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Development of a rapid viability polymerase chain reaction method for detection of *Yersinia pestis*

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Abstract

Due to the occurrence of natural plague outbreaks and its historical usage as a biological weapon, *Yersinia pestis* is considered one of the high-priority biological threat agents. It can remain viable in certain environments including water for > 100 days. Because of its slow-growth characteristic, it usually takes three or more days to detect and confirm the identity of viable *Y. pestis* cells by PCR, serological, or biochemical assays when using the traditional microbiological plate-culture-based analysis, and that too, assuming faster growing microbes present in a water sample do not mask the *Y. pestis* colonies and interfere with analysis. Therefore, a rapid-viability Polymerase Chain Reaction (RV-PCR) method was developed for detection of *Y. pestis*. The RV-PCR method combines 24 h-incubation broth culture in a 48-well plate, and pre- and post-incubation differential PCR analyses, thereby allowing for rapid and high-throughput sample analysis compared with the current plate culture method. One chromosomal and two plasmid gene target-based real-time PCR assays were down-selected, showing ca. 10 genome equivalent detection; the chromosomal assay was then used for RV-PCR method development. A 10^1-cell level (10–99 cells) sensitivity of detection was demonstrated even with complex sample backgrounds including known PCR inhibitors (ferrous sulfate and humic acid), as well as metal oxides and microbes present in Arizona Test Dust (ATD). The method sensitivity was maintained in the presence of dead *Y. pestis* cells up to 10^4 cells per sample. While affording high-throughput and rapid sample analysis, the 48-well plate format used in this method for sample enrichment significantly reduced labor requirements and generation of BioSafety Level-3 (BSL-3) laboratory waste as compared to the usual microbiological plate-culture-based methods. This method may serve as a model for other vegetative bacterial pathogens.

Keywords

Yersinia pestis; Detection; Viability; RV-PCR; Water contamination; Bioterrorism; Plague

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Declarations of interest
None.

1. Introduction

Yersinia pestis, the causative bacterium of plague, was responsible for the catastrophic loss of human life during the pandemics of 541 CE, 1346, and 1855 (Perry and Fetherston, 1997; Slack, 1989). Plague outbreaks continue to be a threat in many parts of the world, especially in Africa, and in particular Madagascar, which reported many fatal cases in 2017 (World Health Organization, 2017). Its high lethality made *Y. pestis* an attractive biological warfare agent (Inglesby et al., 2000; Derbes, 1966) and a potential agent of bioterrorism (Khan et al., 2001; Riedel, 2005). It is a Category A biological select agent in the CDC Critical Agents List generated in conjunction with medical, public health, and intelligence agencies (Centers for Disease Control and Prevention, 2000; Khan et al., 2000). *Y. pestis*, a Gram-negative, non-motile, capsule-forming, non-spore-forming coccobacillus bacterium causes three primary forms of plague disease in humans depending upon the route of infection: bubonic, septicemic, and pneumonic plague. Although rare, it can cause gastroenteritis via ingestion of infected animal meat (Bin Saeed et al., 2005; Leslie et al., 2011). The primary reservoir for *Y. pestis* is small rodents, with fleas typically responsible for animal-to-human transmission (Stenseth et al., 2008). In addition, recent studies have suggested that amoeba in soil or water may be competent environmental reservoirs thus enhancing its survival and transmission (Markman et al., 2018).

While *Y. pestis* is not a water-borne pathogen, it is a potential bio-threat agent for water (Khan et al., 2001). *Y. pestis* in water could cause septicemic and bubonic plague upon entry through open cuts or wounds. Additionally, it is possible that pneumonic plague could occur from inhalation of aerosols derived from water intentionally contaminated with *Y. pestis* (Tang et al., 2006). It has also been shown that a fatal systemic disease occurred when mice were administered *Y. pestis*-contaminated drinking water (Butler et al., 1982). Long-term survival of *Y. pestis* in water can pose a potential problem of prolonged disease transmission. *Y. pestis* has been shown to remain culturable from 2 to 21 days (Wilm, 1897; Pawlowski et al., 2011; Gilbert and Rose, 2012) in tap water, and > 100 days in bottled drinking water (Torosian et al., 2009). Drinking water can be accidentally or intentionally contaminated at its source, during treatment, in the distribution system, or even in bottled form (Khan et al., 2001). Although the implementation of drinking water standards has played a significant role in reducing water-borne illnesses, a serious vulnerability still remains from bioterrorism (Khan et al., 2001).

Current methods for detection of viable *Y. pestis* in water involving direct plating onto solid growth medium are labor-intensive and low throughput, and also require confirmatory analysis via PCR, serological and/or biochemical tests. For example, each sample is processed individually and requires two dilution tubes, nine growth medium plates for plating dilutions, two Microfunnel filters with a plate per filter, and one enrichment culture tube; thus, for 48 samples, one would generate a total of 96 dilution tubes, 48 enrichment cultures, 96 Microfunnel filters, and 528 plates (or more if restreaking from enrichment culture is needed), followed by confirmatory testing from presumptive *Y. pestis* colonies or turbid growth in enrichment cultures for each sample. Here we report development and optimization of a Rapid Viability Polymerase Chain Reaction (RV-PCR) method for rapid detection of viable *Y. pestis*. The RV-PCR method combines shorter sample incubation in

liquid culture (compared to plate culture) with real-time PCR analysis before and after incubation and uses the change in real-time PCR response to specifically detect low concentrations of viable *Y. pestis*. In contrast to the plate culture method, for the RV-PCR method, 48 samples are processed together in one 48-well plate, with two DNA extracts generated per sample, and three PCR analyses conducted per extract, for a total of 288 PCR analyses (less than four 96-well PCR plates) per 48 samples, and no confirmatory testing required since PCR analysis is specific to *Y. pestis*. For two laboratorians, each working an 8-h shift per 24-h period, it would take about 54 h from sample receipt to results reporting for 48 samples using RV-PCR analysis; whereas, for the traditional culture method, it would take from about 88 h, if isolated *Y. pestis* colonies could be detected from all 48 samples, to > 160 h if sample enrichment cultures needed to be analyzed to confirm the absence of viable *Y. pestis* cells.

