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Rapid detection and identification of *Bacillus anthracis* in food using pyrosequencing technology

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ABSTRACT

The development of advanced methodologies for the detection of *Bacillus anthracis* has been evolving rapidly since the release of the anthrax spores in the mail in 2001. Recent advances in detection and identification techniques could prove to be an essential component in the defense against biological attacks. Sequence based such as pyrosequencing, which has the capability to determine short DNA stretches in real-time using biotinylated PCR amplicons, has potential biodefense applications. Using markers from the virulence plasmids (pXO1 and pXO2) and chromosomal regions, we have demonstrated the power of this technology in the rapid, specific and sensitive detection of *B. anthracis* spores in food matrices including milk, juice, bottled water, and processed meat. The combined use of immunomagnetic separation and pyrosequencing showed positive detection when liquid foods (bottled water, milk, juice), and processed meat were experimentally inoculated with 6 CFU/mL and 6 CFU/g, respectively, without an enrichment step. Pyrosequencing is completed in about 60 min (following PCR amplification) and yields accurate and reliable results with an added layer of confidence. The entire assay (from sample preparation to sequencing information) can be completed in about 7.5 h. A typical run on food samples yielded 67–80 bp reads with 94–100% identity to the expected sequence. This sequence based approach is a novel application for the detection of anthrax spores in food with potential application in foodborne bioterrorism response and biodefense involving the use of anthrax spores.

1. Introduction

Bacillus anthracis, the causative agent of anthrax and implicated in the 2001 anthrax attack in the US (Jernigan et al., 2002) has been used as a biological weapon for about 100 years (Tournier et al., 2009). Recent studies on the use of *B. anthracis* spores as a biological weapon following the 2001 anthrax attack have developed scenarios for intentional release including infecting water supplies or releasing aerosolized spores (Levin and Valadares, 2003; Meinhardt, 2005). Earlier reports by the WHO predict that the release of 50 kg of spores upwind of 500,000 civilians could potentially result in 95,000 fatalities and over 10,000 fatalities may result if released in a single subway during rush hour (WHO, 1970). The above reports highlight the vulnerability of the civilian population to intentional release of *B. anthracis* spores. Food is also reported to be a vulnerable target for intentional contamination and the use of *Salmonella enterica* serovar Typhimurium in the tainting of salad bars in the US make the use of biothreat agents such as *B. anthracis* spores a possibility (Torok et al., 1997). The prospect of this vulnerability is even more alarming given that no standards or established guidelines exist for testing foods for these biothreat agents (Kennedy, 2008).

B. anthracis belongs to the *Bacillus cereus* group (Helgason et al., 2000), however, members of this group share a great deal of morphological, biochemical, and genetic similarities (Ash et al., 1991; Priest et al., 1988; Harrell et al., 1995), making differentiation an arduous task. Several reports have explored the use of chromosomal markers for the genotypic characterization of *B. anthracis* (Pearson et al., 2004; Van Ert et al., 2004; Hurtle et al., 2004; Hill et al., 2004; Ellerbrok et al., 2002; Qi et al., 2001). Molecular methods are also increasingly being used for rapid species discrimination. However, some methods used for *Bacillus* spp. such as restriction digests of a target gene (Joung and Cote, 2002) or randomly amplified polymorphic DNA analysis (Yamazaki et al., 1997) are limited in discriminating between a large group of species which exhibit high genetic similarities (Goto et al., 2000). Sequencing has shown to be particularly useful and with the increasing use of sequencing methods and decreased cost after the initial equipment investment, more laboratories are using sequence data for species identification (Turenne et al., 2001). Even though anthrax can be distinguished from closely related *Bacilli* with conventional biochemical tests, such as capsular staining, motility, hemolysis, and observing the presence of intracellular *para*-crystalline formation (Harrell et al., 1995; Helgason et al., 2000; Qi et al., 2001), these tests are time-consuming and may sometimes be inconclusive. Considering that these approaches for species identification can be tedious, expensive, and inaccurate, a rapid and accurate method yielding sequence

