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DETECTION OF BIOLOGICAL WARFARE AGENTS IN MUNICIPAL TAP WATER VIA STANDARDIZED CULTURE METHODS

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PREFACE

The work described in this report was authorized under Interagency Agreement Number EPA/ECBC IAG # DW 21-92153401, National Homeland Security Research Center - U.S. Environment Protection Agency. The work was started in January 2004 and completed in September 2006.

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DETECTION OF BIOLOGICAL WARFARE AGENTS IN MUNICIPAL TAP WATER VIA STANDARDIZED CULTURE METHODS

1. INTRODUCTION

The threat of bioterrorism on U.S. soil has become a stark reality since the tragic events of September 11, 2001 and the subsequent dispersal of anthrax-tainted letters through the U.S. Postal Service. Protecting the nation's civilian population and critical water infrastructure has become a major concern. The Presidential Decision Directive 63 (available at <http://www.fas.org/irp/offdocs/pdd/pdd-63.htm>, accessed June 2009) assigned the United States Environmental Protection Agency (USEPA) the federal lead agency responsible for the task of ensuring the security of the national water infrastructure. USEPA's Office of Research and Development (ORD) and Office of Water (OW) have developed a "Water Security Research and Technical Support Action Plan" (available at http://www.epa.gov/safewater/watersecurity/pubs/action_plan_final.pdf, accessed June 2009). The research plan clearly outlines the need for methods of detection and characterization of contaminants in case of a homeland security related water contamination incident.

In this study, the U.S. Army Edgewood Chemical Biological Center (ECBC) and the USEPA National Homeland Security Research Center (NHSRC) selected, analyzed, and tested existing methods for the detection, isolation, and presumptive identification of selected microorganisms, which may be used as agents of biological warfare or terror in municipal drinking water. The methods used can be found on the American Society for Microbiology (ASM) website (<http://www.asm.org/Policy/index.asp?bid=6342>, accessed June 2009). Research focused on selecting and demonstrating use of microbiological culture methods for detection and presumptive identification of biological contaminants in water that might arise as a result of a terrorist event. These methods are desired to ultimately be used by laboratories examining samples at a sentinel level requiring confirmation at other labs.

This study used and tested existing LRN Level A assays available on the ASM website (<http://www.asm.org/Policy/index.asp?bid=6342>, accessed June 2009) designed for the detection and presumptive identification of various select agent pathogens in clinical samples. In addition, an ultrafiltration (UF) process, as first suggested in the EPA's Response Protocol Toolbox Site Characterization and Sampling Guide (available at http://www.epa.gov/safewater/watersecurity/pubs/guide_response_module3.pdf, accessed June 2009), was used to concentrate 100 L of tap water to a 250 mL volume used subsequently for assessing recovery and identification methods. Ultrafiltration has long been known to be an effective mechanism for concentration of a variety of contaminants to detect these contaminants in water (1). This technique is particularly useful if it is necessary or desirable to concentrate contaminants of various sizes and types, such as the simultaneous collection of viruses, bacteria and protozoa (2-4). The difficulty in using this method is that it may be "too" effective in concentrating microorganisms and other interfering substances from water samples, and may create difficulties in the various assay steps to follow. In a homeland security application, it may be necessary not only to know the identity of a contaminant but also whether the contaminant is viable. Therefore, this study was designed to test viability based culture assays in the context of

ultrafiltration concentration to determine if they are effective at detecting targeted potential microbial contaminants, which may be of concern in incidents of water contamination.

This study consisted of two main phases. Phase I of the study (or “ranging” study) evaluated the specific assays for the targeted bacteria added to a concentrated drinking water sample and aided in the design of Phase II experiments. Phase II evaluated the recovery of target organisms after ultrafiltration combined with potential interference from the drinking water background. This background is expected to contain biotic (including microorganisms and other biological substances) and abiotic substances (including chemicals or metals), which might interfere with the microbial detection and identification. These assessments were determined by spiking both prior to and after UF of 100 L of drinking water. Experiments were also conducted to assess recovery of target microorganisms in drinking water in single and pooled combinations in the presence of background organisms.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions.