Previously, an RV-PCR method was developed for detection of viable *Bacillus anthracis* spores in environmental samples (Létant et al., 2011); however, the sample processing procedure for spores was not conducive to maintaining vegetative cell viability. Therefore, the RV-PCR method was developed for detection of *Y. pestis* cells, which may serve as a model for other vegetative bacterial pathogens, especially in water samples. In case of an incident, both pre- and post-disinfection water samples could be concentrated by ultrafiltration with or without secondary filtration (Kahler et al., 2015; Holowecky et al., 2009) prior to RV-PCR analysis to further improve the sensitivity of detection. The method described here was shown to enable detection of *Y. pestis* cells even in backgrounds of high levels of debris, potential inhibitors, non-target microbial cells/spores, and dead *Y. pestis* cells.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and cell suspension preparation

The pathogenic *Y. pestis* CO92 strain and the attenuated CO92 pgm$^-$ strain lacking the 102-kilobase (kb) *pgm* locus (Buchrieser et al., 1999) were used. The strains were grown on Brain Heart Infusion (BHI) broth (Becton Dickinson BBL™ Cat. No. 237500) or *Y. pestis* Enrichment Broth (YPEB) (T. Doran, D. Hanes, S. Weagant, S. Torosian, D. Burr, K. Yoshitomi, K. Jinneman, R. Penev, O. Adeyemo, D. Williams-Hill, P. Morin [Food and Drug Administration], personal communication). The YPEB medium was used for RV-PCR experiments because it produced higher cell yields for shorter incubation times. Tryptose Blood Agar (TBA) plates without blood prepared from Difco™ Tryptose Blood Agar Base powder (Becton Dickinson, Cat. No. 223220) were used to isolate colonies for liquid culturing. The YPEB medium consisted of the following components (per/L): 25 g Bacto Heart Infusion Broth powder; 6 g Yeast extract; 3 g Soytone; 0.5 g Ferric Ammonium Sulfate; and 8.77 g 3-(*N*-Morpholino) propanesulfonic acid (MOPS). The solution was sterilized by passage through a 0.22-μm filter. *Y. pestis* cell suspensions were prepared from 2 to 3 colonies on TBA plates (started from −80 °C stocks), inoculated into 5-mL YPEB in 50-mL conical tubes. After overnight incubation (18–26 h) at 28 or 30 °C at 180 rpm, cells were harvested (3100 ×g at 4 °C for 15 min) and resuspended in phosphate-buffered saline (PBS, Teknova, Cat. No. P0261). Cells were then adjusted to an optical density at 600nm

(OD_{600}) of ca. 0.1 with PBS, corresponding to ca. 6–7 × 10^6 CFU/mL. Ten-fold serial dilutions were performed in PBS to achieve the desired starting cell density (colony-forming units, CFU/mL) in 2.7 mL sample per well of a 48-well, 5-mL rectangular well plate (E&K Scientific, Cat. No. EK-2044). One part 10 × (10-fold concentrated) YPEB medium (0.3 mL) was added to nine parts cell suspension in PBS (2.7 mL) in 48-well plates, yielding 1 × YPEB. Culture data are shown as either CFU/mL or CFU/2.7 mL sample (corrected for dilution) based on the average (Avg) and standard deviation (SD) of triplicate plates with colony counts within the range of 25–250 CFU/plate. Spent cultures and consummables were autoclaved at 15 p.s.i. for 60 min at 121 °C.

2.2. Sample types and preparation

PBS was used as a substitute for water samples because it maintained cell viability and represented a reproducible matrix in terms of pH and chemical composition to facilitate consistent experimental results during the RV-PCR method development. Materials were added to PBS including i) iron sulfate (heptahydrate; Sigma-Aldrich, Cat. No. 215422) and humic acid (HA; Sigma-Aldrich, Cat. No. 53680-10G) to represent chemical interferences, ii) Arizona Test Dust (ATD; ISO 12103-1, A3 Medium Test Dust; Powder Technology, Arden Hills, MN) to represent chemical, biological (live, non-target microorganisms), and physical challenges (particulates), and iii) dead *Y. pestis* cells to assess the background effect for post-disinfection applications or natural degradation. Iron sulfate and HA solutions were prepared in sterile distilled, deionized (DD) water and added to 2.7 mL water samples at a final concentration of 10 μg Fe^{2+}/mL (27 μg Fe^{2+}/sample) and 50 μg HA/mL (135 μg HA/sample), respectively. These concentrations were at the upper end of the range of values expected for drinking water samples (National Research Council, 1979; World Health Organization, 1996; US EPA, 2005). The dust was previously shown to contain ca. 5 × 10^4 CFU background microbes including fungi and bacterial spores per 10 mg (Rose et al., 2011). Dust was non-sterilized, made into a slurry in DD water, and added to samples at a final concentration of 4 mg/mL (10.8 mg/sample), which is within the range for total suspended solids typically measured in water samples.