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information is greatly needed. Pyrosequencing technology which has the capability to determine short DNA stretches in real-time using biotinylated PCR amplicons (Ronaghi et al., 1996) has recently been used for rapid sequence based detection in several biodefense applications (Amoako et al., 2012b; Loveless et al., 2010; Wahab et al., 2005). The application of pyrosequencing for the detection of *B. anthracis* has been previously reported (Wahab et al., 2005; Ahmod et al., 2011), however, the use of the technology for the specific and accurate detection of *B. anthracis* in complex matrices such as food has not been documented. Here we report the first application of pyrosequencing for the specific detection and confirmation of *B. anthracis* from food matrices such as bottled water, juice, milk and processed meat. The pyrosequencing assays designed are based on chromosomal and virulence plasmid (pXO1 and pXO2) markers. In combination with an immunomagnetic assay previously developed (Shields et al., 2012), we demonstrate excellent detection capability without the need for an enrichment step. The work described here provides a novel tool for biodefense application involving potential foodborne bioterrorism response preparedness.

2. Materials and methods

2.1. Spore preparation

B. anthracis Sterne spores were prepared from overnight cultures of single discrete colonies as previously described (Shields et al., 2012). Briefly, 100 μL of the overnight culture was used to inoculate culture flasks containing 50 mL of blood agar media. Flasks were incubated at 37 °C (without humidity) until sporulation was complete (as determined by malachite green endospore staining). Spores were collected, transferred into 50 mL falcon tubes, and washed with 50% ethanol (pelleted by centrifugation) to remove all remaining vegetative cells. The spores were then resuspended in phosphate buffered saline (PBS) with 1% Bovine Serum Albumin (BSA), aliquoted and stored at −20 °C until use.

2.2. Extraction and quantification of genomic DNA

Genomic DNA was extracted from 65 bacterial isolates (Table 1), 20 of which are *B. anthracis*. All bacterial strains were first cultured from glycerol stocks on Tryptic Soy Agar Plates supplemented with 5% sheep blood (TSBAP) and subsequently a single, discrete colony was subcultured overnight at 37 °C in Tryptic Soy Broth (TSB) prior to DNA extraction. *B. anthracis* DNA was extracted, according to the manufacturer's instructions, using the MasterPure Gram Positive DNA Purification Kit (Epicenter Biotechnologies, Madison, WI, USA), while the DNA from the remaining strains was extracted using the DNeasy Tissue kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. DNA concentrations were then determined using a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and diluted to 5.71 ng/μL (10^6 *B. anthracis* genomic equivalents per microliter) for downstream use. The sources of some of the bacterial strains used are described in a previous report (Amoako et al., 2010).

2.3. Design of oligonucleotide primers for pyromark assays

Genomic signatures on the two virulence plasmids (pXO1 and pXO2) and a region of the *B. anthracis* chromosome were selected as targets for the specific detection of the organism. Consensus sequences from five genomic sequences (Accession numbers: *B. anthracis* str. CDC684 (CP001215), *B. anthracis* str. A0248 (CP001598), *B. anthracis* str. Ames (AE016879), *B. anthracis* str. Sterne (AE017225), *B. anthracis* str. 'Ames Ancestor' (AE017334)), five pXO1 sequences (*B. anthracis* str. A0248 plasmid pXO1 (CP001599), *B. anthracis* str. 'Ames Ancestor' plasmid pXO1 (AE017336), *B. anthracis* virulence plasmid pXO1 (AF065404), *B. anthracis* str. A2012 plasmid pXO1 (AE011190), *B. anthracis* str. CDC684 (CP001216)) and five pXO2 sequences (*B. anthracis* str. CDC684 plasmid pXO2 (CP001214), *B. anthracis* str. 'Ames Ancestor' plasmid pXO2 (AE017335), *B. anthracis* str. A0248 (CP001597), *B. anthracis* plasmid pXO2 (AF188935), *B. anthracis* str. A2012 plasmid pXO2 (AE011191)) were determined using the Geneious Software Suite (version 5.3.5; Biomatters Inc. [http://www.geneious.com]). Consensus sequences for chromosome and plasmid targets were imported into the Pyromark Assay Design software (version 2.0.1.15; Qiagen Inc. [http://www.pyrosequencing.com/dynpage.aspx?id=7257]), which was used to design the sequencing primers based on the sequence analysis method (SQA) for all targets (Table 2). Following design, all primers were examined for specificity in silico using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.4. PCR amplification and specificity