All bacterial strains used in this study are listed in Table 1 below. Vegetative cells were used for each microorganism except for *B. anthracis* in which only spores were used. *B. anthracis*, *B. pseudomallei*, and *B. thailandensis* strains were procured from the U.S. Army Medical Research Institute of Infectious Diseases, while *F. tularensis* and *Y. pestis* strains were procured from the Centers for Disease Control and Prevention and Brigham Young University, respectively. Broth cultures containing media specified for each organism were inoculated from plate media and grown overnight at their optimum temperature (Table 1). Overnight cultures were concentrated via centrifugation (1,400 x g), washed once in cold phosphate buffered saline (PBS) and then resuspended in an equal volume of PBS prior to experimental use. Viable cell densities were determined by measuring absorbance at 600nm (OD₆₀₀) and using turbidometric data previously obtained for each microorganism (data not shown) relating OD to CFU/mL to calculate cell densities. All densities were confirmed by serial dilution and plating onto appropriate media (refer to Table 1).

2.2 Spore Preparation.

Spore preparations of *B. anthracis* AMES and Sterne strain were prepared by inoculating 10 mL of trypticase soy broth (TSB; Culture Media Supplies, Oswego, IL, catalog number CM164-T1020) with inoculum obtained from an appropriate plate culture (see Table 1). The liquid suspension was allowed to incubate for approximately 4 h at 37 °C. After 4 h, the 10 mL cell suspension was used to inoculate 20 TSA plus 5% sheep blood plates (Culture Media Supplies, Oswego, IL, catalog number CM100-P25-5SB) by spreading 0.4 mL of inoculum onto the plates and then incubating the plates at 37 °C for approximately 1 week or until sporulation was 90% or greater as determined microscopically via a simple stain (5). Once the appropriate level of sporulation was achieved, plates were removed from

incubation and placed at 4 °C for approximately 2 h prior to the addition of 15 mL cold PBS onto each plate. Plates were placed onto a platform shaker for 10 min and then spores were teased from the media surface using a smooth cell spreader. The 15 mL of PBS from each plate was then transferred to a 50 mL conical tube and plates were rinsed with 10 mL cold deionized water. All liquid was collected into 50 mL conical tubes. Spores were harvested by centrifugation at 1,400 x g for 10 min at 4 °C, washed three times with cold deionized water and resuspended in a minimal volume of cold deionized water. Samples were enumerated by serial dilution and plating onto sheep blood agar prior to storage at -80 °C. Spore preparations were removed from storage as needed for experimental use.

Table 1. Strains and Growth Conditions for Target Bacteria

Strain	Media ^{a,b}	Culture Temp (°C)	Biosafety Level
<i>Bacillus anthracis</i>			
Sterne	BHI, TSB, SBA/PLET	37	BSL-2
AMES	BHI, TSB, SBA/PLET	37	BSL-3
<i>Burkholderia pseudomallei</i>			
1026B	NA, NB/Ashdown agar	35	BSL-3
<i>Burkholderia thailandensis</i>			
E264	NA, NB/Ashdown agar	35	BSL-2
<i>Francisella tularensis</i>			
LVS	CHA ^c , BHI + 1% cysteine	37	BSL-2
OR960246	CHA ^c , BHI + 1% cysteine	37	BSL-3
<i>Yersinia pestis</i>			
A1122	NA, NB/YSA	28	BSL-2
1866	NA, NB/YSA	28	BSL-3

^aNA = nutrient agar

NB = nutrient broth

YSA = yersinia selective agar

BHI = brain heart infusion

TSB = trypticase soy broth

SBA = sheep blood agar

PLET = polymyxin B lysozyme EDTA thallose acetate agar

CHA = cysteine heart agar

^bMedia are shown as general/selective.

^cHA served as general and selective agar.