2.3. Preparation of killed Y. pestis cell suspensions

Isopropyl alcohol (IPA) exposure was used to generate dead cells for evaluating the RV-PCR method since it has been used to generate dead cells as controls for viable cell staining kits (Molecular Probes, 2004). An overnight culture (100 mL) of *Y. pestis* CO92 pgm^- was diluted to OD_{600} ca. 0.01 in YPEB and incubated with shaking at 30 °C and 180 rpm until an OD_{600} ca. 0.3–0.4 was achieved. The culture was then split into four 20-mL aliquots, harvested by centrifugation (3100 ×*g* at 4 °C for 15 min), the supernatant was removed, and the cell pellets were suspended in 6 mL PBS. For IPA-treated cells, 14 mL 99 + % IPA were added to yield ca. 70% IPA, and 14 mL PBS were added for the control treatment. The cell suspensions were incubated for 2 h at room temperature with gentle mixing every 30 min. The suspensions were then centrifuged at 3100 ×*g* at 4 °C for 15 min. The supernatant was removed and the pellets were washed in 20 mL PBS followed by centrifugation, removal of supernatant, and final suspension in PBS to 20 mL. The IPA-killed cell suspensions were determined from untreated controls to be ca. 4.1 × 10^7 cells per mL. Suspensions were divided into aliquots, stored at 4 °C, and used within 40 days of generation. Before use, the

IPA-treated cell suspensions as well as cell pellets and super-natants (after centrifugation) were heat-lysed and tested by PCR to ensure that DNA was not being lost/degraded over time. Heat lysis was conducted at 95 °C for 5 min followed by placement on ice for 2 min, centrifugation (20,800 ×*g* at 4 °C for 5 min), and removal of liquid for PCR analysis (leaving the cell debris pellet in the tube). The YC2 assay (Table 1) was used for PCR analysis for the different fractions. A comparison of real-time PCR results from heat lysates of cell suspensions, pellets and supernatants from IPA-treated and untreated cells showed similar DNA contents suggesting little to no loss of DNA due to IPA treatment (data not shown). For experiments with different concentrations of dead cells, the IPA-treated suspension was diluted with PBS to achieve the desired dead cell level based on plate counts from the control processed in parallel.

From reference plating, the actual live cells were 460 ± 100 per 2.7-mL water sample for the 10^2 live cell level (100–999 cells) and 46 ± 10 per 2.7 mL water sample for the 10^1 live cell level (10–99 cells), and dead cell levels ranged from 10^4 to 10^6 per sample. Control treatments without dead cells were processed in parallel. Aliquots were processed for DNA recovery at T_0 and T_{24} and analyzed using the YC2 chromosomal assay with undiluted and 10-fold diluted DNA extracts to check for PCR inhibition.

2.4. Rapid-viability PCR method

The RV-PCR method for *Bacillus anthracis* (Létant et al., 2011) employed multiple vacuum filtration steps to first concentrate the spores in a filter cup and then wash twice with different buffers. Unlike ultra-filtration, this filtration resulted in complete drying of the filter and could not be used for vegetative cells while maintaining viability; therefore, the cell suspension in PBS was mixed with 10×-concentrated growth medium. Specifically, a 2.7 mL sample aliquot was added to 0.3 mL 10× YPEB in 5-mL wells of a 48-well plate. After mixing, a 500-μL aliquot was removed from each well before incubation (T_0 aliquot), transferred to a 2-mL Eppendorf tube, and centrifuged at 20,800 ×*g* for 10 min at 4 °C, after which 300-μL supernatant were removed and discarded. The cell pellets in the remaining 200-μL were frozen prior to DNA extraction and PCR analysis. Additionally, 250-μL aliquots were also evaluated with processing as described except only 50-μL super-natant was removed and discarded, and the cell pellets in remaining 200-μL were frozen prior to DNA extraction and PCR analysis. The 48-well plate was then sealed with a sterile AeraSeal™ breathable adhesive seal (Excel Scientific, Cat. No. BS-25), incubated for different time periods from 12 to 40 h at 28 or 30 °C with shaking at 180 rpm, and removal of 500-μL aliquots for the different time points. Aliquots were processed as described and either stored at −20 °C prior to DNA extraction or processed immediately.

The MagneSil® Blood Genomic, Max Yield System (Promega, Cat. No. MD1360) was used for DNA extraction and purification. This kit enables DNA recovery from multiple complex samples simultaneously using a magnetic bead-based cleanup method. The procedure was modified from that used for *B. anthracis* cells (Létant et al., 2011). Briefly, the cell pellet in the remaining 200-μL aliquot was thawed and 800-μL Lysis Buffer were added. The mixture was vortex mixed and incubated for 5 min. Next, 600-μL of paramagnetic particle (PMP) mix were added and mixed by vortexing. The liquid was then removed after placing tubes on

the magnetic rack. One lysis wash step with 360-μL of Lysis Buffer was included, followed by vortex mixing, placing on the magnetic rack, and subsequent liquid removal. Two washes with 360-μL of Salt Wash were then performed, in each case followed by mixing by vortexing and removal of the liquid when on the magnet. Finally, two washes with 500-μL of Alcohol Wash solution were performed with mixing by vortexing and liquid removal. A final wash with 70% ethanol was included to enhance PMP drying. PMPs were air-dried for 2 min and then dried at 80 °C for 20 min. DNA was then eluted by addition of 200-μL Elution Buffer followed by five cycles of vortexing (5–10 s) and heating (1 min) at 80 °C. Each sample DNA extract was kept at room temperature for 5 min prior to mixing and transferring to the magnetic rack. While on the magnet, the DNA extract was recovered and transferred into a clean 1.5-mL Eppendorf tube. If particles remained, the sample DNA extract was centrifuged at 20,800 ×*g* for 5 min at 4 °C, and the supernatant was transferred to a clean Eppendorf tube. The DNA extract was stored at −20 °C until real-time PCR analysis.

