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Background frequency of *Bacillus* species at the Canberra Airport: A 12 month study

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ABSTRACT

Anthrax, caused by *Bacillus anthracis*, is a naturally occurring disease in Australia. Whilst mainly limited to livestock in grazing regions of Victoria and New South Wales, movement of people, stock and vehicles means *B. anthracis* could be present outside this region. Of particular interest is the "background" prevalence of *B. anthracis* at transport hubs including airports. The aim of this study was to determine the background frequency of *B. anthracis* and the commonly used hoax agent *Bacillus thuringiensis* at the Canberra Airport over a 12 month period. Samples were collected daily for seven days each month from August 2011–July 2012 and analyzed using species specific real-time polymerase chain reaction. Fourteen samples (of a total of 575) were positive for the *B. anthracis PL3* genomic marker, 24 for the *cya* (pXO1) plasmid marker and five for the *capB* (pXO2) plasmid marker. Whilst five samples were positive for both *PL3* and *cya*, no samples were positive for all three markers hence there is no evidence to suggest the presence of pathogenic *B. anthracis* strains. *B. anthracis* targets were detected primarily in February 2012 and *B. thuringiensis* peaked in October and November 2011 and again in April and May 2012. This study provides a rapid method to screen for, and differentiate, *Bacillus* species. Armed with this information investigators will be able to discriminate a "threat" from "background" frequencies should the need arise.

1. Introduction

Anthrax, caused by the Gram-positive spore forming bacterium, *Bacillus anthracis*, is a naturally occurring disease with outbreaks primarily occurring in animal populations, such as sheep, goats and cattle. In Australia anthrax is mainly limited to livestock in grazing regions of Victoria and New South Wales in an area known as the 'anthrax belt' [1]. However, as cases of anthrax have occurred outside of the anthrax belt, likely due to the movement of people, stock and vehicles across Australia, *B. anthracis* could naturally be present at varying levels Australia wide. In addition to naturally occurring cases, *B. anthracis* may be used deliberately as a biological agent as was seen most recently in the 2001 USA anthrax letter attacks in which spores were sent in letters through the postal system causing five deaths and the widespread contamination of mail boxes, post offices and buildings [2,3].

B. anthracis possesses two main virulence factors; the anthrax toxin and the capsule which are encoded on the virulence plasmids pXO1 and pXO2, respectively. Toxin formation is controlled by the *lef* gene which encodes for the lethal factor, the *cya* gene which encodes for oedema factor, and the *pag* gene which encodes for the binding protein protective antigen, all of which are on the pXO1 plasmid. The capsule coding genes on the pXO2 plasmid (*capA*, *capB*, and *capC*) are essential for the formation of the poly-D-glutamic acid capsule which is thought to inhibit host defences by preventing phagocytosis of cells by macrophages thereby protecting the bacterial cell (for a review of *B. anthracis* virulence factors see Koehler [14]). Since *B. anthracis* requires both pXO1 and pXO2 plasmids to cause disease [4], and similar plasmids can be found in closely related *Bacillus* species [5,6], detection assays must target both plasmids and a chromosomal target to distinguish pathogenic *B. anthracis* strains from non-pathogenic strains and other *Bacillus* species.

Hoax scares involving white powders can be equally as disruptive, causing panic and confusion, so identification assays need to be capable of quickly distinguishing between a hoax and real threat. Non-pathogenic *Bacillus* species, such as *Bacillus*

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thuringiensis which is found in commercial insecticides, pose a particular challenge for identification as the spores of these *Bacillus* species cannot be microscopically distinguished from those of *B. anthracis.* Whilst these species can be distinguished using culture and microbiological assays, this is time consuming and requires a high level containment laboratory. There are two strains of *B. thuringiensis* commonly found in insecticides which can be differentiated by their plasmid encoded *cry* genes which encodes for a papasporal crystal endotoxin. *B. thuringiensis* subsp. *Kurstaki* contains the *cry1* gene and *B. thuringiensis* subsp. *Israelensis* the *cry4* gene [7].

A variety of methods have been designed for sampling *B. anthracis* and other bacterial spores from the environment, including swabs, wipes, vacuum filters and air sampling, with the publication of methods and their collection efficiencies increasing substantially after the 2001 USA anthrax attacks (reviewed in [8]). Immunological, microbiological and molecular identification methods are available with real-time polymerase chain reaction (qPCR) being commonly applied to food, clinical and environmental samples. The choice of sampling and identification method will vary depending on the type of surface material, area size and available resources. Methods must be efficient, specific, sensitive, rapid, distinguish pathogenic *B. anthracis* from non-pathogenic *Bacillus* species, overcome inhibitors commonly found in the environment such as cleaning products and soil, and be applicable to a field setting. It is important to optimize and validate any chosen method before screening commences.

