ORIGINAL ARTICLE

Decreased time for detection and quantification of virulent *Bacillus anthracis* and *Yersinia pestis* using a BioNanoPore (BNP™) membrane technology

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Abstract

Many aspects of biodefense research require quantitative growth assessments of the test agent. This study evaluated the BioNanoPore (BNP™) technology to quantitate *Bacillus anthracis* and *Yersinia pestis* faster than traditional plate counting methods. The BNP™ technology enabled quantification of *B. anthracis* and *Y. pestis* in phosphate-buffered saline and naïve rabbit blood at 6 and 24 h, respectively. After 6 h of growth, counts for *B. anthracis* ranged from 6·19–6·45 $\log_{10}$ CFU ml^{-1} on BNP™, while counts after 24 h on tryptic soy agar (TSA) ranged from 6·51–6·58 $\log_{10}$ CFU ml^{-1}. For *Y. pestis*, counts on BNP™ at 24 h ranged from 6·31–6·41 $\log_{10}$ CFU ml^{-1} on BNP™ and ranged from 6·44–6·89 $\log_{10}$ CFU ml^{-1} on TSA at 48 h. This study demonstrates that the BNP™ technology provides a more rapid detection of *B. anthracis* and *Y. pestis*, which could aid in the evaluation of potential medical countermeasures and treatments as well as other biological defense applications such as surface sampling or decontamination efficacy.

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Bacillus anthracis and *Yersinia pestis* are Category A select agents due to their capacity to cause illness and death, thus, heightening concerns of using these micro-organisms as potential biological weapons (Inglesby *et al.* 2000, 2002). Such concerns have prompted decades of research investigating and developing vaccines and medical countermeasures against *B. anthracis* and *Y. pestis* infection (Inglesby *et al.* 2000, 2002). Moreover, the 2001 release of *B. anthracis* spores in the mail has prompted growing interests in detection methods, surface sampling, and decontamination of *B. anthracis* spores and other biological agents. Traditional quantitative microbiological methods require a minimum of overnight (16–24 h) culturing for *B. anthracis*, while *Y. pestis* typically requires about 48 h for growth. Therefore, this study evaluated the BioNanoPore (BNP™) technology to quantitate *B. anthracis* and *Y. pestis* within a shorter time period compared to traditional plate counting methods that could be used to expedite data collection in many areas of biodefense research.

All testing was performed under BSL-3 conditions. Cultures of virulent *B. anthracis* Ames or *Y. pestis* CO92 were grown overnight (16–18 h) on an orbital shaker at 200 rev min^{-1} in 10·0 ml of tryptic soy broth (TSB; Remel, Lexena, KS, USA) at 37°C and 26°C, respectively. The cultures were subsequently prepared in sterile phosphate-buffered saline (PBS; Sigma, St Louis, MO, USA) and blood (simulating bacteremia) to evaluate potential matrix effects on quantification. Blood was collected in both EDTA and SPS tubes (Becton Dickinson, Franklin Lakes, NJ, USA) from naïve New Zealand white rabbits and was provided by the Battelle Biomedical Research Center. Each bacterial culture was diluted 1 : 4 in PBS, or blood from EDTA and SPS tubes, and ten-fold serial dilutions prepared in sterile PBS through 10^{-5}. For negative controls, sterile TSB (blanks) was diluted 1 : 4 in PBS, or blood, and ten-fold serial dilutions prepared.

For all samples, 0·1 ml aliquots of each dilution were removed and plated in triplicate on both tryptic soy agar (TSA; Hardy, Santa Maria, CA, USA) and the BNP™ Ultra Fast Identification Technology (NanoLogix, Inc., Hubbard, OH, USA). The *B. anthracis* plated onto TSA was incubated for 20 h at 37°C, and *Y. pestis* samples were cultured on TSA for approximately 48 h at 26°C.

