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Comparison of quantitative PCR and culture-based methods for evaluating dispersal of *Bacillus thuringiensis* endospores at a bioterrorism hoax crime scene

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ABSTRACT

Since the anthrax mail attacks of 2001, law enforcement agencies have processed thousands of suspicious mail incidents globally, many of which are hoax bioterrorism threats. Bio-insecticide preparations containing *Bacillus thuringiensis* (Bt) spores have been involved in several such threats in Australia, leading to the requirement for rapid and sensitive detection techniques for this organism, a close relative of *Bacillus anthracis*. Here we describe the development of a quantitative PCR (qPCR) method for the detection of Bt crystal toxin gene *cry1*, and evaluation of the method's effectiveness during a hoax bioterrorism event in 2009. When combined with moist wipe sampling, the *cry1* qPCR was a rapid, reliable, and sensitive diagnostic tool for detecting and quantifying Bt contamination, and mapping endospore dispersal within a mail sorting facility. Results from the *cry1* qPCR were validated by viable counts of the same samples on *Bacillus*-selective agar (PEMBA), which revealed a similar pattern of contamination. Extensive and persistent contamination of the facility was detected, both within the affected mailroom, and extending into office areas up to 30 m distant from the source event, emphasising the need for improved containment procedures for suspicious mail items, both during and post-event. The *cry1* qPCR enables detection of both viable and non-viable Bt spores and cells, which is important for historical crime scenes or scenes subjected to decontamination. This work provides a new rapid method to add to the forensics toolbox for crime scenes suspected to be contaminated with biological agents.

1. Introduction

Anthrax is a zoonotic infection caused by the bacterium *Bacillus anthracis* [1], that can manifest in three distinct ways depending on the exposure route – cutaneous, gastrointestinal, or pulmonary. The pulmonary disease is the most dangerous, with a mortality rate as high as 95% [2]. Due to the severe nature of the disease, and the robust survival properties of *B. anthracis* endospores, anthrax has been developed as a biological warfare agent [3] and remains a significant concern as an agent of bioterrorism. In the anthrax letter attacks in the USA in 2001, a dried endospore preparation of *B. anthracis* was dispersed through the US Postal system, leading to cases of both inhalational and cutaneous anthrax, and five deaths [4–8].

Much research has been directed towards the rapid detection of *B. anthracis*, with the aims of enabling a rapid response to bioterrorism threats, and minimizing their impact on public health. Current gold standard methods for detecting anthrax involve conventional culture techniques, including the *B. anthracis* selective polymyxin–lysozyme EDTA–thallous acetate (PLET) agar, however, these methods are labour and resource intensive, and require up to 48 h of incubation [1,9]. Nucleic acid detection strategies via polymerase chain reaction (PCR) have been developed, targeting various regions from either or both virulence plasmids, pX01 and pX02, and specific chromosomal markers such as Ba813 [10–15]. Whilst still requiring culture analysis to confirm PCR results, these sensitive and specific assays can be performed in less than 2 h. PCR-based assays are advantageous for forensic investigations involving historical crime scenes or items, as they can detect non-viable organisms. A variety of immunological tests which detect the spores, vegetative cells or toxin proteins of *B. anthracis* have also been developed, however these are generally useful only for clinical applications, and cannot be applied to environmental detection strategies [16]. Rapid, field-based immunological detection of *B. anthracis* can be performed using lateral flow devices [17], however these can lack sensitivity and are prone to false positive results [16].

Several problems arise in applying the above-described methods in the context of a real bioterrorism incident due to a lack of validation of sampling and detection methods [18], and conflicts between the different priorities of traditional forensic analysis, microbiological analysis, and decontamination [19]. The

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development of robust and well-validated anthrax sampling and analysis protocols requires their testing under real-world conditions, where there may be a long lag between the contamination incident and microbiological sampling, and where potentially confounding events may occur, including extensive movement of persons in the contaminated zone, the presence of background organisms, or application of chemical decontamination agents. An acknowledgement of these problems is seen in the report of the United States Government Accountability Office (GAO) on sampling approaches used during the anthrax mailings of 2001 [18]. This report found that the sampling, extraction and analysis techniques applied were not validated, which led to problems with reproducibility and interpretation of results [20].

Bacillus thuringiensis (Bt) is closely related to *B. anthracis*, and it has been suggested that *Bacillus cereus*, *B. anthracis* and *B. thuringiensis* should be considered a single species [21,22]. The phenotypic differences observed between these three organisms have in fact been found to be predominantly due to the presence of plasmids, which vary in copy number and size [23]. Strains of Bt bacteria make crystalline protein (Cry) toxins that have high toxicity towards insects [24,25], making Bt an important biological control agent in agriculture [26]. Endospores of Bt have a similar size, shape and composition to those of *B. anthracis* [27,28], but are non-toxic and readily available commercially [26,29]. These factors make Bt both an excellent surrogate organism for studying *B. anthracis*, and a convincing hoax bioterrorism agent. Whilst Bt is non-harmful to humans, such hoaxes can cause significant public panic, disruptions to infrastructure, and financial damage. In November of 2001, over 260 letters threatening to contain anthrax were sent to Planned Parenthood clinics across the USA, which were subsequently found to contain Bt [30]. Several cases of Bt bioterrorism hoaxes have also occurred in Australia since 2001. The development of improved sampling and rapid detection techniques for Bt spores is important both to inform strategies for the highly similar *B. anthracis*, and to allow a quick and appropriate response to bioterrorism hoaxes. A rapid method for differentiation of *B. anthracis* and *B. thuringiensis* would be a useful initial screening tool for suspected bioterror crime scenes.

