Detection of spores of *Bacillus anthracis* from environment using polymerase chain reaction

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A sensitive PCR based detection of *Bacillus anthracis* spores from environment was standardized. Specific 1247bp amplicon could be detected with template concentration as low as 13 pg. Sensitivity was enhanced to 10 fold by nesting with second set of primers, forming 2080bp amplicon. Extraction of DNA from spores purified from soil samples by aqueous polymer two-phase system followed by partial germination and freeze-thaw treatment yielded best results. Soil sample spiked with spores (8x10⁷/g of sample) could be detected with this method.

*Bacillus anthracis* is a Gram-positive spore-forming bacillus that can cause acute infection in both animals and humans¹. It is primarily a disease of herbivores, which acquires infection after coming into contact with soil borne spores. The distribution of anthrax is worldwide, with foci of anthrax existing in the United States in areas of Kansas and Oklahoma. Loci of anthrax also exist in the southern peninsular India with reports of recurring outbreaks²-³. The disease can be transmitted to humans when spores of *B. anthracis* are introduced by ingestion, inhalation or contact with the skin⁴. Cutaneous anthrax is the most common form of human disease and may occur either in industrial or agricultural settings after exposure to contaminated animals or their products. Virulent strains of *B. anthracis* are encapsulated and cause death in humans and animals by producing various toxins. Poly-D-glutamic acid capsule and toxins are encoded by genes present on two mega plasmids, designated pXO2 and pXO1, respectively⁵-⁶. Previous studies have shown that 60-MDa plasmid pXO2 is essential for capsule formation and cap region located on the plasmid encodes the capsular protein. Isolation and identification of anthrax spores from environmental sample is comparatively difficult, while detection of *B. anthracis* from clinical specimens is simple though time consuming. Polymerase chain reaction (PCR) has been recently employed for detection of spores using primers specific to capsule or toxin genes⁷-¹⁰. In the present investigation, we have reported a sensitive and specific PCR assay for detection of *B. anthracis* spores from the environmental samples (soil). The method was used in combination with a modified spore extraction protocol to facilitate DNA extraction from soil samples.

**Bacterial cultures**—Non pathogenic *B. anthracis* sterne strain was procured from Institute of Veterinary and Preventive Medicine, Ranipet, Vellore (India). Spores of *B. cereus* and *B. subtilis* were procured from Difco, USA.

**Spore preparation of B. anthracis**—*B. anthracis* was grown on Brain Heart Infusion (BHI) agar for 18-24 hr at 37°C. The growth on the plate was scrapped and inoculated into G-medium broth. The culture was allowed to sporulate by incubating the broth at 30°C on a shaker at 200 rpm for four days as described by Stewart and Halvorson⁷. Spores were harvested by centrifugation at 6000g for 10 min at 4°C and suspended in phosphate buffer saline (PBS, pH 7.2) before heating at 60°C for 90 min to inactivate any vegetative cells.

**Purification of spores**—Purification of *B. anthracis* spores was carried out by aqueous polymer two phase system as described by Sacks and Alderton⁸. Briefly, 3.4 ml of potassium phosphate buffer (3M, pH 7.1) was mixed with 1.12 g of polyethylene glycol 4000 (PEG) and the final volume was made up to 10 ml with water including sample (spores). The mixture was homogenized vigorously and centrifuged at 1500 g for 2 min in a swinging bucket rotor at room temperature. The upper phase containing the spores was removed carefully without disturbing the interface.
The experiment was repeated thrice to get the maximal yield of pure spores free from cell debris and vegetative cells. Spores were counted with the help of Neubauer chamber and plate count.

**Extraction of spores from soil**—Spores of *B. anthracis, B. cereus* and *B. subtilis* were spiked in the sterilized garden soil and sand at a concentration ranging from $10^2$ to $10^7$ CFU per gram of soil or sand. The soil sample (1 g) was mixed with distilled water (2 ml) containing 1% TritonX-100 and homogenized on a cyclomixer. The larger soil particles were allowed to settle and relatively clear supernatant, containing spore, was collected. This extraction was repeated three times and the pooled extract was subjected to purification by ‘aqueous polymer two-phase system’ as described earlier. Spores extracted by this method were concentrated by centrifugation at 6000 g for 10 min and suspended in Tris-EDTA (TE) buffer (100 µl).

**Preparation of DNA templates**—Template DNA was extracted from the spores by four different methods either earlier described or modification thereof: (1) plasmid DNA was extracted by the method of Sambrook *et al.* by employing alkali lysis in conjunction with a purification step using polyethylene glycol. This method was used for extraction from culture grown in defined media or spores purified from the spiked soil; (2) total DNA was prepared by QIAamp tissue kit (Qiagen, Hilden, Germany); (3) the method of Zhou *et al.* was employed to extract DNA directly from the spiked soil sample; or (4) template extraction from the spores purified from the soil samples was performed by a modified method of Jackson *et al.*. In the last method, the spores were heat activated for 20 min at 80°C following which they were allowed to germinate partially in 10 ml of buffer containing 10 mM, Tris (pH 8.0), 10 mM L-alanine and 10 mM, CaCl$_2$ at 37°C for 40 min. Heat labile newly germinated spores were subjected to freeze-thaw treatment in liquid nitrogen for 5 cycles. Proteinase-K (45 µl) and SDS (450 µl of 10% stock solution) was added to the suspension and incubated at 42°C for 1 hr. After addition of NaCl to a final concentration of 1M, the nucleic acid was extracted once with phenol:chloroform:isoamyl (24:24:1) and once with chloroform:isoamyl alcohol (24:1). Isopropyl alcohol (0.6 volume) was added to precipitate DNA. After washing with 70% ethanol, the pellet was dissolved in deionised water and used as the template for polymerase chain reaction (PCR). The template was extracted either from 10 ml of buffer suspension or 10g of soil samples and finally redissolved in 200 µl of TE (pH 8.0) buffer. Five microliter of template was used for PCR amplification in each reaction.

