards developed for multiple biothreat organisms	TaqMan® Array Card was capable of detecting all five organisms, with a LOD one order of magnitude greater than the singleplex reactions (10 vs 100 fg/reaction).
Schweighardt et al., 2014 [84]	*F. tularensis* subspecies *tularensis, B. anthracis, Y. pestis, C. botulinum*	Purified DNA	None- purified DNA was acquired for the study	Bead-based liquid hybridization assay, Luminex ® 100TM, targeted ribosomal *rrl* and *F. tularensis* toxicity target	Proof of principle for laboratory samples for simultaneously identifying multiple pathogenic microorganisms. Achieved LODs of 0.1 to 10 ng DNA.

Reference	Organism(s)	Sample Matrix	Sample Preparation Method	Detection Method	Summary
Seiner et al., 2013 [85]	*F. tularensis* subspecies *tularensis* and *holarctica, B. anthracis, Y. pestis*	Purified DNA	Pure genomic DNA purchased for the study.	Multiplexed PCR-based assay for 17 pathogens and toxins	Proof of principle study for FilmArray platform as complete sample-to-answer system, combining sample preparation, PCR and data analysis. LOD at 250 GE.
Turingan et al., 2013 [12]	*F. tularensis, B. anthracis, Y. pestis*	Biowatch filters	Air filters were washed in sterile water, cells were lysed by sonication before DNA was purified by a Qiagen spin column	Microfluidic multiplexed PCR and sequencing assays	Study demonstrated a proof-of-principle for *F. tularensis, B. anthracis, and Y. pestis* detection and subspecies differentiation within environmental aerosol (Biowatch) samples using *B. subtilis.*
Yang et al., 2012 [86]	*F. tularensis* (410101), *B. anthracis, Y. pestis, Brucella* spp, *B. pseudomallei*	Pure cultures	DNA from pure cultures of *F. tularensis* was extracted from cells that were lysed by boiling.	Multiplex PCR targeting *fopA*	Results suggest that the liquid array method would be capable of detecting bioagents of interest from environmental samples. (LOD 0.95 pg DNA/reaction).

Field-deployable detection systems are needed for first responders. Ideally, field-deployable systems would be rugged, sensitive, specific, and easily manipulated within protective gear. Bio-Seeq®, Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.®), and FilmArray® systems are three technologies available for first responders that were discussed in the literature. The Bio-Seeq® instrument is a self-contained, portable, handheld, real-time PCR system that includes a consumable sampling and reaction tube assembly. The consumable assembly includes a sampling swab, buffer, and assay reagents. The operator simply uses the swab to sample a surface, and then inserts the swab into the system and twists to release the prepared buffer. Manual shaking completes the sample processing before inserting the unit into the Bio-Seeq® instrument for PCR analysis [82]. The Bio-Seeq® technology is a novel system for use in high concentration areas as the LOD of *F. tularensis* was determined to be 10^3 cells per reaction when the consumable sampling assembly is used. Furthermore, as the sample DNA is not purified, there could be significant inhibition when used for environmental soils or waters. *F. tularensis* was detectable when spiked into wheat flour, cornstarch, baking soda, and coffee creamer; however, inhibition was noted [82]. R.A.P.I.D. is a field deployable real-time PCR platform for which *F. tularensis* specific primers have been established [81]. A newly developed FilmArray® system utilizes a "Lab-in-a-Pouch" approach for conducting sample-to-answer detection of 17 biothreat agents [85]. A liquid sample is placed within the system pouch, which contains all the reagents required for sample preparation, cell lysis, PCR, and end-point detection. Thus far, the system has only been assessed with *B. anthracis* cells and spores, *Y. pestis* cells, and *F. tularensis* genomic DNA to demonstrate its proof-of-principle [85]. Therefore, future work evaluating field-deployable detecting systems for environmental liquids and soils is needed.

Multiplex real-time PCR detection methods could save both time and valuable resources during a crisis event. Janse et al. [78] developed a multiplex qPCR for simultaneously detecting three genes of *F. tularensis* (*fopA,* IS*Ftu2, pdpD*) while also incorporating an internal positive control (*B. thuringiensis* spores) for both nucleic acid extraction and amplification. The multi-target PCR was initially developed to reduce false positive and false negative results from environmental samples. While this method has not been verified specifically with soils, the authors stated that the method has been utilized for hundreds of solid and liquid samples [78]. More recently, the same research group developed a multiplex asymmetric PCR protocol that amplifies 16 DNA signatures for simultaneous detection of four biothreat agents. Four gene signatures are targeted from *F. tularensis, Y. pestis,* and *Coxiella burnetii*; three signatures are targeted from *B. anthracis,* and a single signature is dedicated to the internal positive control, *B. thuringiensis* [79]. Due to the number of amplified signatures, standard multiplex platforms are unable to differentiate the PCR products. Therefore, two labeling chemistries for microarray detection were compared [79]. Direct hybridization uses in-house labeled primers in the multiplex PCR to generate labeled PCR products, while target-specific primer extension followed by universal hybridization incorporates a unique capture tag sequence during strand extension by DNA polymerase. Both microarray formats allowed multiple pathogens to be simultaneously detected with high specificity and sensitivity [79]. The LOD for *F. tularensis* through either microarray

detection technology was determined to be 12 copies/reaction (target amplicon of 4.1) when targeting the internal spacer region, IS*Ftu2* [79].