Prior to ultrafiltration, all necessary reagents were prepared including: 0.1% sodium polyphosphate (NaPP; Sigma-Aldrich 305553), 10% sodium thiosulfate (Fisher S446), and 0.001 % v/v Tween 80 (Fisher T164). All solutions were prepared in distilled water and filter sterilized. NaPP was made fresh daily, while the sodium thiosulfate and Tween 80 solutions were stored for no longer than one week at 4 °C. The ultrafiltration apparatus (see Figure) was assembled after Lindquist *et al.* (2007). The ultrafiltration apparatus was assembled using the following items: Hmacor HPH 1400 hemoconcentrators (i.e., hollow-fiber ultrafilters; Minntech Corporation catalog no. HPH1400), Masterflex tygon silicone tubing; I/P 26 (Cole Parmer, catalog no. EW-96420-26); filling/venting cap (Cole Parmer, catalog no. EW-06258-10); retentate bottle (Cole Parmer, catalog no. EW-06257-10); 3-way stopcock (Cole Parmer, catalog no. EW-06225-40); Masterflex I/P precision brushless drive (Cole Parmer, catalog no. EW-77410-10); I/P easy-load pump head (Cole Parmer, catalog no. EW-77601-00); and pressure gauge (Cole Parmer, catalog no. EW-68003-02). One hundred liters of tap water (either from EPA or ECBC laboratory faucet) was placed into an appropriately sized sample container followed by the addition of 50 mL of 10% sodium thiosulfate to dechlorinate the sample prior to ultrafiltration. Residual chlorine was measured using a DPD-colorimetric kit to ensure that chlorine residual was < 0.1 ppm. To begin, 1 L of 0.1 % NaPP (blocking agent) was filtered by placing approximately 500 mL of the blocking agent into the 1 L retentate bottle, and the 3 port lid was replaced and secured tightly. Initially all three lines were open (red, yellow, and blue, refer to the Figure), the pump was turned on, the system was primed (air bubbles removed), and the remainder of the blocking solution was added to the retentate bottle, once the priming was complete. Then the blocking solution was recirculated through the filter and reduced to approximately a 250 mL level in the bottle by opening the stopcock to the yellow and blue lines (depicted in the Figure) and removing the covered vent cap. After the blocking solution was filtered through, the pump was turned off (being careful to always close the red line when pump is off to prevent drainage of retentate bottle sample back into the 100 L water sample). Approximately 500 mL of the 100 L of water to be filtered was placed into the retentate bottle, the covered vent cap was replaced, and the pump was turned back on. The stopcock was turned so that all three lines were open. The entire volume of water was then filtered using a filtration rate of approximately 1 L/min while maintaining the pressure below 10 psi. The volume in the retentate bottle was not allowed to drop below 250 mL during the filtration period. When filtration of the 100 L was complete, 150 mL of 0.001 % Tween 80 was aspirated into the filter as a “forward rinse” through the red line (refer to the Figure for proper configuration of the apparatus). The pump speed was reduced to 750 rpm. After the red line was drained (all the eluting solution was aspirated and bubbles appeared in the red line), the 3-way stopcock was turned so that the blue and yellow tubes were open (red line was closed). The vent cover on the retentate bottle lid was quickly removed, and the retentate volume was reduced approximately to 250 mL. The pump was then turned off and the stopcock turned so the blue and red lines were open (yellow closed). The pump was then turned back on to drain the lines. The pump was finally turned off again, the yellow line was opened (red closed), and the pump was reversed and then turned back on to drain the yellow line, making sure to hold the line above the level of the retentate bottle. This completed the filtration, and the 250 mL retentate sample was ready for processing.