The aim of this project was to develop a sampling and identification assay for *B. anthracis* and the two *B. thuringiensis* strains commonly used as insecticides, and screen for these *Bacillus* species at the Canberra Airport over a 12 month period. Using a wipe sampling approach and qPCR this study establishes the background frequency of *B. anthracis*, *B. thuringiensis* subsp. *Kurstaki* and *B. thuringiensis* subsp. *Israelensis* at the Canberra Airport from August 2011 to July 2012, enabling investigators to discriminate a "threat" from "background" should the question arise in the future.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The Anthrax Vaccine (Living Spore Sterne Strain; $pXO1^{+}$ $pXO2^{-}$), manufactured by Pfizer, was the source of *Bacillus anthracis* Sterne strain. Commercially available Nature's Way® Caterpillar Killer Dipel® Bio-insecticide (Yates Australia, referred to hereafter as "Dipel") was the source of *Bacillus thuringiensis* subsp. *Kurstaki*. Dr Liam O'Connor (Pathwest Laboratory Medicine) provided *B. thuringiensis* subsp. *Israelensis*.

Bacterial strains were routinely cultured in nutrient broth or on nutrient agar (Oxoid) prepared according to the manufacturer's instructions. Media was sterilized by autoclaving for 20 min at 103.4 kPa and 121 °C. Broth cultures were grown with aeration in an orbital shaker incubator (Bioline) at 210–240 revolutions per minute (rpm) and 37 °C. Agar plates were incubated under aerobic conditions at 37 °C. All strains were stored in nutrient broth containing 50% (v/v) sterile glycerol (Unilab) at −80 °C.

2.2. Environmental samples

Samples were collected for seven consecutive days each month from August 2011 to July 2012 from five different sites within the Canberra Airport (Canberra, Australian Capital Territory (ACT), Australia). The sites correspond to the movement of people and luggage in and out of the airport, as well as a low traffic control area. Samples were also collected from Australian Air Express freight transport depot (Canberra, ACT, Australia) between August 2011 and January 2012. Samples could not be collected from Australian Air Express for the full duration of the study period due to relocation of their operations. Details of the collection sites are shown in Table 1. A total of 575 samples were collected during the study period.

Samples were collected from a 0.5 m × 1.0 m area using a sterile wipe (Sentry Medical 7.5 cm× 7.5 cm non-woven; catalogue number NWS2323) pre-moistened with 2 mL of sterile phosphate buffer solution (PBS). To minimize sample cross contamination all sampling was carried out using an individual sampling pack for each location. The area was wiped in a horizontal and vertical direction, followed by the perimeter. Wipes were then placed in a 50 mL tube, sealed and transported to the laboratory. A volume of 40 mL of PBS was added to the 50 mL tube containing the wipe and vortexed at maximum speed for 3 min using a Vortex-Genie 2 (SI Scientific Industries) fitted with a horizontal 50 mL attachment. Following a 5 min rest in an upright position, the wipe was removed and the 50 mL tube centrifuged at 4000 × *g* for 15 min at 4 °C in a swinging bucket rotor. The supernatant was removed and the pellet resuspended in 300 μL of sterile PBS and transferred to a 1.5 mL tube. Samples were stored at −20 °C until further use.

2.3. QIAamp DNA extraction mini kit (QIAGEN)

A modified version of the DNA purification from tissue protocol was performed using the QIAamp DNA extraction mini kit [9]. Briefly, 300 μL of Buffer ATL was added to the sample which was then incubated at 80 °C for 20 min. The sample was transferred to a 2 mL tube containing 0.1 mm glass beads (Precellys). A volume of 0.5–1.0 μL of Antifoam Y-30 (Precellys) was added and the sample was then homogenized at 6000 rpm for 3 × 20 s intervals (15 s rest between each interval) in a Precellys 24 Bead-beater (Bertin Technologies). Following homogenization samples were centrifuged at 4000 × *g* for 3 min to pellet the beads and the lysate transferred to a 1.5 mL tube. A volume of 20 μL of proteinase *K* was

Table 1
Study sampling sites at the Canberra Airport and Australian Air Express freight depot.

Sample name	Description	Location	Surface type
People out	People departing Canberra	Approximately 4 metres air-side from the security checking zone, level 2, CA	Tiles (non-porous)
People in	People arriving into Canberra	Directly outside the one-way door at the base of the exit escalator, level 1, CA	Tiles (non-porous)
Luggage out	Luggage departing Canberra	Check-in luggage carousel for all domestic flights, level 2, CA	Smooth rubber (non-porous)
Luggage in	Luggage arriving into Canberra	QANTAS baggage claim carousel, level 1, CA	Textured rubber (non-porous)
Low traffic control	Low traffic area of the airport	Area of low pedestrian traffic underneath the exit escalator, level 1, CA	Tiles (non-porous)
Freight in	Freight arriving into Canberra	Freight storage case, Australian Air Express	Stainless steel (non-porous)
Freight out	Freight departing Canberra	Freight security scanning machine, Australian Air Express	Textured rubber (non-porous)

CA–Canberra Airport.