Rachwal et al. [83] noted the trade-off between achieving multiple organism detection and minimizing LOD. They developed a TaqMan® Array Card that incorporated ten PCR reactions targeting five biothreat agents: *B. anthracis, B. mallei, B. pseudomallei, Y. pestis,* and *F. tularensis.* A comparison of PCR performance of the TaqMan® Array Card and singleplex real-time PCR using pure genomic DNA showed that while the TaqMan® Array Card was capable of detecting all five organisms, its LOD was one order of magnitude greater than the singleplex reactions [83]. In an attempt to minimize LODs and still achieve multiple pathogen identification, Brinkman et al. [75] developed a microarray-based method for simultaneously detecting *Cryptosporidium parvum, C. hominis, Enterococcus faecium, B. anthracis* and *F. tularensis* in concentrated aqueous samples. DNA microarrays can identify thousands of loci within a single sample, and their microarray assay was capable of detecting *F. tularensis* genomic DNA at 20 genomic copies without PCR preamplification. While this method has not been tested with soil samples, the concentrated tap water sample used within the study was equivalent to 33 L of tap water [75]. It is therefore conjectured, that after adequate optimization, soil sample suspensions might be suitable for analysis using this technology.

Other groups have also sought to detect multiple biothreat agents within a single assay. Turingan et al. [12] utilized a microfluidic biochip to develop a multiplexed PCR and sequencing assay for simultaneous detection of three pathogens, 10 loci per pathogen. Schweighardt et al. [84] developed a protocol using a Luminex® system to detect *B. anthracis, Clostridium botulinum, Y. pestis,* and *F. tularensis.* The Luminex® liquid array platform system uses genetically marked beads to simultaneously identify multiple pathogenic microorganisms, and can achieve LODs of 0.1 to 10 nanograms (ng) DNA [84]. Yang et al. [86] assessed a multi-targeted liquid array method for simultaneously detecting *B. anthracis, Y. pestis, B. pseudomallei, Brucella* spp., and *F. tularensis* within a simulated white-power sample. Universal 16S rRNA primers were used for amplification before identification using pathogen-specific hybridization probes. The Bio-Plex assay was then assessed using *B. anthracis* and *Y. pestis* spiked into various household white powders (milk powder, corn starch, wheat flour, instant drink mix). Results suggest that the liquid array method would be capable of detecting bioagents of interest from environmental samples [86]. A multiplex PCR enzyme hybridization assay (mPCR-EHA) has also been developed by He et al. [77] to simultaneously detect variola major, *B. anthracis, Y. pestis,* varicella zoster virus, and *F. tularensis* from within clinical samples. Jeng et al. [80] assessed the utility of high-throughput reverse transcription-PCR coupled to electrospray ionization mass spectrometry analysis (RT-PCR-ESI-MS) for detecting biothreat agents in clinical bronchoalveolar lavage (BAL) fluid specimens. Their analysis determined that RT-PCR-ESI-MS could provide accurate detection of multiple biothreat organisms from within polymicrobial clinical matrices [80]. Development of a qualitative real-time isothermal recombinase polymerase amplification (RPA) assay for *F. tularensis* alone [13] or in combination with *Y.*

pestis, *B. anthracis*, and variola virus [76] shows potential as a field deployable method for quick results (~10 min).

Cooper et al. [2] developed an assay for detecting *F. tularensis* from aqueous samples, but without PCR amplification. The prototype photonic biosensor utilizes label-free single-stranded oligonucleotides to consistently detect *F. tularensis* at low concentrations (minimum concentration tested, 1.7 ng) without PCR amplification. While the method needs to be optimized for field use, the initial studies demonstrate that the method could be a promising tool to rapidly detect *F. tularensis* in the field or with limited laboratory facilities [2]. While each of these technologies are promising, environmental assessments with complex environmental matrices are lacking and will need to be conducted to assess efficacy.

2.5 Combining Culture with PCR to detect live *F. tularensis*

The downfall of PCR techniques are their inability to discriminate between viable and non-viable target microorganisms. This review found two methods for rapid detection of viable pathogenic cells from various matrices by combining culture with PCR. Day and Whiting [87] utilized mammalian macrophage cell cultures to detect *F. tularensis* from contaminated foods. The macrophage cell cultures were exposed to contaminated foods (liquid baby formula, liquid egg whites, and iceberg lettuce mixed 1:1 with PBS) for two-hours to allow cell contact and engulfment of *F. tularensis*. After this initial incubation with the contaminated matrix, the macrophage monolayers are then washed with PBS to remove food particles and reconstituted with macrophage growth medium before an additional five to 18 hours of incubation. The additional incubation allows for proliferation of the engulfed *F. tularensis* within the macrophages. Finally, the macrophage monolayers are scraped from the plates, cleaned, and boiled to lyse the cells. The resulting supernatant is then used directly for real-time PCR analysis [87]. Using this method Day and Whiting [87] were able to detect viable *F. tularensis* from food matrices at a LOD of 10 CFU/mL formula or egg whites and 10 CFU/g lettuce within 22 hours.