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Following incubation of both organisms on TSA, colonies were counted and CFU ml^{-1} determined. The BNP™ technology has two components, consisting of a 50 mm nutrient agar plate containing the BNP™ membrane and a 50 mm non-nutrient agar color indicator plate. The surface of the nutrient agar plate possesses a transparent regenerated cellulose membrane containing a thin film of nutrient agar. This membrane enables exchange of water and nutrients, but is nonpermeable to bacterial cells. The colour indicator plates consist of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0·8–1·0 mg ml^{-1}) dissolved in a 1% agarose matrix. Metabolism of the chromogen by the bacteria leads to coloration (dark purple to black) and subsequent visualization of the microcolonies.

Following spread plate of the *B. anthracis* on the BNP™ nutrient agar, the plates were incubated for 6 h at 37°C after which the BNP™ membrane was aseptically removed, transferred to the indicator plates, and incubated at room temperature for 20–30 min. The *Y. pestis* samples were incubated for 24 h at 26°C after which the BNP™ membrane was aseptically removed, transferred to the indicator plates, and incubated at room temperature for 20–30 min. For *B. anthracis* and *Y. pestis*, dark blue colonies (microcolonies) were visualized, counted, and colony-forming units (CFU) ml^{-1} determined. All data were expressed as mean $\log_{10}$ CFU ml^{-1} for triplicate samples.

In parallel, streak plates from the undiluted 1 : 4 sample of each agent (including negative control blanks) in PBS or blood were prepared on both BNP™ and TSA as a means to determine whether streak plates on BNP™ could be used as a qualitative approach for bacterial viability. These plates were incubated at the respective times and temperatures for each agent as described above and evaluated for growth.

In PBS and naïve rabbit blood, the BNP™ technology enabled visualization and quantification of *B. anthracis* and *Y. pestis* at 6 and 24 h, respectively (Fig. 1). In these matrices, the *B. anthracis* ranged from 6·19–6·45 $\log_{10}$ CFU ml^{-1} on BNP™, while the *B. anthracis* counts on TSA ranged from 6·51–6·58 $\log_{10}$ CFU ml^{-1} (Table 1). The %CV for these samples ranged from 0·2–1·5%. For *Y. pestis*, counts on BNP™ ranged from 6·31–6·41 $\log_{10}$ CFU ml^{-1} on BNP™, while the *Y. pestis* TSA counts ranged from 6·44–6·89 $\log_{10}$ CFU ml^{-1} (Table 1). The %CV for these samples ranged from 0·4–2·3%. All streak plates on BNP™ and TSA were positive for *B. anthracis* and *Y. pestis* (Table 1). All blank samples were negative for growth.

In this study, the BNP™ technology has enabled a more rapid detection and quantification of *B. anthracis* and *Y. pestis* in multiple matrices within a shorter time frame compared to traditional culture methods. When using TSA or BNP™, the mean $\log_{10}$ CFU ml^{-1} counts were similar for *B. anthracis* and *Y. pestis* suspended in PBS or naïve rabbit blood. Although the respective counts for both *B. anthracis* and *Y. pestis* were consistently higher on TSA when compared to BNP™; however, this study only evaluated one time period for culturing the bacteria on BNP™.

An important endpoint measured for evaluating medical countermeasure efficacy is bacterial quantification and identification in the bloodstream, which can help determine appropriate treatment regimen(s). For *B. anthracis* and *Y. pestis*, research models have utilized both quantitative and qualitative methods for determining infectious bacteraemia in blood and tissues (Byrne *et al.* 1998; Engelthale *et al.* 1999; Kao *et al.* 2006; Heine *et al.* 2007). In these studies, growth of these micro-organisms fell within the typical 18–24 h for *B. anthracis* and 48–72 h for *Y. pestis*. Within controlled research settings and real-world scenarios, a more rapid detection of these biological agents (e.g. <24 h), as presented in this study, could aid in the evaluation of potential medical countermeasures and treatments.

