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Evaluation of the rapid analyte measurement platform (RAMP) for the detection of *Bacillus anthracis* at a crime scene

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Abstract

The aim of this study was to evaluate the accuracy and reliability of the rapid analyte measurement platform (RAMP) for presumptive identification of *Bacillus anthracis* spores. Test samples consisted of serial dilutions of spore preparations of several *Bacillus* species, including *B. anthracis*, which were tested, using the RAMP Anthrax test cartridge, according to the manufacturer's instructions. The fluorescence labelled antibody–antigen complexes were detected in the portable reader after 15 min following sample addition. Dilutions of common environmental and household powders were also tested to identify possible false positive results. *B. anthracis* spores were identified reliably in test samples containing more than 6000 spores. The test kits were highly specific, showing no cross reactivity with other *Bacillus* species or any environmental powders tested. The RAMP system for detection of *B. anthracis* spores, from environmental samples, showed consistent results under a variety of analytical conditions, enabling the trained user to provide a rapid, accurate preliminary risk assessment of a suspected bioterrorism incident.
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1. Introduction

The development and availability of hand held devices (HHD) for detection of chemical, biological and radiological (CBR) agents have increased within the last 2 years [1]. In Australia, the use of such devices is limited to specialist police forensic units, allowing scientific officers to detect a number of biological agents in the field and provide rapid assessment and response to potential acts of terrorism.

A potential act of bioterrorism requires both a law enforcement and public health response. The priority lies in determining whether a biological threat agent has been used and, if the threat is credible, identification of the agent and preservation of the crime scene. Providing presumptive information at the scene assists this multi-agency response and is valuable in reducing turn around times for both public health response and site recovery [2].

The RAMP® system (Response Biomedical Corporation, Vancouver, British Columbia) is one of the instruments currently in use in Australia, by both law enforcement agencies and the Public Health Laboratory Network. It is used for the preliminary detection and identification of *Bacillus anthracis* spores in environmental samples that pose a potential threat and require investigation. Previous studies have shown that the RAMP® hand held device for detection of *B. anthracis* is well suited to the requirements of field based testing [2,3]. It is compact, portable, lightweight and easily decontaminated.

The RAMP® Anthrax Assay test cartridge contains an analyte-specific immunochromatographic strip, which uses latex particles tagged with fluorescence-labelled antibodies specific to the surface antigens of anthrax spores. The strip has two internal sites, the test line and the control line. The RAMP® instrument detects the presence of fluorescent beads that attach to the test line when the specific antigen is present. The test result is validated when control antigen coated latex beads attach to the control line and a comparison of the

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fluorescence levels is made by the RAMP® reader. A 'positive' or 'negative' result is obtained in 15 min and is indicated on the screen [1].

This instrument provides a qualitative result with no indication of the spore concentration in the test sample. The aim of this evaluation was to determine the specificity of the RAMP® for detection of *B. anthracis* spores and the working limit of detection.

2. Materials and methods

2.1. Bacterial strains

Two *B. anthracis* strains and three other *Bacillus* species, chosen because of their close genetic relationship with *B. anthracis*, were tested to evaluate the sensitivity and specificity of the RAMP® Anthrax test. These were: *B. anthracis* isolated from a case of veterinary anthrax (VRS 3518, Sydney Australia); *B. anthracis* d-Sterne strain; *B. subtilis* wild strain; two strains belonging to the *B. cereus* group, namely *B. cereus* var. *cereus* (ATCC 14579) and *Bacillus thuringiensis* var. *kurstaki* (Bt). The identity of all strains was confirmed by standard phenotypic culture-based methods [4], including long chain fatty acid analysis using the Sherlock Microbial Identification System (MIS) version 4.0 (MIDI, Inc. Newark, Delaware, USA). Cellular fatty acids (CFA's) were extracted according to manufacturer's instructions. Briefly, cells were grown for 48 h on Trypticase Soy Broth plus agar at 28 °C and Trypticase Soy Agar + 5% blood at 36 °C. A 10 μl of the culture was harvested and the fatty acid methyl esters prepared, separated and identified. Results were compared with those listed on the MIS identification library database.

2.2. Environmental and non-biological samples

Ten environmental and common household powders were tested to evaluate the specificity of the RAMP® Anthrax test namely: plain flour (George Weston Foods Ltd.), bicarbonate of soda, washing powder (ColdPower® Colgate Palmolive Pty Ltd.), flea powder (Novartis Pharmaceuticals,) talcum powder (Johnson and Johnson Pacific, NSW, Australia), cement powder, Dipel®, *B. thuringiensis* spore powder (Arthur Yates & Co. Pty Ltd., Milperra, NSW, Australia), cooking yeast, Coffee Mate® (Nestle Australia Ltd., North Ryde, NSW, Australia), gypsum, caster sugar (CSR Sugars Australia Pty Ltd., Epping, NSW, Australia). A suspension of each powder was prepared at a concentration of 1 g/l in distilled water [5].