In a similar manner, rapid viability (RV)-PCR utilizes an enrichment step and the change in cycle threshold time between two PCR reactions to determine the presence or absence of viable cells [88, 89]. RV-PCR has been used to detect viable *B. anthracis* spores from within dust, water, and dirty air filters [89]. For *B. anthracis* spore samples, spores within the environmental samples are separated from other particles and suspended in a growth medium. Prior to incubation, an aliquot of the sample is collected and processed for genomic identification by real-time PCR. After a minimum of nine hours of incubation an additional aliquot is collected and processed for real-time PCR. Comparing PCR cycle threshold numbers before and after incubation allows the discrimination between viable and non-viable *B. anthracis* spores [89]. While the literature review conducted herein did not find a study where environmental soil or waters were detected by either macrophage cell cultures or RV-PCR, culturing prior to PCR shows promise as a means to detect viable *F. tularensis* at low concentrations. Future work

expanding one or both of these methodologies might provide increased detection capabilities for environmental samples.

3 Conclusions and Identified Data Gaps

Limited work regarding *F. tularensis* detection in soil has been conducted. More information regarding *F. tularensis* detection in environmental waters is available. However, questions remain regarding the complete lifecycle of *F. tularensis* within the environment. The role protozoa and biofilms have in *F. tularensis* persistence needs to be elucidated [15, 31]. Additional information regarding how *F. tularensis* persists in the environment will be helpful in guiding research in the development of appropriate detection technologies targeting *F. tularensis* in microenvironments. Further, once the ecology of *F. tularensis* is understood, proper disinfection technologies for combating sustained *F. tularensis* outbreaks can be developed.

Culturing *F. tularensis* from environmental samples is challenging, yet isolating viable *F. tularensis* cultures from samples is required to evaluate factors such as pathogenicity and antibiotic sensitivity of environmental isolates. It is also the current approach for evaluating the efficacy of decontamination procedures. *F. tularensis* is slow-growing, nutritionally fastidious organism that requires 24 to 72 hours for growth [46] on supplemented medium [15]. Even with selective agars, *F. tularensis* colonies are often out-competed by background organisms present in environmental samples [46]. The review herein found 14 studies that utilized culture analyses with varying success. Future studies focused on the integration of culture and genomic identification could be the future for rapid viable detection [87, 89].

Immunoassay detection of *F. tularensis* can be amenable to hand-held devices, however due to high limits of detection their utility might only be seen in highly concentrated samples [52]. Protein chip immunoarrays can rapidly identify multiple bioagents within a single sample; yet again high limits of detection (~10^6 CFU/mL) limit its utility for screening potential low concentrations in environmental matrices [56]. The overall quality of immunoassays, whether in a single reaction or as part of an immunoarray chip, is dependent upon the specificity of the selected antigens. Some antigens can have cross-reactivity to other microorganism, thus impeding the results [51, 56]. Other immunosensor assays are on the horizon and could offer environmentally applicable methods after further development and optimization [59, 90].

Genomic identification of *F. tularensis* was the most common mode of identification seen in this review. Four genes were repeatedly used to identify *F. tularensis*: *tul4, fopA,* IS*Ftu2*, and *23kDa*. Sampling methods [44], sample purification methods [61], and the PCR primers used within a study can impact the overall findings [67]. A study focused on the specificity of various primers for *F. tularensis* noted that many published primers are not very specific, and therefore evaluate their primers against up-to-date genomic databases before their use is needed during future investigations [67]. It was also noted that no single *F. tularensis* marker was capable

distinguishing all *Francisella* strains; therefore, to maximize resolution multiple markers could be targeted [67].

As mentioned previously in this literature review, extracting DNA from *F. tularensis* is relatively easy when compared to sporulated microorganisms. However, constituents within soil and environmental waters must be removed from DNA samples to increase processing efficiency. UltraClean® DNA extraction kits were widely used for extracting DNA from various environmental sample matrices, and have demonstrated their ability to produce DNA of sufficient quantity and quality for downstream genomic analyses [16, 38, 50, 61, 62, 65]. However, while Whitehouse and Hottel [61] conducted a systematic comparison of DNA extraction kits for isolating *F. tularensis* DNA, laboratory inoculated soils are not equivalent to environmentally contaminated soils. Cells within real environmental samples might be aggregated with other constituents making DNA extraction more complicated [62]. In light of the complexity of soils and the potential for unknown inhibiting compounds in environmental samples, each analytical step should have internal controls. Janse et al. [79] has suggested *B. thuringiensis* spores as a possible agent for both extraction and amplification internal controls. Spores are added prior to sample extraction to ensure that even the most recalcitrant cells within the soil aliquot are lysed, while PCR inhibition is identified by using well studied PCR primers. Primers for the internal control could ideally have the same melting and annealing temperatures. A relatively new mechanism to prevent PCR inhibition is using inhibitor-resistant PCR reagents [66]. While one study herein optimized the use of inhibitor-resistant PCR reagents for detecting *F. tularensis* in soil samples, a detailed comparison of multiple soils with various extraction kits and various inhibitor-resistant PCR reagents might be needed to make generalizations about its applicability. Such an analysis could identify an optimum extraction kit in conjunction with optimum PCR reagents to yield real-time PCR reactions with increased sensitivity.