**Polymerase chain reaction (PCR) amplification**—Two pairs of synthetic oligonucleotides previously published were used to amplify gene segment that encodes edema factor. The first pair of primers amplifies a fragment (1247-bp) while the second pair (nested) was used to amplify fragment (208-bp) nested within 1247-bp fragment. Each PCR cycle started with a 30 sec denaturation step, followed by a 2 min annealing step at 57°C, and a 2 min extension step at 70°C in PCR thermal cycler (Perkin-Elmer Cetus). Each PCR assay consisted of 35 cycles with an additional final extension step of 10 min at 70°C and the final magnesium concentration of 1.5 mM for each assay. PCR product was subjected to agarose (1%) gel electrophoresis and analysed.

The present study is an attempt to develop a rapid PCR based method for detection of *B. anthracis* spores from the environmental samples by testing for the presence of edema factor (EF) gene using two sets of primers previously published. The targeted 1247 and 208 bp fragments were first amplified by template DNA isolated from spores (10$^7$) grown on solid agar medium, separated from vegetative cells by heat treatment in distilled water (60°C, 90 min) followed by several washing with phosphate buffer saline (PBS, pH 7.2). DNA templates were also extracted from spores of standard *B. cereus* and *B. subtilis* and subjected to PCR by same set of primers. The specific 1247 and 208 bp amplicon could be detected by agarose gel electrophoresis of the products and ethidium bromide staining, only with the template from *B. anthracis* and not with those from *B. cereus* and *B. subtilis*. The detection sensitivity of PCR reaction was estimated by diluting the known amount of template DNA extracted from *B. anthracis* sterile (Fig. 1). Amplification was observed with first PCR using as low as 13 pg of template DNA. The sensitivity of the detection was further improved approximately 10-fold by using second set of primers (Fig. 2). The experimental regular and nested PCR was repeated three times with fresh spiking and template preparation.

Difficulty in extracting DNA from *B. anthracis* spores is well documented, and various protocols have been attempted by different workers. Out of several methods of spore lysis attempted, maximum yield was obtained by partial germination of spores as
described in the present study, followed by 10 cycles of freezing and thawing in liquid nitrogen.

Table 1 — Minimum number of \(B.\) \textit{anthracis} spores detectable by PCR when DNA was prepared by different methods.

<table>
<thead>
<tr>
<th>Template extraction method</th>
<th>Spore from agar culture</th>
<th>Purified spores from spiked soil</th>
<th>Direct extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol extraction</td>
<td>(5 \times 10^5/\text{ml})</td>
<td>(5 \times 10^3/\text{g})</td>
<td>NT</td>
</tr>
<tr>
<td>QiAmp kit</td>
<td>(2 \times 10^6/\text{ml})</td>
<td>(1 \times 10^8/\text{g})</td>
<td>NT</td>
</tr>
<tr>
<td>Extraction from soil</td>
<td>NT</td>
<td>NT</td>
<td>(2 \times 10^7/\text{g})</td>
</tr>
<tr>
<td>Partial germination</td>
<td>(10^2/\text{ml})</td>
<td>(8 \times 10^2/\text{g})</td>
<td>NT</td>
</tr>
</tbody>
</table>

The values are mean of three independent experiments. NT—Not tested

In the recent past, few PCR based methods have been attempted for detection of \(B.\) \textit{anthracis} spores, which include a nested PCR method\(^{17}\) and a PCR-ELISA using Streptavidin-coated microtitre plates\(^{18}\). The former method has been used for spores generated on culture media and suspended in definite buffer, ignoring its potential for detection of spores from the environmental samples. PCR-ELISA method, though intended to detect spores from soil samples, involves a non-selective enrichment step and may not be suitable for samples containing complex microbial flora of competitive nature. The present investigation was an attempt to overcome these limitations and can be used for detection of \(B.\) \textit{anthracis} spores from soil samples either by direct isolation of template or isolation after a prior purification of spores from the soil. The aqueous polymer two-phase system employed for spore purification from soil sample consisted of phosphate buffer and PEG. It not only separated spores from soil particles but also removed vegetative cells and debris of different species. This method has been used in our laboratory for immunofluorescence based detection of \(B.\) \textit{anthracis} spores from environmental samples where the efficiency of extraction varied depending on the nature of suspension medium\(^{19}\). The low efficiency of extraction (9-20%) could be the reason for comparatively low sensitivity of detection for spores spiked in soil samples using PCR. Adhesion of spores on the soil particle and/or its entrapment in a more ionic milieu possibly resulted in poor extraction of spores. Extraction of template directly from the soil sample was not beneficial when compared to the template isolation after spore purification. A more comprehensive study is required in this direction, taking into consideration the nature of environmental sample (e.g. varying humus content) and other native microbial flora. We recommend a prior purification and concentration of
sproes before subjecting to partial germination and lysis by freeze-thaw treatment. The template isolated in this manner gives the best PCR results in terms of sensitivity.

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References