A panel of DNA extracted from 65 bacterial strains (20 *B. anthracis* isolates and 45 strains which are either closely or distantly related to *B. anthracis*) was used to screen the pyrosequencing primers for specificity (Table 1). Preliminary examination of the PCR primers was performed using 1× PCR Supermix (Invitrogen Life Technologies, Inc., Carlsbad, CA), 0.5 μM forward and reverse primers (one of which is biotin labeled, see Table 2), and 5.71 ng DNA template in a final volume of 25 μL. Thermocycling conditions were 95 °C for 5 min, followed by 50 cycles of: 58 °C for 20 s, 72 °C for 30 s, 95 °C for 10 s, and a final extension step at 72 °C for 5 min. Pyromark PCR reactions were performed similarly as described above with 1× Pyromark PCR mastermix (Qiagen Inc.) substituted for the Invitrogen PCR Supermix. PCR amplicons were visualized using a QIAxcel system (Qiagen Inc.) and analyzed using the AM320 method, along with the QIAxcel DNA screening kit. PCR in which amplification was successful were then selected for analysis by pyrosequencing.

2.5. Pyrosequencing and data analysis

The 5′ biotin linked PCR products were placed in 24-well plates and bound to streptavidin coated sepharose beads according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The PCR products were denatured and all non-biotin labeled DNA fragments were washed away using the Pyromark Q24 vacuum workstation (Qiagen Inc.). The sepharose beads were then resuspended in an annealing buffer containing 0.3 μM sequencing primer. The pyrosequencing reaction was performed in triplicate using the Pyro Gold Q24 reagents with a predetermined dispensation specific for the target amplicon, using the Pyromark Q24 system.

The raw data files were imported into the Pyromark Q24 software (version 2.0; Qiagen Inc. [http://www.qiagen.com/products/pyromarkq24.aspx]) for analysis. The sequences obtained were then compared to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the sequence search built into the Geneious software suite (MegaBlast algorithm).

2.6. Preparation of spiked food samples

Bottled water, apple juice, whole milk (3.25% milk fat) and processed meat (black forest ham) were purchased from a local grocery store and used for the food spiking experiments as previously described (Shields et al., 2012). Briefly, *B. anthracis* spores were added to 25 mL of liquid foods to achieve a cell inoculation of 6 CFU/mL. The bottled water and milk samples were diluted with 25 mL of BPW containing 1% Tween-20 (BPWT, pH 7.2), and the apple juice sample was diluted with 25 mL of BPW containing 0.2 M Na_2HPO_4 and 1% Tween-20 (pH 8.0). For bacterial capture in solid food, 50 g of processed meat was sliced into 1 cm^2 pieces and inoculated with 6 CFU/g of *B. anthracis* spores. Fifty milliliters of BPWT (pH 7.2) was added (1:1 dilution w/v) and the mixture was stomached. The liquid was further passed through a sponge filter with a 60 μm above the sponge and a 30 μm filter below using a vacuum pump and the filtrate was collected for analysis.

Table 1
Panel of DNA samples screened to determine specificity of the primer sets.