2.3. Bacterial sample preparation

All processes, including spore preparation, serial dilutions, and RAMP® testing were carried out in a Class 1 Biological Safety Cabinet (BSC) within a PC3 laboratory. All procedures were conducted using items contained in the RAMP® Anthrax test kit to allow complete evaluation of the kit from the perspective of the end user.

All strains were passaged three times on horse blood agar (HBA) and incubated aerobically at 37 °C before inoculation onto sporulation agar slopes [4]. The sporulation agar slopes were incubated aerobically for 48–72 h at 30 °C. To determine the degree of sporulation a wet mount was prepared from each culture slope and examined using 400× phase contrast microscopy. Spore slopes were only harvested into sterile water when sporulation and lysis of vegetative cells was complete.

A sterile lysozyme solution (0.5 g/l) (L-6876, Sigma Chemicals, St. Louis, USA) was used to reduce the level of vegetative cell debris surrounding the spores. The spore suspensions were centrifuged at 800 × *g* for 5 min and the spore pellet resuspended with 10 ml of lysozyme solution. The suspensions were agitated for 2 h at 37 °C. Each spore harvest was washed several times to remove the lysozyme solution [6]. Spore preparations were stored at 2–8 °C until required. Several fold dilutions of spore suspensions were prepared for each test organism. The starting concentration of each spore suspension was adjusted to McFarland 4.0 (McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range). McFarland 4 corresponds to approximately 10^7 spores/ml.

To validate the spore concentration present in each dilution, plate counts on each set of dilutions were performed by inoculating 10 and 100 μl, using micropipettes, of each dilution onto 5% HBA and spreading the inoculum evenly over the plate with a sterile hockey stick. The plates were left to dry completely before being incubated aerobically at 37 °C for 24 h. Colonies were then counted and an average of the repetitions was determined. These dilutions were then used to determine the detection limit of the RAMP® Anthrax test.

2.4. Test method

The RAMP® Anthrax test kit contains individual test cartridges specific to the test agent of choice, swabs, buffer tubes and disposable inoculating pipette and tip. In the field, the operator uses the swab to touch the suspect powder then returns it to the buffer tube prior to inoculation of the test cartridge. A portion of the buffer solution is absorbed into the swab therefore, to parallel this procedure in the laboratory; we removed 10 μl of the buffer solution and replaced it with 10 μl of each test dilution.

The RAMP® pipette and tip were used to resuspend the dried reagents and aspirate 70 μl of the mixed sample from the buffer tube. This aliquot was transferred into the test cartridge, which was then placed into the RAMP® reader. Due to the expense of the test kits, each dilution of spore suspension was tested in duplicate or triplicate and powder samples were tested in triplicate. The RAMP® test results were recorded as either positive or negative after 15 min duration.

In order to reduce the risk of aerosolised particles, all bacterial strains were maintained in a liquid medium [7]. To monitor possible laboratory contamination during the procedure and ensure operator safety, horse blood agar plates (HBA) were placed randomly within the biological safety cabinet (BSC) and external to the cabinet around the operator during the experimental phase of this evaluation. The contamination control plates were incubated aerobically at 37 °C for 24 and 48 h. The next day the plates were examined for any growth of the test strains. Colony counts were performed for each contamination control plate and the results recorded following each procedure. The contamination control plates were negative for all but 2 plates, which grew 1 colony each of *B. anthracis*. Both these plates were located within the Class I biological safety cabinet close to the right hand side of the operator.

3. Results and discussion

The bacterial disease caused by *B. anthracis* has long been associated with the infection of grazing animals. Endemic in some areas this bacterium naturally lives in the soil and can infect animals and animal handlers via ingestion, cutaneous infection and inhalation of the bacterial spores. Although infection is not uncommon in humans, inhalational anthrax remains a rare event and is more likely to be associated with a bioterrorist act. Inhalation of greater than 8000 bacterial spores is thought to induce disease, although this value may be less due to the immunosuppressed within our society [8].

The results of the RAMP® Anthrax tests on spore and vegetative bacterial suspensions of five strains of *Bacillus* species are shown in Table 1. All tests on *Bacillus* species other than *B. anthracis* were negative. All tests on *B. anthracis* spore suspensions containing >6000 spores in 10 μl (6×10^5/ml) and one of three tests on a suspension containing ~5000 spores in 10 μl were positive. Tests on a heavy suspension of *B. anthracis* vegetative bacteria (10^8/ml) were also positive. All tests on the 10 common environmental powders (0.1 g/l), performed in duplicate were negative (data not shown). Samples containing >6000 anthrax spores per 10 μl consistently gave positive results, while

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Table 1
Sensitivity and specificity of RAMP® Anthrax test

