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Filtration methods for recovery of *Bacillus anthracis* spores spiked into source and finished water

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Abstract

Spores of *Bacillus anthracis* Sterne strain were recovered from 100 ml and 1 L volumes of tap and source waters using filtration through a 0.45 um filter, followed by overnight culture on agar plates. In a set of experiments comparing sheep red blood cell (SRBC) plates with a chromogenic agar formulation designed by R & F Laboratories, with a spiking dose of 47 plate-enumerated spores in 100 ml tap water, the mean spore recoveries were 34.0 and 30.8 spores, respectively. When a spiking dose of 100 fluorescence activated cell sorter(FACS)-enumerated spores was used in 100 ml potable water, the average recovery with SRBC plates was 48 spores. Detection efforts with spiking doses of 35 and 10 spores in 1 L tap water were successful, but recovery efforts from spiked 1 L volumes of source water were problematic due to the concomitant growth of normal spore-forming flora. Recoveries were also attempted on 10 L volumes of tap water. For a spiking dose of 100 spores, mean recovery from six replicates was 11 spores ($\pm$6.8, range 2–20), and for a spiking dose of 10 spores, mean recovery from six replicates was 2.3 spores ($\pm$3.5, range 0–9). Efforts were also made to ''direct detect'' spores via polymerase chain reaction (PCR) on washes from filters. When spiking 534 spores in 100 ml, 9/9 replicates of spiked tap water, 6/6 source water replicates, and 0/3 unspiked controls were positive by *lef* PCR. When 534 spores were spiked into 1 L tap water, the *lef* PCR was unsuccessful; however, using the nested *vrrA* PCR resulted in 4/9 spiked samples, and 0/3 unspiked controls, testing positive. Our results indicate that an inexpensive and user-friendly method, utilizing filtration apparatus commonly present in many water quality testing labs, can readily be adapted for use in detecting this potential threat agent.
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Keywords: *Bacillus anthracis*; Recovery; Filtration

1. Introduction

In the United States in the Fall of 2001, a terror attack using *Bacillus anthracis* spores distributed through mailing envelopes resulted in the deaths of five people from inhalation anthrax, and infection with either pulmonary or cutaneous anthrax in another 17 individuals (Jernigan et al., 2002). To date, the perpetrator(s) of the attack have not been identified. Conventional speculation on the use of a weaponized *B. anthracis* envisaged scenarios in which an aerosolized formulation was distributed via airplane spraying or some other form of vehicle-mounted, specialized dispersal method. However, this bioterror event provided graphic evidence of the ability of a relatively crude delivery method to cause widespread apprehension among the public, including

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disruption of mail delivery, and has resulted in the implementation of irradiation protocols for mail addressed to upper-level politicians and government officials.

One aspect of a bioterror event involving the use of *B. anthracis* that has not received a great deal of attention is water-borne transmission. We are aware of no official account of such an event, but we believe it prudent to ask if such an event can be dismissed as unlikely. This is particularly relevant in light of the arrest in the US in July, 2002, of two Islamic militants, James and Mustafa Ujaama, on suspicion of plotting to contaminate potable water sources (''Feds Arrest Al Qaida Suspects With Plans to Poison Water Supplies'', by Carl Cameron, Fox News Service, Tuesday July 30, 2002). (The Ujaamas were subsequently released after agreeing to cooperate with authorities in their investigations of other suspected US-based terror cells). Another relevant report, based on an article in an Arabic-language newsmagazine, indicates that Al-Qaida operatives are contemplating poisoning American water supplies (''magazine reports Al-Qaida Threat to US Water Supply'', Associated Press, Baltimore *Sun*, May 30, 2003). In addition to a bioterrorism scenario, covert usage of bioweapons during warfare also cannot be ruled out; during the campaign against Iraq in March/April, 2003, Jordanian police arrested six Iraqis on suspicion of plotting to poison a water tank located in the desert near the border with Iraq. The tank supplied water to US troops (''Jordan arrests Iraqis in Plot to Poison Water, and Probes Others'', by Emily Wax, *Washington Post*, Tuesday April 2, 2003).

There are several aspects of the biology of *B. anthracis* that would make it more suitable than other threat agents for deliberate introduction into source or drinking water supplies: the environmental hardiness of the spores, their resistance to levels of chlorine used to treat drinking water (Burrows and Renne, 1999; Rose et al., 2005), the protean symptomatology of gastrointestinal (GI) tract anthrax (Sirisanthana and Brown, 2002), and the observation that mortality of this form of the infection can approach 50% (Friedlander, 1997). Existing literature on the detection of *B. anthracis* spores in water is scarce; a Russian study published in 1966 indicated that 100 ml river water spiked with the equivalent of 20 spores per 1 ml could be detected by a combination of filtration and overnight culture on peptone/beef serum agar plates (Tomov, 1966). Studies done on other species of the genus *Bacillus* indicate that spores can be recovered from 1–100 ml volumes of source and finished waters using membrane filtration and agar plate culture (Francis et al., 2001; Ostensvik et al., 2004).

We investigated several methods for the detection of *B. anthracis* spores in source and tap water. Our approach was colored by the following considerations: first, the methods should ideally be relatively inexpensive and require modest equipment in order to be feasible for routine use by testing laboratories associated with municipal water facilities. Secondly, they should require skills that are either already present among water quality testing laboratory personnel, or alternatively, require a modest amount of instruction in order to effectively implement. Finally, we looked to design detection protocols that were amenable to ramping-up in terms of scale and number, as might be required during periods when there is reason to perform frequent testing of water samples for the presence of *B. anthracis* (i.e., in response to threats, however believable, that this agent has been introduced into water supplies).

This report describes the use of an overnight, culture-based method, and a more immediate, polymerase chain reaction (PCR)-based ''direct detection'' method, for the detection of *B. anthracis* spores in source and tap water.

2. Materials and methods

2.1. B. anthracis spores

B. anthracis Sterne strain was a kind gift of Dr. Catherine Fenselau, University of Maryland at College Park, MD. This BSL-2 strain lacks the pXO2 plasmid that codes for the capsule genes, and historically was used as a veterinary vaccine. Aliquots (100–200 μl) of spores in sterile, reagent-grade water were quantitated by heat shock at 60–65 °C for 20 min and plating on sheep red blood cell (SRBC) agar plates (Remel, Lenexa, Kansas). The plates were incubated overnight at 37 °C and examined the next morning for the appearance of colony forming units (cfu) with an appearance suggestive of *B. anthracis* Sterne (i.e., gray or whitish colonies with a ''ground glass'' appearance, with weak or absent evidence of hemolytic activity). Another agar formulation, supplied by R&F Laboratories (West Chicago, IL), also was used to culture some of the spores recovered from spiked water samples; with this formulation, the *B. anthracis* colonies initially appear as white colonies with irregular contours and hairlike projections; after ~24 h incubation at 37 °C, the centers assume a dark blue coloration. In contrast, non*B. anthracis* spp. of *Bacillus* (e.g., *B. cereus*) grow as colonies with a well-defined outline and assume a uniform blue coloration throughout the colony.

When the number of colonies exceeded ~50–70/plate, an automated colony-counting platform was used (Q-Count, Spiral Biotech, Norwood, MA).Throughout the manuscript, when quantities of spores are mentioned, this refers to the cfu yielded by plating of the spore quantity in question. However, for spiking experiments conducted at the City of Phoenix Water Services Laboratory, we used spores enumerated via