Microarray detection technologies offer the potential for high-throughput environmental detection. Several groups have utilized microarray technology to simultaneously detect multiple biothreat agents of interest [12, 75, 80, 84, 86] while few have assessed the technology with environmental samples and their associated complexities. Brinkman et al. [75] and Francy et al. [4] have demonstrated the utility of detecting *F. tularensis* genomic DNA from within highly concentrated tap water samples, and thus offers insight into the potential use with other environmental matrices. The introduction of whole genome amplification prior to microarray detection could further improve sensitivity [75]. Future work combining optimized DNA extraction, whole genome amplification with inhibition-resistant polymerases, and multiagent microarray detection could significantly expand biothreat detection capabilities.

Two groups identified by this review have utilized a combination of culture and genomic analysis to rapidly, quantify viable microorganisms. Using macrophage cell cultures to accelerate *F. tularensis* growth before DNA extraction and amplification, Day and Whiting [87] were able to detect viable *F. tularensis* within contaminated foods at a LOD of 10 CFU/mL. RV-PCR is another promising technique that utilizes an enrichment step between two PCR reactions to

quantify the concentration change of a targeted microorganism [89]. While RV-PCR has not been optimized for *F. tularensis* detection, it has been shown to be effective for *B. anthracis* spore detection from within environmental samples. Future work incorporating a macrophage culture step with RV-PCR sample processes could significantly improve viable *F. tularensis* detection capabilities from within environmental soil and waters.

4 References

1. Fujita, O., M. Tatsumi, K. Tanabayashi, and A. Yamada. (2006). Development of a real-time PCR assay for detection and quantification of *Francisella tularensis. Jpn J Infect Dis,* 59(1): 46-51.
2. Cooper, K.L., A.B. Bandara, Y. Wang, A. Wang, and T.J. Inzana. (2011). Photonic biosensor assays to detect and distinguish subspecies of *Francisella tularensis. Sensors,* 11(3): 3004-19.
3. USDA, Soil Survey Staff. (1999). *Soil taxonomy: A basic system of soil classification for making and interpreting soil surveys*. 2nd ed.: Natural Resources Conservation Service. U.S. Department of Agriculture Handbook.
4. Francy, D.S., R.N. Bushon, A.M. Brady, E.E. Bertke, C.M. Kephart, C.A. Likirdopulos, B.E. Mailot, F.W. Schaefer, 3rd, and H.D. Lindquist. (2009). Comparison of traditional and molecular analytical methods for detecting biological agents in raw and drinking water following ultrafiltration. *J Appl Microbiol,* 107(5): 1479-91.
5. Gilbert, S.E. and L.J. Rose. (2012). Survival and persistence of nonspore-forming biothreat agents in water. *Lett Appl Microbiol,* 55(3): 189-94.
6. Balestrazzi , A., M. Bonadei, C. Calvio, A. Galizzi, and D. Carbonera. (2009). DNA extraction from soil: comparison of different methods using spore-forming bacteria and the *swrAA* gene as indicators. *Ann Microbiol,* 59(4): 827-832.
7. Robe, P., R. Nalin, C. Capellano, T.M. Vogel, and P. Simonet. (2003). Extraction of DNA from soil. *Euro Journal Soil Biol,* 39(4): 183-190.
8. Zhou, J., M.A. Bruns, and J.M. Tiedje. (1996). DNA recovery from soils of diverse composition. *Appl Environ Microbiol,* 62(2): 316-22.
9. Kingry, L.C. and J.M. Petersen. (2014). Comparative review of *Francisella tularensis* and *Francisella novicida*. *Front Cell Infect Microbiol,* 4: 35.
10. Keim, P., A. Johansson, and D.M. Wagner. (2007). Molecular epidemiology, evolution, and ecology of *Francisella. Ann N Y Acad Sci,* 1105: 30-66.
11. McCoy, G.W. and C.W. Chapin. (1912). *Bacterium tularense*, the cause of a plaguelike disease of rodents *Public Health Bull* 53: 17-23.
12. Turingan, R.S., H.U. Thomann, A. Zolotova, E. Tan, and R.F. Selden. (2013). Rapid focused sequencing: a multiplexed assay for simultaneous detection and strain typing of *Bacillus anthracis, Francisella tularensis*, and *Yersinia pestis. PLoS One,* 8(2): e56093.
13. Euler, M., Y. Wang, P. Otto, H. Tomaso, R. Escudero, P. Anda, F.T. Hufert, and M. Weidmann. (2012). Recombinase polymerase amplification assay for rapid detection of *Francisella tularensis. J Clin Microbiol,* 50(7): 2234-8.
14. Duncan, D.D., A.J. Vogler, M.J. Wolcott, F. Li, D.S. Sarovich, D.N. Birdsell, L.M. Watson, T.A. Hall, R. Sampath, R. Housley, L.B. Blyn, S.A. Hofstadler, D.J. Ecker, P. Keim, D.M. Wagner, and M.W. Eshoo. (2013). Identification and typing of *Francisella tularensis* with a highly automated genotyping assay. *Lett Appl Microbiol,* 56(2): 128-34.
15. van Hoek, M.L. (2013). Biofilms: an advancement in our understanding of *Francisella* species. *Virulence,* 4(8): 833-46.
16. Berrada, Z.L. and S.R. Telford, 3rd. (2010). Diversity of *Francisella* species in environmental samples from Martha's Vineyard, Massachusetts. *Microb Ecol,* 59(2): 277-83.

