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Rapid viability polymerase chain reaction method for detection of *Francisella tularensis*Staci R. Kane^a, Sanjiv R. Shah^{b,*}, Teneile M. Alfaro^a^a Lawrence Livermore National Laboratory, Livermore, CA, USA^b National Homeland Security Research Center, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC, USA

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ABSTRACT

Francisella tularensis, which causes potentially fatal tularemia, has been considered an attractive agent of bioterrorism and biological warfare due to its low infectious dose, reported environmental persistence, and ability to be transmitted to humans via multiple exposure routes. Due to slow growth on even selective culture media, detection of viable *F. tularensis* from environmental and drinking water samples, usually takes > 3 days. Therefore, a rapid viability polymerase chain reaction (RV-PCR) method was developed to detect and identify viable *F. tularensis* cells in environmental samples. The method uses a change in PCR response during high throughput (48-well) sample incubation in Brain Heart Infusion/Vitox/Fildes/Histidine growth medium to detect viable *F. tularensis* presence, which is at least two times faster than the current plate culture-based method. Using the method, 10^1 to 10^2 live *F. tularensis* cells were detected in simulated complex sample matrices containing chemical and biological interferences.

Francisella tularensis (*F. tularensis*), the pathogen that causes tularemia in humans and animals, could be introduced into water infrastructure due to a natural outbreak, laboratory accident, or intentional contamination. It is known that *F. tularensis*, similar to other vegetative bacterial pathogens, can remain viable and infectious for at least 14 days and up to a year or more in certain environments including water (Anda et al., 2001; Berrada and Telford III., 2011; Gilbert and Rose, 2012; Parker et al., 1951). As reviewed by Rice (2015), drinking water outbreaks of tularemia have been reported in the U.S. and several countries including Bulgaria, Georgia, Germany, Italy, Kosovo, Norway, Russia, Sweden, and Turkey.

F. tularensis is a category A select agent because it can readily be weaponized, has a low infective dose (1–10 cells; Jones et al., 2005; Saslaw et al., 1961), and high morbidity and mortality. Therefore, national security concerns have been raised regarding this potential bioterrorism agent (Oyston et al., 2004). As a result, consensus-based recommendations have been developed for civilian defense if *F. tularensis* is used as a biological weapon (CDC, 2000; Dennis et al., 2001).

Francisella tularensis is a Gram-negative coccobacillus whose main transmission vectors are arthropods (ticks, deer flies), while small mammals (e.g., rabbits, muskrats) serve as reservoir hosts. Protozoa have also been suggested as an important environmental reservoir (Abd et al., 2003). A security concern surrounding this bacterium is its

potential persistence in the environment. Due to its historical usage as a biological weapon (Gürçan, 2014) and the occurrence of natural tularemia outbreaks, there is a need for rapid and sensitive analytical methods for detection of viable *F. tularensis* in environmental samples to ensure public safety and to help mitigate impacts of facility and infrastructure closures following a *F. tularensis* contamination incident.

The current agar plate culture-based methods used for detection of *F. tularensis* and other bioterror agents are labor-intensive and have a low throughput (~30–40 samples processed per laboratory per 8-hr shift), with confirmed results obtained only after several days. In fact, *F. tularensis* detection via culture-based methods and subsequent confirmation takes 3 days or longer (Stewart, 1995). A review of methods for soil and water sample processing and analysis for *F. tularensis* described challenges with using traditional culture with these complex sample types including competition from other faster-growing, non-fastidious microorganisms (US EPA, 2015). In fact, multiple instances were reported where culture identification was unsuccessful.

We recently reported the development of a RV-PCR method for *Yersinia pestis* detection from sample matrices containing similar types of chemical and biological challenge material (Kane et al., 2019). In the current effort, a similar approach was used to develop the RV-PCR method for *F. tularensis*, including high-throughput sample incubation with 48-well plates (E&K Scientific, Santa Clara, CA; Cat. No. EK-2044);

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Table 1
Nucleotide sequences of the primer/probe sets used for *Francisella tularensis* RV-PCR analysis.