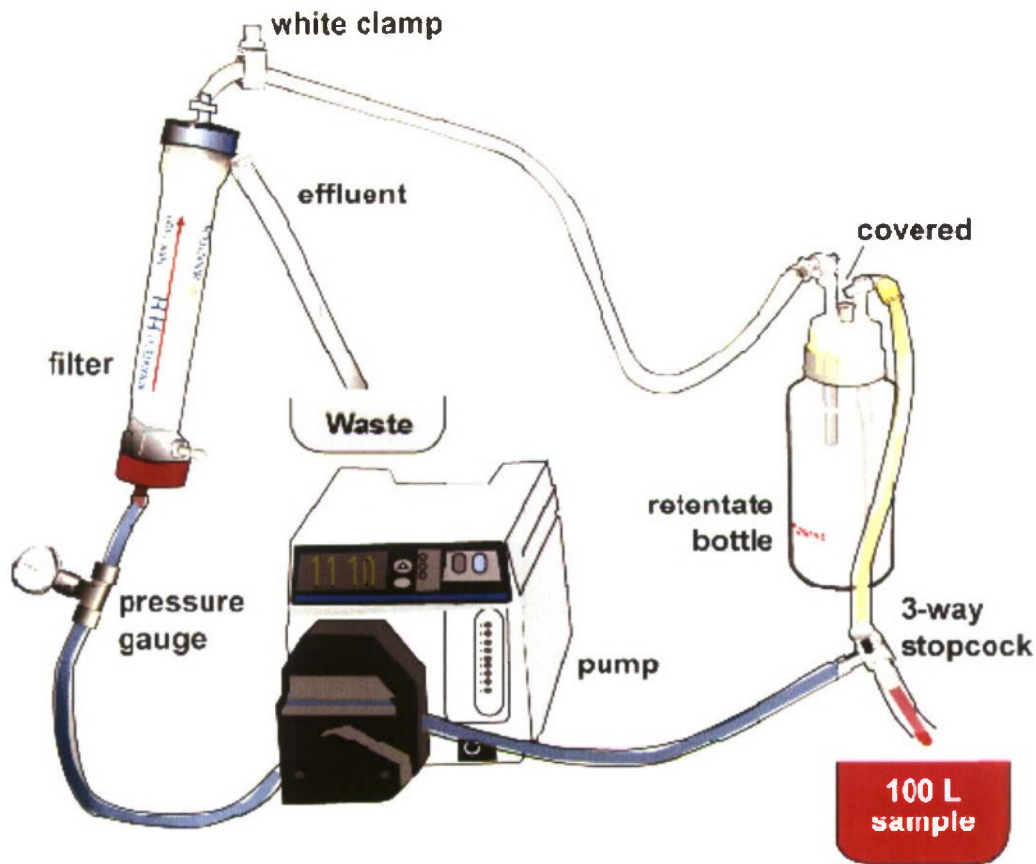


Figure. Ultrafiltration Setup Showing Relative Positioning of Pump, Hollow-fill Fiber Column and Collection of Retentate.

2.4 Spiking and Recovery.

For spiking after ultrafiltration, the appropriate number of microorganisms were directly added to the 250 mL retentate volume. For spiking during ultrafiltration, a 5 mL syringe (no needle) containing the requisite number of microorganisms (determined as described above) was used to slowly inject the inoculum into the tubing (blue line) between the three way valve and pump head (see Figure). Spiking was done in this manner to avoid contaminating the large volume carboy of water with select agent pathogens. This protocol ensured that all pathogens remained contained within a biosafety cabinet. The addition of vegetative cells or spores was performed in three pulses. After spiking (either during or after ultrafiltration), 100 μ L aliquots of the 250 mL retentate was plated onto appropriate selective media and incubated under appropriate aeration conditions (refer to Table 1). After incubation, plates were examined for the presence of “putative” isolates (colonies that resembled the target organism as determined by colony morphology criterion). Those colonies were subcultured onto nutrient rich microorganism specific media and incubated (refer to Table 1). Subculturing in this manner was

repeated until a pure colony culture was achieved. The pure culture was subjected to various biochemical tests described below to presumptively identify the organism.

2.5 Biochemical Tests.

The following biochemical tests were performed: Gram stain, motility, catalase, oxidase, indole, antibiotic susceptibility, and urease. Gram staining was performed using standard laboratory Gram stain procedure and stains (5). The motility test was performed using standard motility medium per manufacturer's instructions (Culture Media Supplies, Oswego, IL, catalog no. CM247-S920). Catalase test was performed per manufacturer's instructions by transferring inoculum from a pure plate culture grown on TSA (Culture Media Supplies, Oswego, IL, catalog no. CM100-P25) onto the surface of a clean, dry glass microscope slide using a sterile inoculating loop. A drop of 3% H₂O₂ was then immediately dropped onto the portion of smeared inoculum on the slide and observed for gas bubbles, indicating a positive test. Oxidase test was performed per manufacturer's instructions by smearing a portion of inoculum obtained from a pure plate culture grown on TSA onto filter paper impregnated with 1% tetramethyl-*p*-phenylenediamine dihydrochloride (BD Biosciences, Sparks, MD, catalog no. 231746) and observing for a rapid color change to blue or purple within 10 s indicating a positive reaction. Indole test was performed per manufacturer's instructions by saturating a piece of filter paper contained in the bottom of a Petri dish with indole reagent (BD Biosciences, Sparks, MD catalog no. 266641) followed by transferring a portion of inoculum from a pure plate culture grown on TSA onto the filter paper. Then the paper was observed for a rapid development of a blue color indicating a positive reaction. Colistin and polymyxin B resistance was performed per manufacturer's instructions by streaking a Mueller-Hinton agar plate (Culture Media Supplies, Oswego, IL, catalog no. CM101-P25) with an inoculum using a sterile swab dipped into PBS cell suspension that was at 0.5 McFarland turbidity standard. Inoculum was continually added and vortexed until turbidity equaled that of a 0.5 McFarland standard. A colistin and polymyxin B disk was placed in the inoculated area of the plate. All plates were allowed to incubate for 24 to 48 h (refer to Table 1) prior to examining for zone of inhibition around the disk. No clear zone indicated resistance to polymyxin B or colistin, while presence of a clear zone indicated susceptibility to the antimicrobial agents. Urease test was performed per manufacturer's instructions by using urea agar slants in which a loopful of test organism was transferred to the surface of the urea agar slant (Hardy Diagnostics, Santa Maria, CA, catalog no. L65) from a pure plate culture grown on TSA and incubated for 15 min (refer to Table 1). After 15 min of incubation, the slants were observed for a color change to pink in the inoculated area indicating a positive reaction. If no color change was observed, the slant was replaced in the incubator and observed again after 24 h of incubation.