17. Meric, M., M. Sayan, D. Dundar, and A. Willke. (2010). Tularaemia outbreaks in Sakarya, Turkey: Case-control and environmental studies. *Singapore Med J*, 51(8): 655-9.
18. Nakazawa, Y., R.A. Williams, A.T. Peterson, P.S. Mead, K.J. Kugeler, and J.M. Petersen. (2010). Ecological niche modeling of *Francisella tularensis* subspecies and clades in the United States. *Am J Trop Med Hyg*, 82(5): 912-8.
19. Reintjes, R., I. Dedushaj, A. Gjini, T.R. Jorgensen, B. Cotter, A. Lieftucht, F. D'Ancona, D.T. Dennis, M.A. Kosoy, G. Mulliqi-Osmani, R. Grunow, A. Kalaveshi, L. Gashi, and I. Humolli. (2002). Tularemia outbreak investigation in Kosovo: Case control and environmental studies. *Emerg Infect Dis*, 8(1): 69-73.
20. Dennis, D.T., T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A.D. Fine, A.M. Friedlander, J. Hauer, M. Layton, S.R. Lillibridge, J.E. McDade, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, and K. Tonat. (2001). Tularemia as a biological weapon: medical and public health management. *JAMA*, 285(21): 2763-73.
21. Grunow, R. and E.J. Finke. (2002). A procedure for differentiating between the intentional release of biological warfare agents and natural outbreaks of disease: its use in analyzing the tularemia outbreak in Kosovo in 1999 and 2000. *Clin Microbiol Infect*, 8(8): 510-21.
22. Broman, T., J. Thelaus, A.C. Andersson, S. Backman, P. Wikstrom, E. Larsson, M. Granberg, L. Karlsson, E. Back, H. Eliasson, R. Mattsson, A. Sjostedt, and M. Forsman. (2011). Molecular detection of persistent *Francisella tularensis* subspecies *holarctica* in natural waters. *Int J Microbiol*, 2011.
23. Johansson, A., A. Ibrahim, I. Goransson, U. Eriksson, D. Gurycova, J.E. Clarridge, 3rd, and A. Sjostedt. (2000). Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol*, 38(11): 4180-5.
24. Whitehouse, C.A., K.E. Kesterson, D.D. Duncan, M.W. Eshoo, and M. Wolcott. (2012). Identification and characterization of *Francisella* species from natural warm springs in Utah, USA. *Lett Appl Microbiol*, 54(4): 313-24.
25. Forsman, M., E.W. Henningson, E. Larsson, T. Johansson, and G. Sandstrom. (2000). *Francisella tularensis* does not manifest virulence in viable but non-culturable state. *FEMS Microbiol Ecol*, 31(3): 217-224.
26. Mitscherlich, E. and E.H. Marth. (1984). *Microbial Survival in the Environment*. Berlin: Springer-Verlag. 741-742.
27. Goethert, H.K. and S.R. Telford, 3rd. (2009). Nonrandom distribution of vector ticks (*Dermacentor variabilis*) infected by *Francisella tularensis*. *PLoS Pathog*, 5(2): e1000319.
28. Davis-Hoover, W., W.G. Wade, Y. Li, T.D. Biggs, and P.G. Koga. (2006). Persistence of *Bacillus anthracis* spores and *Clostridium botulinum* and destruction of *Francisella tularensis* and *Yersinia pestis* in municipal solid waste landfill leachates, in *Fourth Intercontinental Landfill Research Symposium*: Gällivare (Lapland), SWEDEN, 2006.
29. Kuske, C.R., S.M. Barns, C.C. Grow, L. Merrill, and J. Dunbar. (2006). Environmental survey for four pathogenic bacteria and closely related species using phylogenetic and functional genes. *J Forensic Sci*, 51(3): 548-58.