2.5. Y. pestis CO92 real-time PCR analysis

Y. pestis CO92 DNA standards were generated from harvested 5-mL YPEB cultures. A Master Pure™ Complete DNA and RNA (ribonucleic acid) Purification Kit (Epicentre® Biotechnologies Inc. Cat. No. MC85200) was used to extract DNA from pure culture following the manufacturer's protocol. DNA was measured using the high sensitivity Quant-iT™ DNA assay (Invitrogen, Cat. No. Q32854) with a Qubit™ fluorometer (Cat. No. Q33216). Standard DNA concentrations prepared in PCR-grade water ranged from 1 fg/μL to 1 ng/μL. Each PCR plate contained seven 10-fold dilutions, ranging from 5 fg per 25-μL PCR to 5 ng per 25-μL PCR.

Candidate real-time PCR primer-probe sets (72 total) were analyzed *in silico* using GenBank and other sequence databases for predicted specificity to virulent *Y. pestis* strains and lack of cross-reactivity with near neighbors. Assays were then tested against 12 target DNA templates (*Y. pestis* strains Pestoides B, F and G; Nairobi; Shasta; A1122; Java 9; Nicholisk 41; Harbin 35; KIM 27; Antigua; CO92) and two near neighbor DNA templates (*Y. pseudotuberculosis* strains Yp III and PB1/+) to evaluate specificity and sensitivity of assays for the chromosome (YC2 assay), the pMT1 plasmid (YpMT1 assay), and the pPCP1 plasmid (YpP1 assay) (Table 1). The down-selected YpP1 assay targeted the plasminogen activator/outer membrane protease (Pla) gene, while the YpMT1 assay targeted the caf1R gene, a positive regulator of the F1 operon (encoding the F1 capsule antigen) involved in virulence. The YC2 assay targeted a gene encoding an outer membrane auto-transporter barrel domain protein, with similarity to Type V secretory pathway adhesin AidA. Autotransporter proteins of a type V secretion system and these systems have been linked to virulence in Gram-negative bacteria (Derbise et al., 2010).

The PCR mix contained TaqMan® 2× Universal PCR Master Mix (ThermoFisher, Cat. No. 4304437) including deoxynucleotide triphosphates (dNTPs), a 6-Carboxyl-X-Rhodamine (ROX), AmpErase® UNG (uracil-N-glycosylase), as well as forward and reverse primers and a probe labeled at the 5′ end with FAM (6-carboxyfluorescein) and at the 3′ end with Black Hole Quencher® (BHQ-1) (Table 1). PCR-grade water was used to make the mix

volume up to 20-μL per reaction and 5-μL of sample DNA extract or DNA standard were added. The following thermal cycling conditions were used on an Applied Biosystems® (ABI) 7500 Fast Real-Time PCR instrument: 2 min at 50 °C for UNG incubation, 10 min at 95 °C for DNA polymerase activation, and 45 amplification cycles (5 s at 95 °C for denaturation and 20 s at 60 °C for annealing/extension). Both undiluted and 10-fold diluted sample DNA extracts (prepared in PCR-grade water) for both T_0 and later time points were analyzed to check for PCR inhibition. Three replicate samples were analyzed per experimental condition, and three replicate PCR analyses were conducted per sample replicate. DNA extracts from different time points from the same samples were analyzed on the same plate to minimize variability. The ROX dye in the ABI Universal Master Mix was used to normalize the fluorescent reporter signal. Automatic baseline and threshold settings were used throughout after reviewing the response curves to confirm their appropriateness. Spent PCR plates and consumables were disinfected in a permitted autoclave operated at 15 p.s.i. for 60 min at 121 °C.

2.6. RV-PCR data interpretation

The RV-PCR criteria for positive detection of *Y. pestis* cells was evaluated, namely ΔC_T (C_T [T_0] − C_T [T_f]) ≥ 6, (where f = final incubation time, h). In most cases a 24-h incubation was used, such that $T_f = T_{24}$. For cases where no PCR response was obtained (non-detect results), the C_T values were set to 45 to calculate ΔC_T (since 45 PCR cycles were used). A $\Delta C_T \geq 6$ represented an increase in DNA concentration of approximately 2-log, due to the presence of viable cells in the original sample that propagated during incubation. For individual sample replicates within an experiment, the RV-PCR result was considered positive when the average of at least 2 of 3 PCR replicates for T_0 and T_f had $\Delta C_T \geq 6$. If a single PCR replicate was positive and the other two replicates were non-detect, the T_0 or T_f aliquot was considered negative or non-detect (NDT) and the C_T was set to 45, in order to calculate ΔC_T. The RV-PCR method sensitivity of detection was equivalent to the *Y. pestis* cell level where 100% of the samples had a $\Delta C_T \geq 6$; however, this did not account for losses from sampling and sample handling.