In August of 2009, a threatening letter containing powder was received and opened at a government mail sorting facility in Sydney, Australia. Initial microbiological and molecular biological analysis of the powder, including several screening assays for *B. anthracis* (all returned negative results, data not shown), revealed the presence of Bt spores and crystal toxin genes (data not shown), indicating that this was a bioterrorism hoax event. The aims of the current study were to develop a quantitative PCR (qPCR) assay for *cry1* crystal toxin genes, to compare the efficacy and sensitivity of the *cry1* qPCR assay compared to a culture-based endospore detection method, and to evaluate the usefulness of qPCR and culture methods in a case study of the high profile mailroom immediately after the 2009 incident. The *cry1* qPCR assay was originally developed for use during controlled studies regarding recovery efficiencies of various sampling materials of *Bacillus* spores from porous surfaces and items. The assay was applied in this case to gain insights into the dispersal patterns of *Bacillus* endospores in real-world environments during bioterrorism incidents, and to use this information to better inform forensic sampling, decontamination, and emergency responses.

2. Materials and methods

2.1. Mailroom processes during incident

A mailroom worker occupying the desk space shown in grid C3 of Fig. 1 opened a powder-containing letter at 10:45 am on the 6th of August 2009. Immediately following detection of the suspicious mail item, the staff member placed a large plastic bucket over the item to prevent further contamination of the area, and then triggered the duress alarm (both bucket and alarm are in grid D1 of Fig. 1). As per

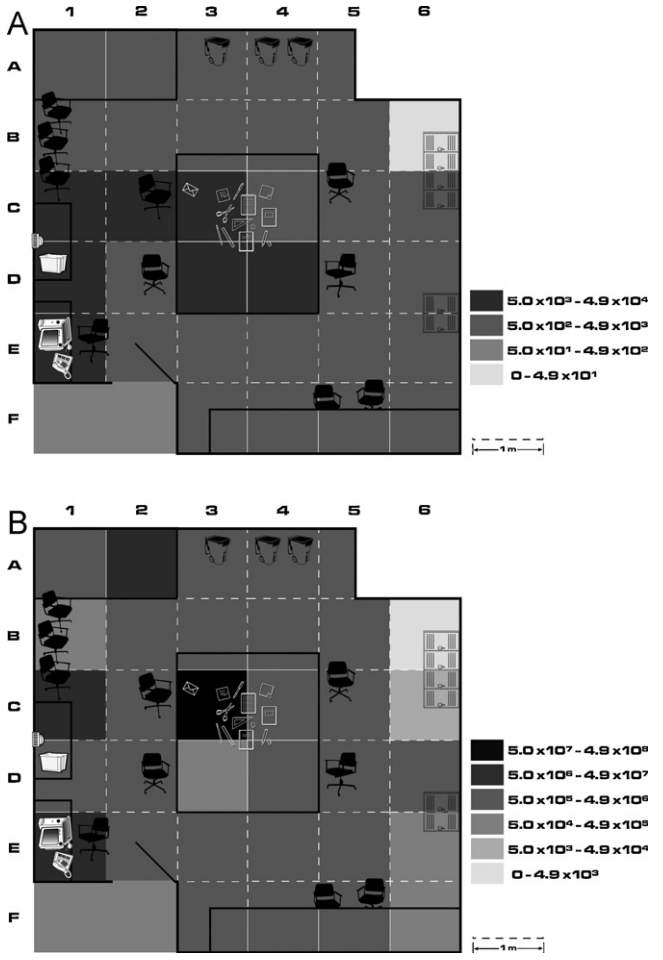

Fig. 1. (a) Map of affected mailroom, showing colony counts on PEMBA medium recovered from each 1 m × 1 m grid square. For ease of visualisation, colony counts have been graded into 4 levels, represented by different shades of gray. (b) Map of affected mailroom, showing *cry1* gene copies recovered from each 1 m × 1 m grid square. For ease of visualisation, gene counts have been graded into 6 levels, each represented by a different shade of gray.

standard office procedures the mailroom was isolated, the air conditioning was turned off, and NSW Emergency Services were notified. Three other staff members present remained in the room until Emergency Services arrived approximately 10 min later. A team of three NSW Fire and Rescue (NSWFR) personnel were the first emergency team to enter, and they instructed all staff in the mailroom to decontaminate exposed skin (face and hands) with soapy water, and then removed them from the scene. Decontamination of mailroom staff was done in a small kitchen opposite the mailroom (grid D2 of Fig. 2). The NSWFR then performed some environmental monitoring for toxic chemicals, and were decontaminated with 5% sodium hypochlorite solution as they exited the mailroom (Fig. 1, grids F1 and F2) (this took approx. 30 min). A second entry team, composed of two NSW Police Forensic (NSWPF) and one NSWFR worker then entered the room to process the suspicious letters (this took approx. 30 min). The items were photographed, and some initial analysis performed on the powder present in the letter, including testing for *B. anthracis* by means of the RAMP Immunoassay (Response Biomedical Corporation, Vancouver, BC), which returned a negative result. The suspicious letters were then packed inside two sets of plastic bags, and sealed within a drum for decontamination and subsequent transport to the NSWPF laboratory for further analysis. It is estimated approximately 0.1 g of powder was present in the letter, which represents approximately 1.0×10^8 spores based on viable counts of a commercial Bt product (data not shown). A limited surface decontamination was performed in the mail room, which involved topical application of 5% bleach solution to the table area where the letter was initially opened. NSWPF and NSWFR personnel were surface decontaminated upon exiting the mailroom (Fig. 1, grids F1 and F2). The mailroom remained closed for the rest of the day, but was returned to normal service the next morning, following identification of the white powder as Bt.

2.2. Microbiological sampling of mailroom after incident

Two days after the incident occurred, forensic scientists re-visited the government building to investigate levels of endospore contamination of the