Assay ID	Forward Primer ^a	Reverse Primer ^a	Probe ^a	Amplicon Length (bp)	Reference
F4	TTGCTCCAAGTAGCTGCAAGATT	CCAAGTGTCTTGGTGGTGGTA	FAM-TGCTGCCGAGATGTTTCATATTAACGTGATGC-BHQ	125	This study Kugeler et al. (2006)
F5	GAGACATCAATTAAAAGAAGCAATACCTT	CCAAGAGTACTATTTCCCGTTGGT	FAM-AAAATTCTGCTCAGCAGGATTTTGATTTGGTT-BHQ	105	

^a Sequences are listed in 5' to 3' orientation. Acronyms: bp, base pair; FAM, fluorescein; BHQ, Black Hole Quencher.

however, differences included 1) use of optimized Brain Heart Infusion (BHI)/Vitox/Fildes/Histidine (BVFH) growth medium (Morris et al., 2017), which was concentrated 6-fold rather than 10-fold as described for the *Y. pestis* growth medium, 2) addition of 0.6-mL of 6-fold concentrated BVFH medium to 3-mL sample to yield 1 × concentration, 3) incubation at 37 °C instead of 30 °C, and 4) use of a 30-h incubation period instead of 24-h, since *F. tularensis* has a longer doubling time than *Y. pestis* (approx. 6 h (Heine et al., 2016) compared to < 2 h (Chauvaux et al., 2007), respectively).

The 3-mL samples were prepared with phosphate buffered saline (PBS; Teknova, Inc., Hollister, CA; Cat. No. P0261) with or without challenge materials including 1) 4 mg/mL Arizona Test Dust (ATD; ISO 12103-1, A3 Medium Test Dust; Powder Technology, Arden Hills, MN) shown to contain ~5 × 10⁴ colony-forming units (CFU) per 10 mg including fungi and bacterial spores (Rose et al., 2011), 2) 10 µg/mL Fe²⁺ as iron sulfate heptahydrate (Sigma-Aldrich, St. Louis, MO; Cat. No. 215422), and 3) 50 µg/mL humic acid (Sigma-Aldrich, Cat. No. 53680-10G) as a surrogate for natural organic matter. Ferrous iron and humic acid are known PCR inhibitors (Teng et al., 2008; Tsai and Olson, 1992) and these concentrations were at the upper end of the range of values expected for drinking water samples (Farrah et al., 1978; NRC, 1979; WHO, 1996; US EPA, 2005). PBS was used as a substitute for water samples because it maintained cell viability and represented a reproducible matrix; in addition, its use is consistent with the EPA protocol for collection, concentration and resuspension of vegetative pathogens from large volume water samples (US EPA, 2017). *F. tularensis* Schu S4 cells were grown in BVFH medium at 37 °C (180 rpm) to an OD₆₀₀ (optical density at 600 nm) of ~1.5 which was diluted 10-fold in BVFH medium (to ~1 × 10⁹ CFU/mL), followed by centrifugation (3000 × g for 10 min at 4 °C), washing one time in PBS buffer, and repeated centrifugation. The cell pellet was then suspended and diluted in PBS (with or without challenge material) to obtain average cell densities from 12 to 2500 CFU/3-mL based on serial dilution and plating of the inoculum onto Chocolate Agar (Hardy Diagnostics, Santa Maria, CA; Cat. No. E14) with incubation at 37 °C for 3 days.

The 6 × BVFH medium was prepared as follows: 1) 22.2 g Bacto™ Brain Heart Infusion Broth Base powder (BD Biosciences, Franklin Lakes, NJ; Cat. No. 237500) and 0.6 g L-histidine (Sigma-Aldrich, Cat. No. H8000-5G) were dissolved in 28 mL Millipore water and brought to boiling for 1 min followed by cooling the solution; 2) 12 mL of sterile Vitox Supplement (Thermo Fisher Scientific, Waltham, MA; Cat. No. SR0090A) was added, 3) 60 mL of sterile Fildes Enrichment (Thermo Fisher Scientific, Cat. No. R45037) was added, and 4) the solution was filter-sterilized with a disposable filtration system. The 6 × solution was stored at 4 °C and brought to room temperature before use. Concentration of the growth medium enabled use of a larger volume water sample (3-mL plus 0.6 mL 6 × BVFH medium per 5-mL well) and demonstrated reproducible growth even at low inoculum levels, where 10¹ CFU/3-mL increased approx. 2 to 2.6-log for 30-h incubation. The 6 × BVFH was mixed with the water sample (PBS with or without challenge material) by pipettor, 0.5-mL aliquots were removed before (T₀ aliquot) and after sample incubation at 37 °C with shaking at 180 rpm for 30-h (T₃₀ aliquot). The 48-well plate was sealed with a sterile AeraSeal™ breathable adhesive seal (Excel Scientific, Victorville, CA; Cat. No. BS-25) during incubation. The aliquots were processed as previously described (Kane et al., 2019) using a modified Promega paramagnetic particle (PMP)-based DNA extraction protocol (MagneSil® Blood Genomic, Max Yield System; Promega, Madison, WI; Cat. No. MD1360) yielding T₀ and T₃₀ DNA extracts for PCR analysis.