Bacterial strain	Primer set					
	Cya	*gerXB*	*capBCAD*	*acpB*	*prophage lambda1*	*prophage lambda3*
Bacillus anthracis isolate 15	+	+	−	−	+	+
Bacillus anthracis isolate 35	+	+	+	+	+	+
Bacillus anthracis isolate 44	+	+	+	+	+	+
Bacillus anthracis isolate 53	+	+	+	+	+	+
Bacillus anthracis isolate 56	+	+	+	+	+	+
Bacillus anthracis isolate 59	+	+	+	+	+	+
Bacillus anthracis isolate 79	+	+	+	+	+	+
Bacillus anthracis isolate 127	+	+	+	+	+	+
Bacillus anthracis isolate 128	+	+	+	+	+	+
Bacillus anthracis isolate 131	+	+	+	+	+	+
Bacillus anthracis isolate 155	−	−	−	−	+	+
Bacillus anthracis isolate 158	−	−	+	+	+	+
Bacillus anthracis isolate 179	+	+	+	+	+	+
Bacillus anthracis isolate 240	+	+	+	+	+	+
Bacillus anthracis isolate 252	+	+	+	+	+	+
Bacillus anthracis isolate 552	+	+	+	+	+	+
Bacillus anthracis isolate 9616	+	+	+	+	+	+
Bacillus anthracis 03-0191	+	+	+	+	+	+
Bacillus anthracis Sterne strain	+	+	−	−	+	+
Bacillus anthracis Ames strain	+	+	+	+	+	+
Bacillus cereus ATCC 31101	−	−	−	−	−	−
Bacillus cereus ATCC 14579	−	−	−	−	−	−
Bacillus cereus ATCC 15816	−	−	−	−	−	−
Bacillus cereus ATCC 10876	−	−	−	−	−	−
Bacillus subtilis NWBL 0060	−	−	−	−	−	−
Bacillus coagulans ATCC 7050	−	−	−	−	−	−
Bacillus thuringiensis ATCC 10792	−	−	−	−	−	−
Bacillus brevis ATCC 8246	−	−	−	−	−	−
Bacillus licheniformis ATCC 12759	−	−	−	−	−	−
Bacillus circulans ATCC 61	−	−	−	−	−	−
Proteus vulgaris ATCC 13315	−	−	−	−	−	−
Klebsiella pneumoniae ATCC 13993	−	−	−	−	−	−
Shigella dysenteriae ATCC 11835	−	−	−	−	−	−
Escherichia coli O157:H7 EDL 933	−	−	−	−	−	−
Escherichia coli ATCC 25922	−	−	−	−	−	−
Pasteurella haemolytica Z13	−	−	−	−	−	−
Clostridium perfringens ATCC131124	−	−	−	−	−	−
Acinetobacter baumanii strain 14B	−	−	−	−	−	−
Vibrio vulnificus Z89	−	−	−	−	−	−
Citrobactor brackii ATCC 12012	−	−	−	−	−	−
Salmonella typhimurium 71 − 471	−	−	−	−	−	−
Aeromonas hydrophila Z22	−	−	−	−	−	−
Pseudomonas aeruginosa ATCC 27853	−	−	−	−	−	−
Streptococcus pneumoniae ATCC 49619	−	−	−	−	−	−
Streptococcus pyogenes ATCC 19615	−	−	−	−	−	−
Enterococcus faecalis ATCC 29212	−	−	−	−	−	−
Micrococcus lysodeikticus Z9	−	−	−	−	−	−
Staphylococcus aureus ATCC 25923	−	−	−	−	−	−
Campylobacter coli NML 06 − 0224	−	−	−	−	−	−
Campylobacter jejuni ATCC 11168	−	−	−	−	−	−
Legionella pneumophila ATCC 33153	−	−	−	−	−	−
Listeria monocytogenes NTCC 7933	−	−	−	−	−	−
Listeria grayi ATCC 19120	−	−	−	−	−	−
Listeria innocua ATCC 33090	−	−	−	−	−	−
Listeria ivanovii ATCC 19119	−	−	−	−	−	−
Listeria murrayi ATCC 25401	−	−	−	−	−	−
Listeria seelingeri ATCC 35967	−	−	−	−	−	−
Listeria welshimeri ATCC 35897	−	−	−	−	−	−
Yersinia enterolytica ATCC 23715	−	−	−	−	−	−
Yersinia frederiksenii ATCC 33641	−	−	−	−	−	−
Yersinia intermedia ATCC 29909	−	−	−	−	−	−
Yersinia kristensenii ATCC33638	−	−	−	−	−	−
Yersinia enterolytica (food isolate)	−	−	−	−	−	−
Yersinia pestis CO92	−	−	−	−	−	−
Yersinia pseudotuberculosis ATCC 39833	−	−	−	−	−	−

2.7. Immunomagnetic capture of B. anthracis Sterne spores from spiked food samples

Following the preparation of spiked food samples, 50 mL of the prepared food sample was mixed with 1 mg (50 μL) of Pathatrix (Life Technologies Inc., Carlsbad CA, USA) beads functionalized with rabbit anti-*B. anthracis* polyclonal antibody (Tetracore, Rockville MD, USA). The specificity of the antibody has been demonstrated in our previous paper (Shields et al., 2012). The beads were mixed, captured and washed according to the *i*CropTheBug method as previously described (Shields et al., 2012). Extracted beads were resuspended in 150 μL of TE. DNA was extracted using the Epicenter MasterPure DNA

Table 2
Pyrosequencing primers for the rapid identification of *B. anthracis*.