2.7. Biosafety

All manipulations with *Y. pestis* cultures were done under Biosafety Level 3 (BSL-3) conditions in an CDC-permitted facility, including use of a certified Class II biosafety cabinet with thimble connection and ducted exhaust and the following personal protective equipment (PPE): Hood Powered Air Purifying Respirator (PAPR), Tyvek coverall with hood and boots, shoe covers, and double latex or nitrile gloves. Aerosolization risk was mitigated by use of aerosol barrier tips during pipetting and use of gasketed safety cups for centrifugation. Secondary containment was used for capped culture tubes and sealed 48-well plates during incubation. Waste was subjected to two rounds of sterilization using a permitted autoclave, documented at 15 p.s.i. and 121 °C for ≥60 min prior to disposal.

3. Results

3.1. RV-PCR method development for detection of viable Y. pestis

The RV-PCR method developed here uses a differential PCR response before and after sample enrichment to detect viable *Y. pestis* cells based on a change in cycle threshold (C_T) over time or ΔC_T (de-fined by C_T [T_0] − C_T [T_{24}]) ≥ 6. This represents an increase in cells containing DNA of ca. 2 $\log_{10}$.

For real-time PCR assay evaluation for RV-PCR, the YpP1, YpMT1, and YC2 assays showed that 50-fg genomic *Y. pestis* CO92 DNA (ca. 10 genome copies) was detected 100% of the time (n = 21 runs per assay) and the 5-fg level was detected for about 75% of PCR runs (n = 21 per assay). Since the plasmid copy numbers can vary, the YC2 assay (showing similar detection sensitivity) was used for method development. For *Y. pestis* growth, Brain Heart Infusion (BHI) broth and *Y. pestis* Enrichment Broth (YPEB) were evaluated in 48-well plates. The BHI broth yielded poor growth (data not shown). There was a 3.6 to 4-log increase after 24-h incubation for the different *Y. pestis* cell levels on YPEB (Table 2). Similar *Y. pestis* log increases were only observed with BHI broth if inoculated from three sequential overnight cultures and the incubation period was increased to 40 h. Therefore, the YPEB medium was selected for *Y. pestis* RV-PCR method development.

Two aliquot volumes from 48-well cultures were evaluated for DNA extraction, 250-μL and 500-μL, in order to achieve sufficient detection levels for RV-PCR analysis. In addition, two incubation periods, 12 h and 24 h, were tested for both aliquot volumes (for time points T_0 and T_{12} or T_0 and T_{24}). Culture data showed an average ca. 4.2–4.7 log increase over a 24-h period and ca. 1.7–2.0-log increase during 12-h incubation (Table 3). As expected, RV-PCR analysis showed that the 500-μL aliquot volume for DNA extraction gave higher average ΔC_T values than the 250-μL aliquot volume, although differences were not statistically significant (p-values ranged from 0.1 to 0.9). Based on these data, the 500-μL aliquot volume was used for DNA extraction for subsequent RV-PCR method evaluation. Although the average ΔC_T values were approximately 6 or greater after 12-h incubation with 10× YPEB diluted to 1× with phosphate-buffered saline (PBS), a longer incubation period was selected for water samples containing challenge material.

3.2. RV-PCR method performance with complex samples

Throughout the course of method development and evaluation, PBS was used as a substitute for actual water samples in order to maintain cell viability and reduce sample variability to obtain reproducible results. As per EPA protocol (US EPA, 2017), a large volume water sample (1–2 L) is typically collected and concentrated onto filter media, after which bacterial contaminants are recovered from the filter by washing with PBS for subsequent analysis. Results for RV-PCR method evaluation with PBS containing Arizona Test Dust (ATD) (10.8 mg/sample) or humic acid (HA; 135 μg/sample) plus iron (27 μg Fe^{2+} as $FeSO_4$ per sample) are shown in Table 4. For the 10^2-cell level, there was little to no PCR inhibition for the control treatment with similar ΔC_T values (C_T [T_0] − C_T [T_{24}]) for undiluted and 10-fold diluted DNA extracts; however, the treatment with iron and HA showed inhibition for 1 of the 3 replicates for undiluted extracts, while the 10-fold diluted extract had all three

replicates with $\Delta C_T > 6$. The ATD treatment showed similar ΔC_T values to those of the control treatment with all values > 6, for either undiluted or 10-fold diluted extracts. For the 10^1-cell level, there were lower ΔC_T values for the ATD treatment compared to the control treatment, especially when comparing the 10-fold diluted DNA extracts. The ΔC_T values for the ATD treatment met the criterion for positive detection ($\Delta C_T \geq 6$) for all but one sample replicate for 10-fold diluted extracts ($\Delta C_T = 4.1$, while the undiluted extract value was 12.2). For the Fe/HA treatment, 1 of 3 sample replicates showed PCR inhibition (i.e., non-detect), although this was resolved with 10-fold dilution of the sample DNA extract. The data suggested that debris such as the reference test dust could cause growth inhibition, resulting in higher T_{24} C_T values, likely due to the presence of indigenous organisms. The test dust was reported to contain fungal spores as well as *Bacillus* spores and other bacteria (Rose et al., 2011) that may be faster growing than *Y. pestis*. However, at these low cell levels, the RV-PCR method still showed the ability to accurately detect live *Y. pestis* cells in complex backgrounds, with consistent detection at the 10^1-cell level. The negative controls showed non-detect results for all replicates at both time points (data not shown).