In some cases, a duplicate aliquot was removed and processed using an automated Roche MagNA Pure Compact instrument with Nucleic Acid Isolation Kit I (Roche, Indianapolis, IN; Cat. No. 03730964001) protocol for comparison purposes and to show that part of the method could be automated. Briefly, the sample aliquots were treated by heat lysis at 70 °C for 10 min, cooling for 3 min, addition of 300 µL Roche chemical lysis buffer, followed by vortexing tubes for 10 s at

1800–2000 rpm, and incubation at 22–25 °C for 30 min (with 3–5 s vortexing every 5 min). These crude sample lysates were then processed on the Roche instrument using the pre-programmed DNA-Bacteria Purification Protocol yielding 100 µL purified DNA extracts. This validated inactivation procedure per the Centers for Disease Control and Prevention (CDC) select agent regulations (CDC and USDA, 2018) was approved via LLNL Institutional Biosafety Committee review prior to transfer of crude sample lysates to the robot (within the same laboratory) for subsequent extraction and DNA purification.

Two real-time PCR assays targeting the *pdpD*, pathogenicity determinant protein D unique to Type A *F. tularensis* were used (Table 1) including the F4 assay designed at LLNL and an assay designed at the CDC (Kugeler et al., 2006), referred to here as F5. Real-time PCR analysis was conducted using an Applied Biosystems® 7500 Fast Real-Time PCR System (Foster City, CA) as previously described (Kane et al., 2019). In addition, Platinum™ Taq DNA Polymerase (Plat Taq; Invitrogen, Carlsbad, CA; Cat. No. 10966026) was included at 0.25 µL per reaction (1.25 U) to determine its effect on assay sensitivity. Three replicate samples were analyzed per experimental condition, and three replicate PCR analyses were conducted per sample replicate. DNA extracts from T₀ and T₃₀ from the same samples were analyzed on the same plate to standardize the analysis conditions. Both undiluted and 10-fold-diluted DNA extracts (prepared in PCR water) for both T₀ and T₃₀ were analyzed to check for PCR inhibition (i.e., if the difference between C_T values for 10-fold diluted and undiluted extracts is negative and/or significantly less than three).

For cases where no PCR response was obtained (non-detect [NDT] results), the C_T values were set to 45 (since 45 PCR cycles were used), to calculate ΔC_T. A ΔC_T ≥ 6 represented an increase in DNA concentration of approximately 2-log (100-fold) or greater because of the presence of viable cells in the original sample that propagated during incubation. For individual replicates within an experiment, the RV-PCR result was considered positive when at least two of three replicates met the algorithm requirement. Specifically, if two or three of three PCR replicates were NDT, then a C_T value of 45 was assigned as the average T₀ C_T or T₃₀ C_T (as appropriate) to calculate ΔC_T; whereas, if two or three of three replicates showed C_T values ≤ 45, the average of those values ≤ 45 was used to calculate ΔC_T. The RV-PCR method sensitivity of detection was equivalent to the *F. tularensis* cell level where 100% of the spiked samples had ΔC_T ≥ 6. This value was essentially an analytical sensitivity of detection for the RV-PCR method and this work did not investigate losses that could occur from sampling and sample handling (i.e., transport, storage) prior to RV-PCR analysis.

Real-time PCR assay optimization showed that addition of 1.25 U Plat Taq per reaction resulted in better sensitivity, with average C_T values reduced with the enzyme addition by an average 9.8 and 8.9 C_T units for F4 and F5 assays, respectively. Both assays showed detection of 5 fg Schu S4 genomic DNA with Plat Taq added; therefore, it was included along with the AmpliTaq Gold® DNA polymerase from the MasterMix. In addition, DNA extracts from the automated Roche MagNA Pure® Compact showed an average 1.8 ± 0.9 lower C_T values than the manual Promega MagneSil® DNA kit extracts. Although both assays consistently demonstrated < 30 genome equivalent sensitivity, other *F. tularensis* assays could readily be integrated into the RV-PCR method, as well.

The resulting RV-PCR method showed good sensitivity, using the criterion of ΔC_T values ≥ 6 for positive detection even in the presence of chemical and biological backgrounds (Table 2). For samples containing Fe and humic acid, 10¹ Schu S4 CFU-level were detected, whereas, 10² CFU-level were detected for samples with ATD (containing background microbes). In general, PCR inhibition did not appear to contribute to higher C_T values since the difference between T₃₀ C_T values for undiluted and 10-fold diluted extracts from ATD-spiked samples showed roughly a three C_T difference (data not shown), as expected for a 10-fold lower DNA concentration. It is suggested that both undiluted and 10-fold diluted DNA extracts (T₀ and T₃₀) should be analyzed for

Table 2

RV-PCR results for different starting cell levels per sample with and without chemical and biological backgrounds.