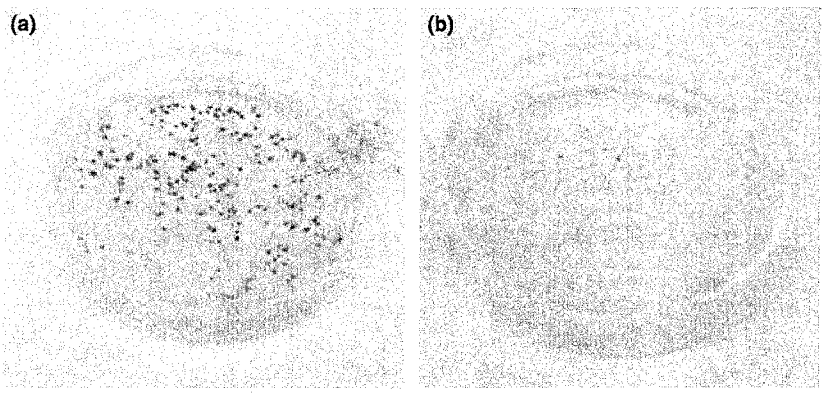

Figure 1 Representative photos of *B. anthracis* Ames (a) and *Y. pestis* CO92 (b) colonies (dark spots) on the BNP™ indicator plates after 6 and 24 h incubation, respectively.

Table 1 Quantitative and qualitative assessments of *B. anthracis* Ames and *Y. pestis* CO92 on the BioNanoPore (BNP™) technology and tryptic soy agar (TSA); $n = 3$/sample

	BNP™		TSA	
Organism/matrix	Streak plate	Mean $\log_{10}$ CFU ml^{-1} (range; %CV)	Streak plate	Mean $\log_{10}$ CFU ml^{-1} (range; %CV)
Bacillus anthracis Ames*				
PBS	+	6·19 (6·15–6·25; 0·8%)	+	6·55 (6·52–6·58; 0·5%)
PBS Blank	–	0	–	0
Blood (EDTA Tube)	+	6·45 (6·43–6·46; 0·2%)	+	6·58 (6·48–6·67; 1·5%)
Blood (EDTA Tube) Blank	–	0	–	0
Blood (SPS Tube)	+	6·45 (6·40–6·49; 0·7%)	+	6·51 (6·41–6·59; 1·4%)
Blood (SPS Tube) Blank	–	0	–	0
Yersinia pestis CO92†				
PBS	+	6·41 (6·31–6·46; 1·3%)	+	6·66 (6·59–6·72; 1·0%)
PBS Blank	–	0	–	0
Blood (EDTA Tube)	+	6·31 (6·16–6·44; 2·3%)	+	6·89 (6·83–6·93; 0·7%)
Blood (EDTA Tube) Blank	–	0	–	0
Blood (SPS Tube)	+	6·41 (6·34–6·45; 0·9%)	+	6·44 (6·41–6·46; 0·4%)
Blood (SPS Tube) Blank	–	0	–	0

*Culture time was 6 h on BNP™ and 20 h on TSA.
†Culture time was 24 h on BNP™ and 48 h on TSA.

The BNP™ technology could also be used for other biological defense applications such as surface sampling or determining the efficacy of decontaminating *B. anthracis* and *Y. pestis*. For example, surface sampling and quantification of *B. anthracis* spores on nonporous surfaces involved the culturing of bacterial samples overnight prior to counting (Rose *et al.* 2004; Hodges *et al.* 2006). Additionally, studies evaluating the surface decontamination efficacy of vapour-phase hydrogen peroxide have implemented growth times ranging from 18–24 h and 40–48 h for *B. anthracis* and *Y. pestis*, respectively (Rogers *et al.* 2005, 2008). The use of BNP™ could aid in a more rapid assessment of surface sampling or decontamination of different biological agents. Although this study provides data for *B. anthracis* and *Y. pestis* grown on BNP™ for a single time period, future work may require optimal growth time determinations for each organism. Moreover, this technology will also need to be further evaluated to determine the feasibility of using BNP™ to quantitate bacteria from a mixed population.

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