30. EPA and CDC. (2012). Method development for optimum recovery of *Yersinia pestis* from transport media and swabs. Environmental Protection Agency and Centers for Disease Control and Prevention. EPA/600/R-12/620.
31. Abd, H., T. Johansson, I. Golovliov, G. Sandstrom, and M. Forsman. (2003). Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Appl Environ Microbiol,* 69(1): 600-6.
32. Sjostedt, A. (2006). Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. *Microbes Infect,* 8(2): 561-7.
33. Visvesvara, G.S. (2010). Free-Living amebae as opportunistic agents of human disease. *J Neuroparasitology,* 1:(Article ID N100802) (13 pages).
34. Kantardjiev, T. and T. Velinov. (1995). Interaction between protozoa and mircoorganisms of the genus *Francisella. Problems Infect Dis,* 22: 34-35.
35. Svensson, K., M. Granberg, L. Karlsson, V. Neubauerova, M. Forsman, and A. Johansson. (2009). A real-time PCR array for hierarchical identification of *Francisella* isolates. *PLoS One,* 4(12): e8360.
36. Durham-Colleran, M.W., A.B. Verhoeven, and M.L. van Hoek. (2010). *Francisella novicida* forms in vitro biofilms mediated by an orphan response regulator. *Microb Ecol,* 59(3): 457-65.
37. Mahajan, U.V., J. Gravgaard, M. Turnbull, D.B. Jacobs, and T.L. McNealy. (2011). Larval exposure to *Francisella tularensis* LVS affects fitness of the mosquito *Culex quinquefasciatus. FEMS Microbiol Ecol,* 78(3): 520-30.
38. Offermans, M.T.C. and N.D. Zegers. (2007). Test Results 7th NATO-SIBCRA BW Round Robin Trial 2006. The Netherlands. TNO Defence, Security, and Safety, Delft, Netherlands. TD2007-0043.
39. Sellek, R., O. Jimenez, C. Aizpurua, B. Fernandez-Frutos, P. De Leon, M. Camacho, D. Fernandez-Moreira, C. Ybarra, and J. Carlos Cabria. (2008). Recovery of *Francisella tularensis* from soil samples by filtration and detection by real-time PCR and cELISA. *J Environ Monit,* 10(3): 362-9.
40. Şimşek, H., M. Taner, A. Karadenizli, M. Ertek, and H. Vahaboğlu. (2012). Identification of *Francisella tularensis* by both culture and real-time TaqMan PCR methods from environmental water specimens in outbreak areas where tularemia cases were not previously reported. *Euro J Clin Microbiol & Infect Dis,* 31(9): 2353-2357.
41. Anda, P., J. Segura del Pozo, J.M. Diaz Garcia, R. Escudero, F.J. Garcia Pena, M.C. Lopez Velasco, R.E. Sellek, M.R. Jimenez Chillaron, L.P. Sanchez Serrano, and J.F. Martinez Navarro. (2001). Waterborne outbreak of tularemia associated with crayfish fishing. *Emerg Infect Dis,* 7(3 Suppl): 575-82.
42. Petersen, J.M., J. Carlson, B. Yockey, S. Pillai, C. Kuske, G. Garbalena, S. Pottumarthy, and L. Chalcraft. (2009). Direct isolation of *Francisella* spp. from environmental samples. *Lett Appl Microbiol,* 48(6): 663-7.
43. EPA and CDC. (2011). Comparison of ultrafiltration techniques for recovering biothreat agents in water. Environmental Protection Agency and the Centers for Disease Control and Prevention. EPA 600/R-11/103.
44. Johansson, A., L. Berglund, U. Eriksson, I. Goransson, R. Wollin, M. Forsman, A. Tarnvik, and A. Sjostedt. (2000). Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia. *J Clin Microbiol,* 38(1): 22-6.

45. Walker, R.E., J.M. Petersen, K.W. Stephens, and L.A. Dauphin. (2010). Optimal swab processing recovery method for detection of bioterrorism-related *Francisella tularensis* by real-time PCR. *J Microbiol Methods,* 83(1): 42-7.
46. Versage, J.L., D.D.M. Severin, M.C. Chu, and J.M. Petersen. (2003). Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *J Clin Microbiol,* 41(12): 5492-5499.
47. Humrighouse, B.W., N.J. Adcock, and E.W. Rice. (2011). Use of acid treatment and a selective medium to enhance the recovery of *Francisella tularensis* from water. *Appl Environ Microbiol,* 77(18): 6729-32.
48. Berrada, Z.L. and S.R. Telford. (2011). Survival of *Francisella tularensis* Type A in brackish-water. *Arch Microbiol,* 193(3): 223-6.
49. Petersen, J.M., M.E. Schriefer, K.L. Gage, J.A. Montenieri, L.G. Carter, M. Stanley, and M.C. Chu. (2004). Methods for enhanced culture recovery of *Francisella tularensis. Appl Environ Microbiol,* 70(6): 3733-3735.
50. Barns, S.M., C.C. Grow, R.T. Okinaka, P. Keim, and C.R. Kuske. (2005). Detection of diverse new *Francisella*-like bacteria in environmental samples. *Appl Environ Microbiol,* 71(9): 5494-500.
51. Pohanka, M. and P. Skladal. (2009). *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis*. The most important bacterial warfare agents - review. *Folia Microbiol,* 54(4): 263-72.
52. Berdal, B.P., R. Mehl, H. Haaheim, M. Loksa, R. Grunow, J. Burans, C. Morgan, and H. Meyer. (2000). Field detection of *Francisella tularensis. Scand J Infect Dis,* 32(3): 287-91.
53. Peruski, A.H., L.H. Johnson, 3rd, and L.F. Peruski, Jr. (2002). Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. *J Immunol Methods,* 263(1-2): 35-41.
54. Grunow, R., P. Miethe, W. Conlan, E.-J. Finke, S. Friedewald, and M. Porsch-Ozcurumez. (2008). Rapid detection of *Francisella tularensis* by the immunoaffinity assay ABICAP in environmental and human samples. *J Rapid Methods Autom Microbiol,* 16(1): 30-54.
55. Grunow, R., A. Kalaveshi, A. Kuhn, G. Mulliqi-Osmani, and N. Ramadani. (2012). Surveillance of tularaemia in Kosovo, 2001 to 2010. *Euro Surveill,* 17(28).
56. Huelseweh, B., R. Ehricht, and H.J. Marschall. (2006). A simple and rapid protein array based method for the simultaneous detection of biowarfare agents. *Proteomics,* 6(10): 2972-81.
57. Pohanka, M. and P. Skládal. (2007). Piezoelectric immunosensor for the direct and rapid detection of *Francisella tularensis. Folia Microbiol,* 52(4): 325-330.
58. O'Brien, T., L.H. Johnson 3rd, J.L. Aldrich, S.G. Allen, L.-T. Liang, A.L. Plummer, S.J. Krak, and A.A. Boiarski. (2000). The development of immunoassays to four biological threat agents in a bidiffractive grating biosensor. *Biosens Bioelectron,* 14(10–11): 815-828.
59. Sharma, N., A. Hotta, Y. Yamamoto, O. Fujita, A. Uda, S. Morikawa, A. Yamada, and K. Tanabayashi. (2013). Detection of *Francisella tularensis*-specific antibodies in patients with tularemia by a novel competitive enzyme-linked immunosorbent assay. *Clin Vaccine Immunol,* 20(1): 9-16.

