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A Novel Technique for Fast Detection of *Bacillus anthracis* Spore by Multiplex PCR

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Bacillus anthracis **is a spore-forming bacterium that causes anthrax disease. Its spore resists against physical and chemical agents. Molecular detection of *B.anthracis* spore requaiers the genomic DNA which is embded in the spores. in this study we developed a rapid detection of spor by Multiplex PCR using rapid physical disruption of spore in a short time without genomic DNA extraction. Genome of non-virulent strain of *B.anthracis* spores was released by MiniBead Beater-8 system. 3 pairs of primers were used in Multiplex PCR. PCR products were analyzed in agarose gels electrophoresis. Detection limit , specificity and accuracy of the test were determined through various process. This method enables to disrupt spores during 3 minutes and perform detection process with specific primers by Multiplex PCR method. Gel electrophoresis analysis showed 3 fragments of PCR products. digested 1083 bp fragment created 696 bp and 386 bp fragments which confirmed the test accuracy. Detection limit was 7680 spores/ml or 7 spore/μl. The results showed that the optimized physical disruption method has a proper velosity and there is no need for DNA extraction. Finally this method is capable to detect *B.anthracis* spores with high sensitivity and specificity.**

Key words: Detection- *Bacillus anthracis* - Spore - Multiplex PCR.

Bacillus anthracis as an endospore-forming, aerobic, gram-positive, rod-shaped bacterium with a width of 1-1.2μm and a length of 3-5μm. *Bacillus anthracis* is the etiologic agent of anthrax that occurs in livestock, wildlife, and humans (Spencer, 2003; Van Ert *et al.*, 2007). The high lethality, high transmission, low cost, and ability to aerosolization *B.*anthracis spore and use in threatening letters make this agent attractive to terrorists and other criminals to use in bioterrorism scenarios (Hess, 2012; Okinaka *et al.*, 1999; Pile *et al.*, 1998). After the anthrax letter event of September 2001 considerable attempt has been done to develop rapid detection technologies for presence of *B.anthracis* spore, such as polymerase chain reaction method (Bell *et al.*, 2002). Coetaneous anthrax has a very low mortality but gastrointestinal and inhalation anthrax are very lethal if untreated (Klietmann & Ruoff, 2001; Swartz, 2001). Due the threat of *B. anthracis* spore contamination in white powder in the letters and high possibility of aerosolization the disruption of spore to obtain DNA must be necessary for rapid detection and diagnosis. However bacterial culture represents a gold standard for *B. anthracis* identification but definitive identification of isolated colonies such as *B. anthracis* and detection of Virulent from non virulent strains may take a few days or more and requires specialized testing, including direct fluorescent antibody (DFA) staining of the capsule and cell wall

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polysaccharide and lysis of colonies by gamma phage (Bell *et al.*, 2002; Popovic *et al.*, 2005). In addition to its chromosome, *B. anthracis* has two large basic plasmid containing pathogenesis genes (virulence and capsule). The pXO1 plasmid is 174 kb in size and carries toxin genes includede pag, lef, and cya. The pXO2 plasmid is 95 kb in size and carries the capsule genes coding for capA, capB, and capC (Keim *et al.*, 1997; Knisely, 1966; Kolstø *et al.*, 2009). The agent of anthrax and other bacillus family are distinct in capsule and immobility. The etiologic agent of anthrax is spore producing bacteria that resistant to hard physical conditions (Hugh Jones, 1999). 3methods of Spore disruption divided into chemical, mechanical, and thermal treatments. Physical disruption methods are preferred. Most chemical agents inhibit PCR reaction requiring removal in subsequent, additional steps. A completely automated system for spore detection should ideally include a rapid physical disruption method and the ability to manipulate, without manual intervention and easy to use in PCR reaction without further DNA extraction steps (Belgrader *et al.*, 2000; Kuske *et al.*, 1998). Lysis of an organism to obtain its genomic material is an important step in sample preparation for nucleic acid testing. However, lysis is a significant challenge for thick-walled microorganisms such as *Bacillus anthracis* spores and *Mycobacterium tuberculosis* cells (Hurley *et al.*, 1987; Käser *et al.*, 2009; Vandeventer *et al.*, 2011). Typical commercial kits for DNA extraction require many manual steps including the introduction of the sample, enzymes, and buffers with various mixing, incubation, centrifugation, and tube transfer steps. However, the focus of this research is on mechanical lysis systems that are typically required to gain access to the nucleic acid in hard-to-lyse samples, including bio threat letters containing spores of *B. anthrax*. For biodefense systems there is a clear need to minimize sample volume and lower costs. In this paper, we describe development of an automated spore disruption system and multiplex PCR method for the rapid detection of *B.anthracis*.

