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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.