Target	Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Sequencing primer (5′–3′)	Max read length (bp)
pXO1	*Cya*	F: GTGGTGTGGCTACAAAGGGATTGAATGT Biotin-R: TCTCGACAGCTAATTGTTGACCATGCTTCT	123	CAAAGGGATTGAATGTT	94
	gerXB	F: TGGAGTTTGGTCCATTTGTGGCCG Biotin-R: AAACCCCTACAAGCCACTGGTACAC	129	GTGGCCGCAAATCAG	97
pXO2	*acpB*	Biotin-F: CACAGGAGAAAGTGAAAGTTGGGCAGA R: AGGGGTTGAACCTAAGTCAAGAGGTATTGT	166	AGTCAGCATTAACATCGT	107
	capBCAD	Biotin-F: GTCTTGCAAGCTACCCTTACCATGCG R: ACGAACGTTTATGGCCCCCACTGTAT	139	Same as reverse primer	113
Chromosome	*prophage lambda1*	F: GGGGGATTAATTGCAAAAGCGTGTT Biotin-R: CCCATGGCGTTCCCATAGTTATGA	94	TTGCAAAAGCGTGTT	69
	prophage lambda3	F: GGTGATGCGTCAAAAGCCGATGGA Biotin-R: TTACGTGGGCCAAATTGTCCCGTTT	122	AAAAGCCGATGGAAC	94

Extraction kit and subjected to the pyrosequencing method described above.

3. Results

3.1. Specificity of selected B. anthracis pyrosequencing targets

All primer sets generated by the Geneious software were first tested for specificity to *B. anthracis* through in silico comparison to the GenBank nucleotide database. The six resulting primer sets presented in Table 1 showed no cross-reactivity with other bacterial strains in silico, and were selected for further analysis through in vitro screening against a DNA panel consisting of 65 bacterial strains (Table 1). As shown, all six targets (two each for pXO1, pXO2 and the chromosome) were specific for *B. anthracis*, with no amplification from the other bacterial strains tested. Additionally, the *B. anthracis* strains which lacked pXO1 (isolate 155 and isolate 158), along with those which lack pXO2 (isolate 15, isolate 155 and *B. anthracis* Sterne strain), were correctly identified as such.

3.2. Evaluation of B. anthracis pyrosequencing targets

The chromosomal and plasmid targets that were identified as specific for *B. anthracis* were compared based on the read length obtained from pyrosequencing. The average read length for each target across all 20 *B. anthracis* strains was 48 bp for *acpB*, 42 bp for *capBCAD*, 68 bp for *gerXB*, 64 bp for *cya*, 54 bp for *prophage lambda3* and 36 bp for *prophage lambda1*. These read lengths were within the range recommended by the PyroMark manufacturer (http://www.qiagen.com/faq/faqview.aspx?faqid=2216&S). From this data, an optimal set of targets (*gerXB* for pXO1, *acpB* for pXO2 and *prophage lambda3* for the chromosome) which had comparatively higher read lengths, was selected for the specific detection of *B. anthracis* using pyrosequencing technology.

Fig. 1 shows the alignment of pyrosequencing reads obtained from 20 *B. anthracis* isolates examined with each of the optimal targets (*gerXB*, *acpB* and *prophage lambda3*). The data collected from three additional targets (*cya*, *capBCAD* and *prophage lambda1*) also showed alignment with the consensus sequence (data not shown). As can be seen, the sequences align very well to the consensus sequence obtained from the GenBank database. All *B. anthracis* strains tested in this study align to the consensus and show greater than 97% identity, indicating positive identification of *B. anthracis*.

3.3. Detection of B. anthracis Sterne spores in spiked food matrices

Using the pXO1 and chromosome targets, we successfully detected and identified *B. anthracis* Sterne spores in liquid food matrices (water, apple juice, milk), and processed meat when experimentally inoculated at 6 CFU/mL (150 CFU per 25 mL), and 6 CFU/g (300 CFU per 50 g), respectively (Table 3). DNA extracted from the food spiking assays produced long, quality sequencing reads (67–80 bp) which yielded BLAST results of over 94% sequence identity.