MATERIALS AND METHODS

Preparation of bacterial strains

Standard strains of *Bacillus subtilis* (PTCC:1365), *Bacillus cereus* (PTCC:1247) obtained from scientific and industrial research organization of Iran and *Bacillus anthracis* traditional vaccine strain (sterne 34F2) which contain only the pXO1 plasmid were obtained from veterinary organization of Iran. Bacteria were cultured on sheep blood agar (SBA) under the biological safety cabinet (BSC) level 2con at optimum temperature for growth (37°C) at least for 18-24 hours. The colonies were checked by gram staining (Käser *et al.*, 2009).

Preparation of spore crops

all steps were performed under sterile conditions. Bacteria was cultured on LB broth (Luria -Bertani Medium) for 24 hours at 37°C, and Ammonium salt sugar for producing spore for 5 to10 days at 37°C, (Warriner and Waites, 1999) and was examined microscopically (from colonies on the media, slide prepared and malachite green staining, approximately 85 to 90% of the cells were free spores. To prepare bacterial suspensions bacterial colonies from the media agar surface were washed with 5to10 ml of cold distilled water and washed three times in cold distilled water by centrifugation In 15 ml falcon tube at 3700 g for 30 min. The supernatant fluid was transferred to other tubes containing formaldehyde and discarded. Approximately 1 g (wet weight) of cells was obtained from each falcon tube by this procedure.

Purification of spore crops

To 0.5 to 1.5 g (wet weight) of cells 5 ml of cold distilled water was added after that the cells were homogenized by shaker for 3 min. To increase the yield of precipitation, consisting of clumped cell suspension, equal volume of 98 degrees ethanol was added and incubated for 12 hours at 4ÚC. Sediment was washed twice with 5 ml distilled water . The tube content was centrifuged at 3700 g for 30 min and the supernatant fluid was discarded of distilled water and dissolved in 3 ml of distilled water as uniformed suspension. To a clearance suspension of any remaining vegetative bacteria, suspension was heated at 60ÚC in water bath for 45 min. To obtain very condensed spores the suspensions were centrifuged at 3700 g for 10 min and stored at - 72ÚC without any significant decrease in the cell count (Powers, 1968; Warriner & Waites, 1999).

Lysis of *B. anthrasis* spores and DNA extraction

for disruption of *B.anthrasis* spores we

used BioSpec Mini bead beater- 8 to rupture *Bacillus subtilis*, *Bacillus cereus* and *Bacillus anthracis* traditional vaccine strain. For this purpose the tube was filled with 0.1mm beads supplied by the manufacturer of the Mini-Beadbeater-8. The tubes were inserted into the arms of the Mini- Beadbeater-8. The conical tubes have the advantage of being transferable to an Eppendorf centrifuge. The tubes containing 1ml of the cells and beads were spun for 1 min at 2500 RPM and other tube for 3 min at 5000 RPM, then aqueous layer containing the nucleic acids was removed for further purification and quality control. To clear the yield of the last section from any residual RNA it's incubated with 1.5 mg/ml RNase A (Fermentas) for 2 min at 37°C. Then samples were immediately transferred to fresh tubes and kept on ice. The malachite green staining method was used to confirm the disruption of spores and presence of non spore forming bacteria. The spore stocks were stored at -20°C