3.3. RV-PCR method performance in a dead Y. pestis cell background

For application to post-disinfection or natural degradation scenarios with high levels of dead cell backgrounds, the RV-PCR method was evaluated with low levels of live *Y. pestis* cells in the presence of different levels of isopropanol (IPA)-killed *Y. pestis* cells; this disinfection method effectively killed cells without DNA damage or loss, thus providing the most challenging test case. The RV-PCR method results for 10^1 and 10^2 live cell levels with 10^4–10^6 dead cells in 2.7 mL water samples are shown in Table 5, as both undiluted and 10-fold dilutions of T_0 and T_{24} DNA extracts. A comparison of undiluted and diluted extracts from each sample showed an average 3.4 ± 0.9 C_T difference (i.e., no PCR inhibition) and demonstrated similar trends in ΔC_T values for different live and killed cell level combinations. For undiluted DNA extracts, the 10^2 live cell level with up to 10^5 dead cells and the 10^1 live cell level with up to 10^4 dead cells were both consistently detected with a 24-h incubation period (ΔC_T values ≥ 6), whereas, higher dead cell backgrounds produced generally negative results (avg. ΔC_T values of 4.6 ± 0.8 for the 10^2 live/10^6 dead cell level treatment and 5.7 ± 0.5 for the 10^1 live/10^5 dead cell level treatment). For 10-fold diluted DNA extracts, the 10^2 live cell level treatment had average ΔC_T values ≥ 6 for all dead cell backgrounds; however, the individual ΔC_T values were 7.5, 4.9, and 6.8 for 10^5 dead cells, showing 2 of 3 were positive. The replicate ΔC_T values for the 10^6 dead cell/10^2 live cell treatment were 11.0, 5.5, and 3.7, showing 1 of 3 were positive. For the 10^1-live cell level treatment, 3 of 3 were positive with a 10^4 dead cell background (Avg. $\Delta C_T = 7.8 \pm 1.0$), while none were positive with 10^5 dead cells (Avg. $\Delta C_T = 4.9 \pm 0.5$). For these levels of dead cells (10^4–10^6), the control treatments with no live cells showed non-detect results for triplicate analyses as expected.

4. Discussion

A RV-PCR method which was developed for detection of viable *Y. pestis* cells, employs PCR analysis before and after incubation of a water sample mixed with concentrated growth medium. With optimized procedures for high throughput culturing and DNA extraction/

purification, the method showed good accuracy and sensitivity of detection even with samples containing potential inhibitors. Consistent growth was observed with YPEB in place of BHI broth (≥4-log cell growth over 24 h) using 10× YPEB diluted to 1× concentration with a water sample. Presence of iron and HA did not impact the 10^1-cell level sensitivity of detection with a 24-h sample incubation (especially with 10-fold DNA extract dilution). While the presence of reference dust (ca. 10 mg/sample) apparently showed growth inhibition, the 10^1-cell level sensitivity of detection was still maintained.

In case of *Y. pestis* water contamination, large volume samples could be concentrated prior to analysis (Kahler et al., 2015; Holowecky et al., 2009), potentially also concentrating insoluble growth and PCR inhibitors, as well as dead *Y. pestis* cells (from disinfection or natural degradation). The RV-PCR method with 24-h sample incubation showed detection of 10^1- to 10^2-cell levels in killed cell backgrounds up to 10^4 and 10^5, respectively, while for higher killed cell backgrounds, detection was inconsistent; this could potentially be mitigated with a longer sample incubation period (i.e., 30 h). While these dead cell levels were quite high, they could be present upon sample concentration, especially for post-disinfection scenarios. In addition to a longer incubation for concentrated water samples, both undiluted and 10-fold diluted DNA extracts could be used to address inhibition and minimize false positive/false negative results. The method did not produce false positive results for high concentrations of dead cells alone.

This effort served to bracket the conditions where RV-PCR analysis could be used for detection of live *Y. pestis* cells in pre- and post-disinfection, or natural degradation scenarios. Furthermore, the method could be a model for vegetative cells of other bacterial pathogens including both bioterrorism and public health threats. The RV-PCR method is expected to have an advantage over traditional culture methods since isolated *Y. pestis* colonies (or other fastidious bacterial pathogens) may be difficult to detect in samples containing high concentration of non-target microbes. Furthermore, since *Y. pestis* has been shown to become viable but not culturable (VBNC) in water (Pawlowski et al., 2011; Gilbert and Rose, 2012; Suchkov et al., 1997), it is likely that VBNC cells could be more readily detected from liquid culture used in RV-PCR analysis than from solid media used in traditional plate culture (Wai et al., 2000; Miller and Davey, 1965). The former method could provide better conditions for cell resuscitation, and thus, avoid false negative results. In addition, the RV-PCR method could provide results in less than half the time of the traditional culture method, which requires 72 h or longer for confirmed results (Riedel, 2005). Integration of automated DNA extraction procedures with RV-PCR analysis could further reduce the labor and time-to-results. Finally, RV-PCR uses a single multi-well plate for 48 samples and controls, thereby generating less waste and comprising a smaller laboratory footprint for analysis relative to the traditional culture method that uses numerous petri plates, and dilution and enrichment culture tubes per sample. While the reported *Y. pestis* real-time PCR assays demonstrated ca. 10 genome-equivalent detection sensitivity, other *Y. pestis* real-time PCR assays could be integrated into the RV-PCR method as well as other quantitative PCR platforms. Future work to evaluate the RV-PCR method with actual water samples including tap/drinking water, both small (50–100 mL) and large volume (100 L concentrated using ultra-filtration) samples; ground water; source water; and waste water will significantly expand the method utility. Additionally, testing of other variables including

sample holding time at different temperatures will guide real-world sample analysis requirements. Due to the shortened time to results, this viability detection method would enhance response capabilities for bioattacks or natural plague outbreak scenarios. More rapid results with the same or improved accuracy compared to plating methods will aid decision-makers in planning disinfection/decontamination efforts and determining their efficacy, thereby enabling safe, timely restoration.