4. Discussion

Recent outbreaks of *E. coli*, *S. enterica* serovar Typhimurium, and *L. monocytogenes* in North American and European food supplies demonstrate the vulnerability of food (Tauxe et al., 2010; King et al., 2012) and highlight food as a soft target for intentional contamination. These outbreaks indicate that rapid detection methods with greater accuracy are required to address any potential food contamination involving biothreat agents. However, to date a critical gap still remains, as no standards, established guidelines or routines exist to test for these threats in food (Kennedy, 2008). In recent years there has been some development of methods for the detection of biothreat agents in food (Wielinga et al., 2011a; Amoako et al., 2010; Shields et al., 2012; Reekmans et al., 2009; Woubit et al., 2012; Amoako et al., 2012a), however, there is still much to be done.

Since the release of anthrax spores in the mail in 2001, the development of advanced methodologies for the detection of biothreat agents (including *B. anthracis*) has been evolving rapidly. Some of the recent advances in detection and identification techniques (Amoako et al., 2012b; Walker et al., 2010; Qu et al., 2010; Laue and Bannert, 2010; Janse et al., 2010; Lian et al., 2010; Sapsford et al., 2011; McGrath et al., 2011; Wielinga et al., 2011b) could prove to be an essential component in the defense against biological attacks. In particular, methods that offer greater speed and accuracy such as those based on pyrosequencing (Wahab et al., 2005; Amoako et al., 2012b; Ahmod et al., 2011) will contribute significantly to an effective response against biological attacks. A crucial factor in accurate biothreat agent identification is the capability to discriminate between the target agent and its near neighbors. Sequencing based methods such as pyrosequencing offer a better resolution in discriminating between closely related microbial species. There is the added layer of confidence in the use of sequence based methods since the sequence information generated can be used for both confirmation of pathogen presence and trace-back to source. To our knowledge there is no report on the use of sequence based genomics approaches for the direct detection of *B. anthracis* in food. We have demonstrated in this study the first application of pyrosequencing for the rapid detection, identification and confirmation of *B. anthracis* in food matrices such as milk, bottled water, juice and processed meat. The combined use of immunomagnetic separation (Shields et al., 2012) and pyrosequencing showed positive detection when liquid foods (bottled water, milk, juice), and processed meat were experimentally inoculated with 6 CFU/mL and 6 CFU/g, respectively, without an enrichment step. Sequencing runs are completed in about 60 min after PCR amplification and this rapid turnaround time (about 7.5 h from sample to sequence results), together with the accurate sequence information generated (>94% sequence identity was sufficient to positively identify the presence of *B. anthracis*), will enable