Preparation of Primers

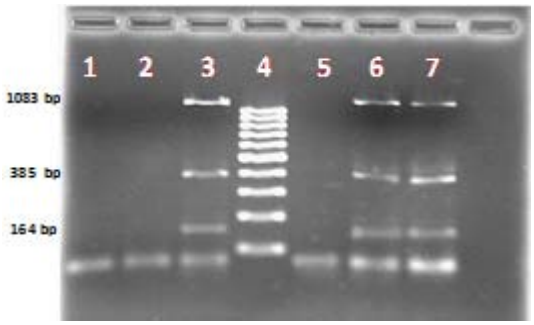
Specific primers for detection of *B. anthrasis* genome and plasmid were selected from pervious published work (11). Primer number 1 is from plasmid pXO1 and gives fragment of 1083 bp. Primer number 2 from plasmid pXO1 with 385 bp fragments and primer position number 3 from chromosomal sequence that gives fragment of 164 bp.

Multiplex PCR analysis of *B. anthrasis* samples

For Multiplex PCR reaction 1 µl of sample (disrupted spore) was added to 23.8 µl of master mix. The final materials concentrations in the optimized reaction as follows: 1.05x standard *Taq* buffer, 0.21 mM concentrations of dNTPs, 2.5U of *Taq* DNA polymerase/µl, 3.15 mM $MgCl_2$, then three pair primers 3.36PM concentrations of each set of primers the forward and reverse (F/R) primers were added to the master mix. Then final volume was adjusted by distilled water to 23.8µl. Multiplex PCR was performed using the eppendorf mastercycler gradient (Germany). The initially denature was carried out 94°C for 1 min. The thermocycling conditions consist of: repeated fo30 cycles of 94°C for 30s, 52°C for 30s, and 72°C for 40s. The final Extension was performed at 72°C for 40s.the PCR product was separated on 2% agarose gel electrophoresis, and were stained by ethidium bromide and visualized on gel documentation apparatus.

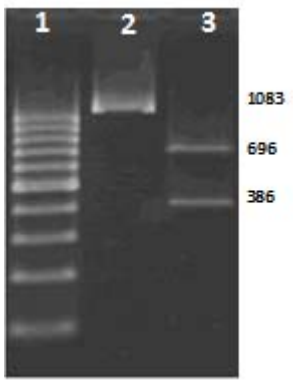
Enzymatic Digestion of PCR Product

For confirmation of the PCR product larger fragment were digested by HindIII restriction enzymes according to below reaction: PCR product (5µl), Hind III enzyme (1µl), buffer 10X (2µl), double distilled water (12µl) were incubated at 37 ° C for three hours and separated on 2% agarose gels under 90 volts.



Lanes: 1, PCR products of spore suspension of 1/64 dilution before disruption; 2, PCR products of spore suspension of 1/32 dilution before disruption; 3, Positive control (DNA extracted from strains Sterne 34f2); 4, 100 bp DNA molecular size ladder (Fermentase); 5, negative control (double distilled water); 6, PCR products of disrupted spore suspension of 1/32 dilution; 7, PCR products of disrupted spore suspension of 1/64 dilution.

Fig. 1. Result of multiplex PCR on gel electrophoresis before and after spore disruption.



Lanes: 1, 100 bp DNA molecular sizes ladder (Fermentase); 2, the 1083 bp PCR product; 3, product digested by *Hind* III enzyme.

Fig. 2. The 1083 bp PCR product digested by Hind III enzyme.