60. Gillings, M.R. (2014). Rapid extraction of PCR-competent DNA from recalcitrant environmental samples. *Methods Mol Biol,* 1096: 17-23.
61. Whitehouse, C.A. and H.E. Hottel. (2007). Comparison of five commercial DNA extraction kits for the recovery of *Francisella tularensis* DNA from spiked soil samples. *Mol Cell Probes,* 21(2): 92-6.
62. Klerks, M.M., A.H. van Bruggen, C. Zijlstra, and M. Donnikov. (2006). Comparison of methods of extracting *Salmonella enterica* serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control. *Appl Environ Microbiol,* 72(6): 3879-86.
63. MoBio Laboratories. (2010). *UltraClean® Soil DNA Isolation Kit*. MoBio Laboratories, Inc. p. 21.
64. Bader, D.E., G.R. Fisher, and L.J. McLaws. (2003). Molecular genetic analysis of killed biological agents in sample unknowns: NATO SIBCA Exercise III. TR-2003-043.
65. Bader, D.E., G.R. Fisher, and L.J. McLaws. (2004). Analysis of a simulated biological/chemical agent mixture in soil. TM 2004-164.
66. Trombley Hall, A., A. McKay Zovanyi, D.R. Christensen, J.W. Koehler, and T. Devins Minogue. (2013). Evaluation of inhibitor-resistant real-time PCR methods for diagnostics in clinical and environmental samples. *PLoS One,* 8(9): e73845.
67. Ahlinder, J., C. Ohrman, K. Svensson, P. Lindgren, A. Johansson, M. Forsman, P. Larsson, and A. Sjodin. (2012). Increased knowledge of *Francisella* genus diversity highlights the benefits of optimised DNA-based assays. *BMC Microbiol,* 12: 220.
68. Buzard, G.S., D. Baker, M.J. Wolcott, D.A. Norwood, and L.A. Dauphin. (2012). Multi-platform comparison of ten commercial master mixes for probe-based real-time polymerase chain reaction detection of bioterrorism threat agents for surge preparedness. *Forensic Sci Int,* 223(1-3): 292-7.
69. Escudero, R., A. Toledo, H. Gil, K. Kovacsova, M. Rodriguez-Vargas, I. Jado, C. Garcia-Amil, B. Lobo, M. Bhide, and P. Anda. (2008). Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. *J Clin Microbiol,* 46(9): 3139-43.
70. Forsman, M., A. Nyrén, A. Sjöstedt, L. Sjökvist, and G. Sandström. (1995). Identification of *Francisella tularensis* in natural water samples by PCR. *FEMS Microbiol Ecol,* 16(1): 83-92.
71. Garcia Del Blanco, N., M.E. Dobson, A.I. Vela, V.A. De La Puente, C.B. Gutierrez, T.L. Hadfield, P. Kuhnert, J. Frey, L. Dominguez, and E.F. Rodriguez Ferri. (2002). Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. *J Clin Microbiol,* 40(8): 2964-72.
72. Matero, P., H. Hemmila, H. Tomaso, H. Piiparinen, K. Rantakokko-Jalava, L. Nuotio, and S. Nikkari. (2011). Rapid field detection assays for *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis. Clin Microbiol Infect,* 17(1): 34-43.
73. Oyston, P.C., A. Sjostedt, and R.W. Titball. (2004). Tularaemia: bioterrorism defence renews interest in *Francisella tularensis. Nat Rev Microbiol,* 2(12): 967-78.
74. Brett, M.E., L.B. Respicio-Kingry, S. Yendell, R. Ratard, J. Hand, G. Balsamo, C. Scott-Waldron, C. O'Neal, D. Kidwell, B. Yockey, P. Singh, J. Carpenter, V. Hill, J.M. Petersen, and P. Mead. (2014). Outbreak of *Francisella novicida* bacteremia among inmates at a Louisiana correctional facility. *Clin Infect Dis,* 59(6): 826-33.