3. RESULTS

Prior to beginning Phase I or II of this study, preliminary experiments were conducted to first assess recovery efficiency for microorganisms among background organisms, individually and in combination, when spiked in a UF retentate sample. It was found that each microorganism could be detected among background when spiked alone in a UF sample by plating onto selective media (refer to Table 1) and observing colony morphology. When microorganisms were combined, it was found that *B. anthracis* spores made it difficult to detect

suspect colonies of *F. tularensis* because the *B. anthracis* colonies outgrew *F. tularensis* on the media tested. However, strains of *F. tularensis*, *Y. pestis*, and *B. thailandensis* could be spiked together in a UF sample without any potential interference (data not shown). Based on these findings, it was decided that water samples should be spiked with *B. anthracis* spores separately, and the remaining organisms could be pooled to reduce the number of UF runs needed for the study. BSL2 strains were used in these experiments (Table 1).

For phase I of this study or “ranging” study, 250 mL UF samples were generated by the USEPA in Cincinnati, OH, and shipped immediately on ice to ECBC for use. Parameters such as pH, temperature, turbidity, and conductivity were recorded for each UF sample along with an estimate of the background heterotrophic count obtained by serial dilution and plating onto general nutrient rich media (data not shown). Phase I studies involved spiking a 250 mL UF sample with *B. anthracis* spores or the remaining microorganisms in combination (*F. tularensis*, *Y. pestis*, and *B. thailandensis*) followed by recovery on selective media (see Table 1) and identification by biochemical tests (listed previously in Section 2). Surrogates or avirulent strains were used for all Phase I/ranging studies (BSL2 strains; see Table 1). This phase represented bacteria recovered from a background of heterotrophic microorganisms naturally present in drinking water that were highly concentrated as a result of the UF process. The recovery of microorganisms at this stage represented idealized (no loss during the UF process) number of target microorganisms against background drinking water flora. UF samples were spiked at three concentrations: high (1,000 CFU/L of initial sample volume), medium (100 CFU/L), and low (10 CFU/L) with each target microorganism. For the high concentration, the initial target of 1,000 CFU/L in unfiltered water corresponded to a working stock titer of 400 CFU/mL in the 250 mL UF sample (or 100,000 CFU in total). Other microorganism concentrations (medium, low) corresponded to 40 and 4 CFU/mL for medium and low, respectively) in the 250 mL UF sample. For Phase I, 2 replicate UF concentrations were performed for the high concentration sample for each microorganism, while the number of separate UF samples performed for the medium and low concentrations differed slightly depending on the target microorganism.

A limit of detection (LOD) is the minimal number of targeted microorganisms added to either 100 L of water or retentate after ultrafiltration for a >95% confirmed detection and positive identification. Results from Phase I are shown in Tables 2 and 3 below. As shown, *B. anthracis* Sterne was detected for each replicate (low, medium, and high). The titer of 10 CFU/L was the lowest concentration performed for *B. anthracis* Sterne and therefore represents the lowest bound for the LOD for this organism given this study design. *B. thailandensis* was not detected at the low titer, but was detected six out of eight times at the medium titer (100 CFU/L), making the LOD between 100 and 1000 CFU/L. *F. tularensis* was detected two out of two times at the high titer (1,000 CFU/L); however, it was not detected at the medium titer but was detected once at the low titer of 10 CFU/L. *Y. pestis* was not detected at medium and low titers. For this purpose, further titers were performed for *Y. pestis* and *F. tularensis* to try and determine a more accurate LOD (Table 3). Additional titers tested showed that *Y. pestis* was detected between 500 and 750 CFU/L. Therefore, the LOD for *Y. pestis* appears to be between 500 and 750 CFU/L. Additional concentrations tested for *F. tularensis* did not aid in determining a more accurate LOD for that organism, which can only be considered to be <1000 CFU/L. All microorganisms described as detected were observed as