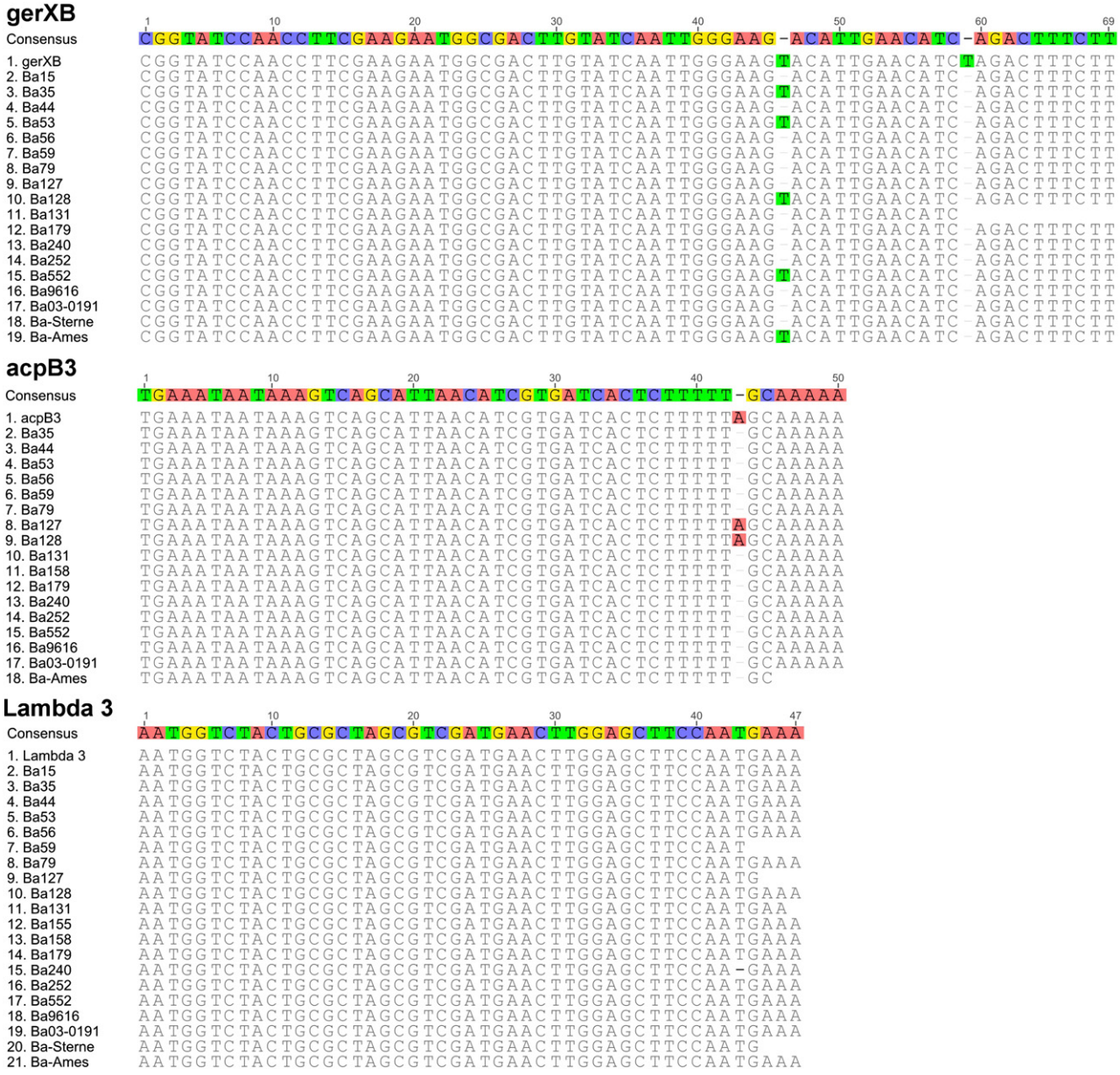

Fig. 1. Alignments of the sequence data collected during pyrosequencing assays for the downselected targets (*gerXB* for pXO1, *acpB* for pXO2, and *prophage lambda3* for the chromosome). Assays were run in triplicate and consensus read sequences are shown for each isolate.

Table 3
Pyrosequencing read lengths and BLAST identities for *B. anthracis* Sterne spores in the food matrices.

Food matrix	Target	Read length (bp)	% Identity (BLAST)
Water	*cya*	77	100%
	gerXB	80	>94%
	prophage lambda1	67	>95%
	prophage lambda3	72	>97%
Apple juice	*cya*	77	100%
	gerXB	80	>96%
	prophage lambda1	67	>95%
	prophage lambda3	72	>97%
Milk	*cya*	77	100%
	gerXB	80	>96%
	prophage lambda1	67	>97%
	prophage lambda3	72	>97%
Sliced ham	*cya*	77	100%
	gerXB	80	>96%
	prophage lambda1	67	>97%
	prophage lambda3	72	>98%

rapid informed decisions to be made during a foodborne contamination and thereby minimize the effect on public health. The sequence identities were not verified by regular sequencing to determine if there were errors since our goal was to determine the presence of *B. anthracis* and not sequence variation such as SNPs or mutations. In addition to the high sequence identity, the genetic targets used (based on the two virulence plasmids pXO1 and pXO2) have the ability to provide information on the virulence potential of the implicated anthrax strain as well. The generation of two targets for each virulence plasmid provides an extra measure of confidence and will compensate for any potential mutation that may occur in any of the target primer regions. Furthermore, the inclusion of a chromosomal target also enables the detection and identification of strains that have lost either of the two plasmids.

5. Conclusion

In conclusion, this report has for the first time demonstrated the power of pyrosequencing combined with Immunomagnetic separation

in the detection of *B. anthracis* from both simple and complex food matrices including bottled water, apple juice, milk and processed meat. The levels of detection observed after experimental inoculation without an enrichment step are unprecedented and together with the one hour time for completing pyrosequencing runs following PCR amplification offer an effective detection system for anthrax in food. The current work is novel for detection of anthrax spores in food and provides a system with potential application in a foodborne bioterrorism response involving the use of anthrax spores. In food biodefense application, culture confirmation takes too long, hence the ability to rapidly sequence a PCR fragment using pyrosequencing provides an added layer of confidence and helps to make rapid informed decisions on food contamination.

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