75. Brinkman, N.E., R. Francisco, T.L. Nichols, D. Robinson, F.W. Schaefer, 3rd, R.P. Schaudies, and E.N. Villegas. (2013). Detection of multiple waterborne pathogens using microsequencing arrays. *J Appl Microbiol,* 114(2): 564-73.
76. Euler, M., Y. Wang, D. Heidenreich, P. Patel, O. Strohmeier, S. Hakenberg, M. Niedrig, F.T. Hufert, and M. Weidmann. (2013). Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *J Clin Microbiol,* 51(4): 1110-7.
77. He, J., A.J. Kraft, J. Fan, M. Van Dyke, L. Wang, M.E. Bose, M. Khanna, J.A. Metallo, and K.J. Henrickson. (2009). Simultaneous detection of CDC category "A" DNA and RNA bioterrorism agents by use of multiplex PCR & RT-PCR enzyme hybridization assays. *Viruses,* 1(3): 441-459.
78. Janse, I., R.A. Hamidjaja, J.M. Bok, and B.J. van Rotterdam. (2010). Reliable detection of *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis* by using multiplex qPCR including internal controls for nucleic acid extraction and amplification. *BMC Microbiol,* 10: 314.
79. Janse, I., J.M. Bok, R.A. Hamidjaja, H.M. Hodemaekers, and B.J. van Rotterdam. (2012). Development and comparison of two assay formats for parallel detection of four biothreat pathogens by using suspension microarrays. *PLoS One,* 7(2): e31958.
80. Jeng, K., J. Hardick, R. Rothman, S. Yang, H. Won, S. Peterson, Y.H. Hsieh, B.J. Masek, K.C. Carroll, and C.A. Gaydos. (2013). Reverse transcription-PCR-electrospray ionization mass spectrometry for rapid detection of biothreat and common respiratory pathogens. *J Clin Microbiol,* 51(10): 3300-7.
81. McAvin, J.C., M.M. Morton, R.M. Roudabush, D.H. Atchley, and J.R. Hickman. (2004). Identification of *Francisella tularensis* using real-time fluorescence polymerase chain reaction. *Mil Med,* 169(4): 330-3.
82. O'Connell, K.P., P.E. Anderson, J.J. Valdes, and J.R. Bucher. (2004). Testing of the Bio-SEEQ® (Smiths detection handheld PCR instrument): sensitivity, secificity, and effect of interferents on *Francisella tularensis* assay performance. Aberdeen Proving Ground, MD: U.S. Army Research, Development and Engineering Command. ECBC-TR-380.
83. Rachwal, P.A., H.L. Rose, V. Cox, R.A. Lukaszewski, A.L. Murch, and S.A. Weller. (2012). The potential of TaqMan Array Cards for detection of multiple biological agents by real-time PCR. *PLoS One,* 7(4): e35971.
84. Schweighardt, A.J., A. Battaglia, and M.M. Wallace. (2014). Detection of anthrax and other pathogens using a unique liquid array technology. *J Forensic Sci,* 59(1): 15-33.
85. Seiner, D.R., H.A. Colburn, C. Baird, R.A. Bartholomew, T. Straub, K. Victry, J.R. Hutchison, N. Valentine, and C.J. Bruckner-Lea. (2013). Evaluation of the FilmArray® system for detection of *Bacillus anthracis, Francisella tularensis* and *Yersinia pestis. J Appl Microbiol,* 114(4): 992-1000.
86. Yang, Y., J. Wang, H. Wen, and H. Liu. (2012). Comparison of two suspension arrays for simultaneous detection of five biothreat bacterial in powder samples. *J Biomed Biotechnol,* 2012: 831052.
87. Day, J.B. and R.C. Whiting. (2009). Development of a Macrophage Cell Culture Method To Isolate and Enrich *Francisella tularensis* from Food Matrices for Subsequent Detection by Real-Time PCR. *J Food Protect,* 72(6): 1156-1164.
88. Kane, S.R., S.E. Letant, G.A. Murphy, T.M. Alfaro, P.W. Krauter, R. Mahnke, T.C. Legler, and E. Raber. (2009). Rapid, high-throughput, culture-based PCR methods to

analyze samples for viable spores of *Bacillus anthracis* and its surrogates. *J Microbiol Methods,* 76(3): 278-84.
89. Letant, S.E., G.A. Murphy, T.M. Alfaro, J.R. Avila, S.R. Kane, E. Raber, T.M. Bunt, and S.R. Shah. (2011). Rapid-viability PCR method for detection of live, virulent *Bacillus anthracis* in environmental samples. *Appl Environ Microbiol,* 77(18): 6570-8.
90. Rydzewski, K., T. Schulz, E. Brzuszkiewicz, G. Holland, C. Luck, J. Fleischer, R. Grunow, and K. Heuner. (2014). Genome sequence and phenotypic analysis of a first German *Francisella* sp. isolate (W12-1067) not belonging to the species *Francisella tularensis. BMC Microbiol,* 14(1): 169.

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