Organism	CFU/ml	Concentration, spores/bacteria/10 μl	Results +ve/total
Bacillus anthracis spores[a]	10^9	10^7	3/3
	2×10^7	250,000	3/3
	6.2×10^5	6200	3/3
	5.1×10^5	5100	1/3
	4.8×10^5	4800	0/3
	2×10^4	200	0/3
B. anthracis spores[b]	4.8×10^7	480,000	3/3
B. anthracis vegetative	10^8	10^6	3/3
B. subtilis spores	10^9	10^7	0/2
	10^5	10^3	0/2
Bacillus thuringiensis spores	10^{10}	10^8	0/2
	10^4	10^3	0/2
B. cereus spores	10^{10}	10^8	0/2
	10^5	10^2	0/2
Distilled water	Nil	Nil	0/3

Number of bacterial isolates restricted by current National and International legislation on Bioterrorist agents.
[a] *B. anthracis* clinical isolate VRS 3518.
[b] *B. anthracis*, d-Sterne strain.

samples containing fewer that 5000 spores per 10 μl were consistently negative. There were no false positive results with any of the other *Bacillus* species tested, even at high concentrations, or with the common powders tested (Table 2).

This evaluation demonstrates that the RAMP® Anthrax test is specific for *B. anthracis* and sensitive enough to detect spores at concentrations likely to be found in visible, suspicious powders. For example the letters carrying anthrax spores, which resulted in many cases of clinical anthrax and five deaths in the USA in October 2001, contained more than 10^9 spores/g [9]. Therefore a limit of detection of 6000 spores should be adequate to detect moderately concentrated samples.

The RAMP detection system should be used only when a suspect powder is present in visible quantities, as trace amounts may not meet the limit of detection leading to false negative results.

Table 2
Household powders

Test sample	Dilution (mg/ml)	Result (neg)
Caster sugar	0.1	3/3
Plain flour	0.1	3/3
Bicarbonate soda	0.1	3/3
Washing powder[a]	0.1	3/3
Flea powder	0.1	3/3
Talcum powder	0.1	3/3
Cement powder	0.1	3/3
Dipel powder[b]	0.1	3/3
Cooking yeast	0.1	3/3
Coffee mate	0.1	3/3
Gypsum	0.1	3/3

[a] ColdPower®.
[b] *B. thuringiensis* spore powder.

The RAMP® Anthrax test did not display any false positive results with the other *Bacillus* species tested including *B. thuringiensis*, a close relative of *B. anthracis* and which is widely available commercially as a bio-insecticide for the control of crop-destroying and disease carrying insects and a close relative of *B. anthracis* [4]. The RAMP Anthrax test is designed to quantify an antigen–antibody reaction; however it is unclear whether the method detects both spores and vegetative cells of *B. anthracis* or spores only. To assess the ability of the kit to detect the presence of vegetative cells of *B. anthracis*, serial dilutions of an overnight culture of *B. anthracis* were prepared for the evaluation. Prior to testing, the suspension was examined using 40× phase contrast microscopy to ensure no spores were present. The RAMP Anthrax test detected vegetative cells at a concentration of 10^8 CFU/ml, suggesting the presence of an antigen common to the vegetative cell wall and the spore of *B. anthracis*. This finding suggests that the RAMP would detect the presence of *B. anthracis* in a crude mixture of vegetative cells and spores and in the presence of other *Bacillus* species likely to be encountered in the environment.

The limitation of this study is that we tested only pure spore suspensions, without the presence of any potential interference from environmental contamination or a ‘‘carrier’’ substance, which could inhibit or cross-react with the test in practice. Tests on individual powders were promising, demonstrating no interference with the immunoassay reaction. Each cartridge has an internal control to ensure that both the flow rate and fluorescence detection has occurred should this internal control not activate an error reading will be displayed on the reader screen. No errors within both the test cartridge and the fluorescence reader were detected and no false positive results occurred on any of the test samples or household powders. The household powders selected for this study reflect the identity of many non-credible and/or hoax threats (New South Wales Police Forensic Counter Terrorism, personal communication).

At present the field-based protocol does not include any sample preparation prior to testing in the RAMP®. However, to reduce the effect of environmental inhibition in the sample and the risk of false negative and system errors a new buffer dilution step has been recently introduced to the test method. An aliquot of the initial buffer tube sample is transferred to a new buffer tube and the test is continued from this tube. Further research will be conducted to establish whether a sample clean up process would aid in reducing environmental inhibitors within the sample, and improve the sensitivity of the instrument. This will be achieved by trialling a clean up method designed to remove large particles from the sample thereby creating a more homogeneous sample, which may contain bacterial spores of a known size. The subsequent effect on sensitivity could be trialled by testing a range of powders contaminated with a known quantity of spores and comparing the clean up step with the standard method.

4. Conclusions

Hand held detection devices for rapid detection of biological agents are becoming more widely available as technology

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improves and the need for more accurate risk assessment at the incident site increases. However, the use of presumptive tests in the field should be limited to scientifically trained operators, as part of a layered detection approach. The RAMP® instrument and tests kits are valuable tools for both first responders and public health laboratories [10], enabling a more rapid and effective response to threats and acts of bioterrorism.

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