suspicious colonies on selective media and were then sub-cultured to obtain a pure culture followed by identification using LRN Level A methods (biochemical tests). The biochemical tests performed for each microorganism for presumptive identification are shown in Table 4 below.

Based on Phase I results, Phase II of the study sought to determine the limit of detection for each of the selected pathogen in municipal water while tolerating losses due to the UF process. Phase II used virulent BSL3 strains for all experiments and involved spiking the microorganisms during UF. All experiments for Phase II were conducted at ECBC using ECBC tap water. Based on the results obtained in Phase I of the study (spiking after UF), the titers represented below in Table 5 were chosen for the BSL3 microorganisms for Phase II of the project (spiking during UF). It was found that *B. anthracis* AMES spores could be detected down to 250 spores/L and *B. pseudomallei* was detected down to 500 CFU/L. The minimum titer of *F. tularensis* that was detectable was 5000 CFU/L, while the minimum detectable concentration of *Y. pestis* was 1,000,000 CFU/L. For both phases, proper controls were included. Negative controls consisted of uninoculated ultrafiltrate, and positive controls included inoculated buffer or culture media.

Table 2. Phase I Recovery of Target Microorganisms in 250 mL Spiked Ultrafiltration Retentate^a

Microorganism	Titers ^b			Total
	Low	Medium	High	
	10	100	1,000	
<i>Bacillus anthracis</i>	8/8 (100)	4/4 (100)	2/2 (100)	14/14 (100)
<i>Burkholderia thailandensis</i>	0/3 (0)	6/8 (75)	2/2 (100)	8/13 (62)
<i>Francisella tularensis</i>	1/4 (25)	0/4(0)	2/2 (100)	3/10 (30)
<i>Yersinia pestis</i>	0/4 (0)	1/4 (25)	2/2 (100)	3/10 (30)

^aResults reported as number of times detected/number of replicates performed followed by percent recovery as determined by dividing the number of times detected by the number of replicates. Variation was < 0.5 log between replicates.

^bAll titers are reported as CFU/L.

Table 3. Phase I Additional LOD Determinations^a

Microorganism	Titer ^b					Total
	250	375	500	750	1,000	
<i>Francisella tularensis</i>	0/1 (0)	NA ^c	0/1 (0)	0/1 (0)	0/1 (0)	0/4(0)
<i>Yersinia pestis</i>	0/1 (0)	0/1 (0)	1/2 (50)	1/1 (100)	NA ^c	2/5(40)

^a Results reported as number of times detected/number of replicates performed followed by percent recovery in parenthesis as determined by dividing the number of times detected by the number of replicates.

^b All titers are reported as CFU/L.

^c NA=not applicable.

Table 4. LRN Level A Methods Used for Identification of Target Microorganisms

Micro-Organism	Gram Reactivity	Motility	Catalase	Oxidase	Indole	Antibiotic Susceptibility	Urease
<i>Bacillus anthracis</i>							
Sterne	+	-	NA	NA	NA	NA	NA
AMES	+	-	NA	NA	NA	NA	NA
<i>Burkholderia pseudomallei</i>							
1026B	-	+	+	+	-	Resistant	NA
<i>Burkholderia thailandensis</i>							
E264	-	+	+	+	-	Resistant	NA
<i>Francisella tularensis</i>							
LVS	-	NA	+	-	NA	NA	-
OR960246	-	NA	+	-	NA	NA	-
<i>Yersinia pestis</i>							
A1122	-	NA	+	-	NA	NA	-
1866	-	NA	+	-	NA	NA	-

NA = not applicable

Table 5. Phase II Recovery of Target Microorganisms with Spiking During Ultrafiltration^a