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Table 1

Nucleotide sequences of the primer/probe sets used for *Y. pestis* RV-PCR analysis.

Assay	Forward primer[a]	Reverse primer[a]	Probe[a,b]	Amplicon length (bp)
YC2	CAACGACTAGCCAGGCGAC	CATTGTTCGCACGAAACGTAA	TTTTATAACGATGCCTACAACGGCTCTGCAA	78
YpP1	TGGGTTCGGGCACATGATA	CCAGCGTTAATTACGGTACCATAA	CTTACTTTCCGTGAGAAGACATCCGGCTC	101
YpMT1	GGTAACAGATTCGTGGTTGAAGG	CCCCACGGCAGTATAGGATG	TCCCTTCTACCCAACAAACCTTTAAAGGACCA	99

[a]Sequences are listed in the 5′ to 3′ orientation.

[b]Probes were labeled at the 5′ end with FAM (6-carboxyfluorescein) and at the 3′ end with Black Hole Quencher® (BHQ-1); bp = base pair.

Table 2

Growth of *Y. pestis* cells in 48-well plates (1× YPEB).

Starting cell level (CFU/mL)	Actual CFU/mL/timepoint Avg (SD)[a]		Log increase
	0 h	24 h	
10^0	2.8 (0.1) × 10^0	2.4 (1.2) × 10^4	4.0
10^1	2.8 (0.1) × 10^1	1.7 (0.6) × 10^5	3.9
10^2	2.8 (0.1) × 10^2	1.5 (0.1) × 10^6	3.8
10^3	2.8 (0.1) × 10^3	8.5 (2.0) × 10^6	3.6

[a]Data points show the average (Avg) and standard deviation (SD) from triplicate analyses for inoculum reference plating (0 h) and after 24 h incubation, corrected for dilution.

Table 3

Effect of aliquot volume on *Y. pestis* cell growth and ΔC_T for RV-PCR analysis.

Starting CFU level/sample[a]	Aliquot volume µL	YC2 assay ΔC_T[b] Avg (SD)		Avg change in log cell density	
		$T_0 - T_{12}$	$T_0 - T_{24}$	$T_0 - T_{12}$	$T_0 - T_{24}$
10^3	250	6.1 (0.8)	13.2 (2.3)	2.1	4.4
	500	6.3 (0.6)	15.3 (0.6)	2.0	4.2
10^2	250	5.9 (1.9)	16.0 (0.7)	1.9	4.6
	500	6.9 (1.1)	16.8 (0.9)	1.7	4.6
10^1	250	7.3 (1.1)	17.7 (0.7)	1.6	4.7
	500	9.6 (0.8)	19.8 (0.6)	1.8	4.4

[a] Per 2.7-mL water sample, there were ca. 38, 380, and 3800 cells, from reference plating, for the 10^1-, 10^2-, and 10^3-cell levels, respectively.

[b] Average (Avg) and standard deviation (SD) were based on triplicate samples.

Table 4

RV-PCR results for 10^1 or 10^2 *Y. pestis* cell level per sample with and without chemical or biological backgrounds.

Treatment/Starting CFU level[a]	Sample replicate	YC2 assay C_T Avg (SD)[b]				ΔC_T (T_0 - T_{24})[c]	
		Undiluted		10-fold diluted		Undiluted	10-fold diluted
		T_0	T_{24}	T_0	T_{24}		
Control/10^2	1	38.8 (0.7)	23.6 (0.5)	NDT	28.2 (0.2)	15.2	16.8
	2	37.0 (0.2)	24.3 (0.8)	39.4 (0.4)[d]	23.2 (1.8)	12.7	16.2
	3	37.9 (0.8)	23.0 (0.4)	39.5 (2.1)[d]	26.6 (0.7)	14.9	12.9
	Avg (SD)	37.9 (0.9)	23.6 (0.8)	39.4 (1.4)	26.0 (2.4)	14.3 (1.4)	15.3 (2.1)
Fe/HA 10^2	1	NDT	27.7 (0.6)	NDT	30.4 (0.5)	17.3	14.6
	2	43.6 (1.0)[d]	31.1 (3.3)[d]	41.4 (0.4)[d]	28.3 (2.8)	12.7	13.1
	3	41.8 (0.3)	38.5 (6.5)	40.2 (0.1)[d]	26.8 (1.3)	3.3	13.4
	Avg (SD)	42.8 (1.4)	32.4 (6.0)	40.8 (0.7)	28.5 (2.2)	11.1 (7.1)	13.7 (0.8)
ATD/10^2	1	NDT	31.3 (0.3)	NDT	32.8 (0.4)	13.7	12.2
	2	42.9 (0.3)[d]	27.0 (0.4)	NDT	29.7 (0.3)	15.9	15.3
	3	NDT	29.5 (0.5)	NDT	32.4 (0.2)	15.5	12.6
	Avg (SD)	NA	29.2 (1.9)	NDT	31.6 (1.5)	15.0 (1.2)	13.4 (1.7)
Control/10^1	1	39.1 (0.3)	24.4 (0.2)	NDT	29.8 (0.7)	14.7	15.2
	2	40.6 (0.3)	32.7 (0.5)	NDT	33.9 (3.3)	7.9	11.1
	3	NDT	23.6 (0.3)	NDT	28.0 (0.2)	21.4	17.0
	Avg (SD)	39.9 (0.9)	26.9 (4.4)	NDT	30.6 (3.1)	14.7 (6.8)	14.4 (3.0)
Fe/HA 10^1	1	42.3 (0.1)[d]	33.2 (1.4)	NDT	31.1 (0.1)	9.1	13.9
	2	NDT	33.9 (0.2)	NDT	30.3 (0.1)	11.1	14.7
	3	NDT	NDT	42.3 (1.7)[d]	28.3 (0.1)	0.0	14.0
	Avg (SD)	NA	37.3 (5.8)	NA	29.9 (1.3)	6.7 (5.9)	14.2 (0.4)
ATD/10^1	1	NDT	33.8 (0.4)	40.0 (0.03)[d]	35.9 (0.5)	12.2	4.1
	2	NDT	34.2 (0.2)	NDT	37.6 (0.8)	10.8	7.4
	3	40.5 (1.3)	32.7 (0.4)	NDT	35.5 (0.3)	7.8	9.5