Treatment	Starting CFU/3-mL Sample ^a	ΔC _T Range	Positive RV-PCR Samples ^c
Control	2500 ± 170	7.2–9.3	3 of 3
	1200 ± 350	7.4–9.3	3 of 3
	250 ± 17	6.8–8.1	3 of 3
	120 ± 35	8.9–9.3	3 of 3
	25 ± 1.7	10.6–12.3	3 of 3
	12 ± 3.5	10.3–12.7	3 of 3
Fe/Humic acid	2500	11.5–17.2	3 of 3
	1200 ± 350	14.5–22.7	3 of 3
	250 ± 17	12.5–13.9	3 of 3
	120 ± 35	15.4–18.3	3 of 3
	25 ± 1.7	16.4–16.8	3 of 3
	12 ± 3.5	15.9–17.8	3 of 3
ATD ^b	2500 ± 170	9.2–10.7	3 of 3
	1200 ± 350	5.5–8.3	2 of 3
	250 ± 17	6.4–7.5	3 of 3
	120 ± 35	7.2–8.2	3 of 3
	25 ± 1.7	0–4.9	0 of 3
	12 ± 3.5	0–0	0 of 3

Acronyms: ATD, Arizona Test Dust; C_T, cycle threshold.

^a CFU values are averages and standard deviations corrected for dilution, expressed with 2 significant figure accuracy.

^b The ΔC_T range includes the highest ΔC_T value per ATD sample replicate, either from undiluted or 10-fold diluted DNA extracts.

^c The number of positive samples per three replicate samples.

samples with complex matrices to assess PCR inhibition and provide more data, potentially reducing false negative rates.

Growth of *F. tularensis* cells may have been enhanced by iron and/or nutrients associated with the humic acid formulation. These data suggest that the BVFH growth medium could be further optimized, although this could also enhance growth of competing microbes. Conversely, in the presence of ATD, *F. tularensis* growth appeared to be inhibited for low cell levels (10¹ CFU/3-mL sample) leading to non-detect results (although growth was not monitored in this case), which could not be attributed to PCR inhibition (i.e., both undiluted and 10-fold diluted DNA extracts were non-detect). Additional testing is needed to assess RV-PCR method performance with a range of real-world water samples, concentrated from larger volumes to improve detection limits, as well as testing with a dead *F. tularensis* cell background for natural decay or post-decontamination scenarios.

While traditional culture methods are considered the gold standard for viability analysis, this effort demonstrated that RV-PCR could detect down to the 10²-CFU level per 3-mL sample with background microbes in < 36 h (sample processing and analysis time), compared to > 72 h required for confirmed results from plate culture analysis. This CFU level would be difficult to detect on agar plates where faster-growing background microbes would outcompete *F. tularensis* for colony development. Additionally, the RV-PCR method uses a single 48-well plate for 48 samples (and controls) for growth compared with the culture method that uses 11 Chocolate Agar plates, dilution tubes, and an enrichment culture tube per sample, with additional plates to re-streak from the enrichment culture for presumptive *F. tularensis* colony isolation. Therefore, more incubator space (larger laboratory footprint) would be required for plates and tubes compared with 48-well plates used for RV-PCR analysis. Furthermore, by incorporating automated DNA extraction, less consumable waste is generated and DNA extracts can be obtained more rapidly with reduced labor required. Whereas, only 30–40 samples can be processed per 8-h shift for 2 laboratorians using the current culture-based method, more than twice this sample number (2 plates of 48 samples each) can be processed during this 2-person shift using the RV-PCR method. Assuming follow-on labor requirements for culture analysis, taking place over 3 or more days (i.e., presumptive colony/culture observation, restreaking for isolation, and

confirmation by PCR) and RV-PCR analysis after 30-h incubation (DNA extraction and PCR analysis) are comparable, we estimate a > 4-fold increase in sample throughput for RV-PCR analysis, with lower costs due to reduced labor requirements.

With proper training and proficiency demonstration, the RV-PCR method for detection of viable *F. tularensis* from water samples could help enhance the laboratory capability for rapid, reliable, and high-throughput sample analysis to effectively respond to an intentional, accidental, or natural contamination incident resulting in water infrastructure contamination. Additionally, subsequent to following sample-type-specific processing and concentration steps, this RV-PCR method could be used for other sample types during a response to a wide-area *F. tularensis* contamination incident, although further verification testing would be required (i.e., for different sample types and potential interferences).

Declaration of Competing Interest

None

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