Microorganism	Titer ^b				
	<u>100</u>	<u>250</u>	<u>2,500</u>	<u>5,000</u>	<u>10,000</u>
<i>Bacillus anthracis</i>	0/3 (0)	2/2 (100)	2/3 (66)	2/2 (100)	2/2 (100)
<i>Burkholderia pseudomallei</i>	<u>500</u>	<u>1,000</u>	<u>2,500</u>	<u>5,000</u>	<u>10,000</u>
	½ (50)	2/2 (100)	1/1 (100)	2/2 (100)	4/7 (57)
<i>Francisella tularensis</i>	<u>1,000</u>	<u>5,000</u>	<u>10,000</u>	<u>1,000,000</u>	
	0/1 (0)	1/1 (100)	2/2 (100)	3/3 (100)	
<i>Yersinia pestis</i>	<u>100,000</u>	<u>500,000</u>	<u>1,000,000</u>		
	0/3 (0)	0/1 (0)	3/3 (100)		

^aResults reported as number of times detected/number of replicates performed followed by percent recovery in parenthesis as determined by dividing the number of times detected by the number of replicates.

^bAll concentrations reported as CFU/L.

4. DISCUSSION

The principal objective of this study was to demonstrate the utility of LRN Level A protocols for the identification and presumptive confirmation of putative colonies isolated following spiking of a select list of bio-threat agents, concentration, and recovery from municipal water samples. This project focused on analyzing and testing methods for recovery of microorganisms to assess the sensitivity of the standardized culture methods. It was necessary to develop and test a technology addressing the titer of spiked samples from large volumes of sample water due to the realization that a threat material would be rapidly diluted soon after its introduction in the water system. Therefore, an ultrafiltration process was used in this study to concentrate 100 L of tap water to a 250 mL final retentate sample. This process was established in the Response Protocol Toolbox available on the USEPA website (http://www.epa.gov/safewater/watersecurity/pubs/guide_response_module3.pdf, accessed June 2009).

The present study consisted of two phases in which the first phase or “ranging” study focused on direct evaluation of the effectiveness of specific assays for identification of target organisms among background microorganisms. The primary goal of the ranging study was to identify and address any initial issues with the LRN Level A assays selected. The ranging study was also to determine rough order of magnitude concentrations for each target that could be used to spike the large volumes of water in Phase II prior to transitioning to the more costly and hazardous BSL3 Phase II operations.

As expected, for each bio-threat agent, the ability to detect the bacteria was greater in Phase I than Phase II. It was noticed during Phase I that *F. tularensis* was not easily detected, particularly at the medium concentration. This was believed to be due to overwhelming background organisms and the lack of a 'better' selective media. These factors in combination made identifying the organism based on colony morphology extremely difficult. In contrast, *F. tularensis* was detected once at the low concentration, but this was probably due to the notation of a marked decrease of background organisms present in that particular ultrafiltrate sample, thereby making identification of putative colonies easier. Also, an apparent discrepancy between the detectable levels of *Y. pestis* obtained in Phase I compared to Phase II could be due to the difference in water quality. The difference water quality was between water at the USEPA in Cincinnati, OH, used for Phase I as compared to ECBC tap water used in Phase II of the study. ECBC water was noted to have less background organisms present when compared to USEPA water, which aided in easier detection of *F. tularensis*. However, recovery of *Y. pestis* was more difficult in ECBC tap water as compared to recovery in water samples received from the USEPA. This resulted in a much higher titers being required for detection (even when taking the loss of recovery due to UF into account). This potential difference could be attributable to the fact that two different strains of *Y. pestis* were used or possibly represented a difference in water quality between EPA and ECBC.

In summary, the concentration procedure proved effective in concentrating large volumes of sample water contaminated with microorganisms to a minimal working volume for testing. Methods used for identification were successful at presumptively identifying each target and approximate LODs were obtained. These detection methods and concentration protocol must be further refined and tested in a multi-laboratory validation study, preferably in different municipalities across the nation to further support the use of these procedures in response to a terrorist event. It is envisioned that in a situation where the nation's water supply is compromised, on-site ultrafiltration could be performed followed by shipment of a small volume (250 mL) water sample to an LRN Level A laboratory for processing, using protocols developed under this study for 'ruling in' or 'ruling out' threats. The specific identification, however, would be performed by a national reference lab with BSL-3 level capability.

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