Treatment/Starting CFU level[a]	Sample replicate	YC2 assay C_T Avg (SD)[b]				ΔC_T (T_0 - T_{24})[c]	
		Undiluted		10-fold diluted		Undiluted	10-fold diluted
		T_0	T_{24}	T_0	T_{24}		
	Avg (SD)	NA	33.6 (0.7)	NA	36.3 (1.1)	10.3 (2.2)	7.0 (2.7)

[a] Per 2.7-mL water sample, there were ca. 29 cells and 290 cells, from reference plating, for the 10^1- and 10^2-cell levels, respectively.

[b] Average (Avg) and standard deviation (SD) are from triplicate PCR analyses. Average C_T values were only calculated if two or three sample replicates had positive PCR results.

[c] Avg. ΔC_T was calculated as the average of the individual sample ΔC_T values rather than based on the difference of average T_0 C_T and T_{24} C_T values.

[d] Values are from two PCR replicates; the third replicate was non-detect. NDT = Non-detect. NDT set to 45 to calculate ΔC_T. ATD = Arizona Test Dust. HA = Humic acid. NA = Not applicable; the average C_T was only calculated from two or three samples with positive PCR results.

Table 5

RV-PCR results for 10^1 and 10^2 live *Y. pestis* cell level/sample with different killed target cell concentrations.

Sample type		Undiluted extracts YC2 assay C_T Avg ± SD[b]		ΔC_T Avg ± SD (Positive Replicates)	10-fold diluted extracts YC2 assay C_T Avg ± SD[b]		ΔC_T Avg ± SD (Positive Replicates)
Live cell level[a]	Dead cell level	T_0	T_{24}		T_0	T_{24}	
10^2	0	38.3 ± 1.8	21.6 ± 0.6	16.7 ± 1.2 (3 of 3)	39.3, NDT, NDT	26.5 ± 1.6	16.6 ± 1.5 (3 of 3)
	10^4	33.4 ± 0.3	22.5 ± 0.7	10.9 ± 1.1 (3 of 3)	37.2 ± 1.3	26.7 ± 1.8	10.5 ± 1.6 (3 of 3)
	10^5	30.6 ± 0.9	23.2 ± 0.6	7.4 ± 0.8 (3 of 3)	32.7 ± 2.2	26.3 ± 1.1	6.4 ± 1.4[d] (2 of 3)
	10^6	26.4 ± 0.3	21.8 ± 0.9	4.6 ± 0.8 (0 of 3)	31.8 ± 2.7	25.1 ± 1.1	6.7 ± 3.8[d] (1 of 3)
10^1	0	NDT, 40.4, NDT	24.3 ± 0.8	19.2 ± 2.5 (3 of 3)	NDT, NDT, NDT	27.7 ± 0.7	17.3 ± 0.8 (3 of 3)
	10^4	33.8 ± 0.4	26.3 ± 0.7	7.5 ± 0.8 (3 of 3)	37.1 ± 0.8	29.3 ± 1.0	7.8 ± 1.0 (3 of 3)
	10^5	30.3 ± 0.5	24.6 ± 0.6	5.7 ± 0.5 (1 of 3)	33.2 ± 0.4	28.3 ± 0.6	4.9 ± 0.5 (0 of 3)
0	0	NDT, NDT	NDT, NDT	0 ± 0[c] (0 of 2)	NDT, NDT	NDT, NDT	0 ± 0[c] (0 of 2)
	10^4	34.5 ± 0.3	33.4 ± 0.4	1.1 ± 0.3[c] (0 of 2)	36.0 ± 1.6	36.5 ± 0.4	− 0.5 ± 2.1[c] (0 of 2)
	10^5	30.2 ± 0.6	29.9 ± 0.5	0.4 ± 0.6 (0 of 3)	33.8 ± 0.7	33.1 ± 0.7	0.7 ± 0.4 (0 of 3)
	10^6	26.2 ± 0.9	26.3 ± 0.5	−0.1 ± 0.5 (0 of 3)	29.5 ± 0.7	29.2 ± 0.1	0.3 ± 0.7 (0 of 3)

[a] Per 2.7-mL water sample, there were ca. 46 cells and 460 cells, from reference plating, for the 10^1- and 10^2-cell levels, respectively.

[b] Average (Avg) and standard deviation (SD) based on triplicate PCR analyses of undiluted and 10-fold diluted extracts from triplicate samples except where noted. NDT = Non-detect. NDT set to 45 to calculate ΔC_T. The Avg ± SD for T_0 or T_{24} was not calculated if some of the replicates were NDT.

[c] Avg ± SD based on two sample replicates.

[d] Denotes case where average ΔC_T value exceeds the criterion for positive detection, although individual replicate ΔC_T values were not all ≥6.