Sensitivity and Specificity

The serial dilutions of the disrupted bacterial spore sample were prepared from zero to 1/1024. To spores counting another sample of bacterial spores was cultivated in BA media. After this process the PCR optimization carried out on *Bacillus cereus*, *Bacillus subtitles*, *E.coli O157: H7* and *Shigella dysenteriae.*

RESULTS

Culture of bacterial strains

After incubation, gram stain analyses were performed and gram positive bacilli were seen**.** The yield of spore production and highly purified spores was confirmed by malachite green staining and microscopic examination, green spores and red vegetative bacilli were observed.

Multiplex PCR

The PCR products from 1/32 dilution had 3 separate bands of (1083 bp (pa), 385 bp (lf) and 164 bp (chr) (Fig. 1).

PCR Product confirmation

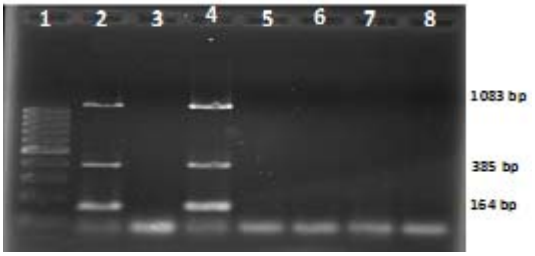
The results of restriction enzyme digestion of the larger fragment (1083bp) By *Hind* III enzyme revealed, two correct pieces of 696bp and 386bp that confirms the PCR product accuracy produced by specific primers (Fig. 2).

Sensitivity and Specificity

The sensitivity of PCR for detection of diluted sample of disrupted spores was shown in 1/512 dilution. At this dilution the number of spores was 7×10^3/ml. To investigate the specificity of the Multiplex PCR, extracted DNA from *Bacillus anthracis*, *Bacillus cereus*, *Bacillus subtilis*, *E.coli O 157*, and *Shigella dysenteriae*, were checked by specific primers and only *Bacillus anthracis* produced desired product (Fig. 3,4).

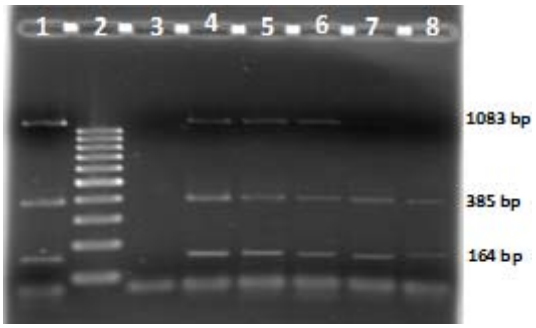
DISCUSSION

Previously the conventional culture-based methods are considered as gold standard for identification of *B. anthracis.* The manual testing methods are time-consuming and confirmatory testing frequently requires referring samples to reference laboratories for isolation of different virulent and non virulent strains (Bell *et al.*, 2002). Several studies were demonstrated value of molecular methods in specific detection of *B.anthracis.* The results of our study showed that disrupted spore suspension of traditional vaccine strain (sterne 34F2) by means of multiplex PCR correctly identified the *B.anthracis* containing pXO1 plasmid. During a suspected bioterrorism attacks or for monitoring cleanup after



Lanes:1,100 bp DNA molecular size ladder (Fermentase); 2, positive control (non diluted disrupted spore); 3, negative control (double distilled water); 4, PCR products of disrupted spore suspension of 1/32 dilution from *Bacillus anthracis*; 5,PCR products of disrupted spore suspension of 1/32 dilution from *Bacillus cereus*; 6, PCR products of disrupted spore suspension of 1/32 dilution from *Bacillus subtilis*; 7, PCR products of disrupted suspension of 1/32 dilution from *E.coli O 157*; 8, PCR products of disrupted s suspension of 1/32 dilution from *Shigella dysenteriae.*

Fig. 3. Specificity of multiplex PCR for diluted sample of disrupted spores.



Lanes:1, positive control (dilution at zero of disrupted bacteria); 2, 100 bp DNA molecular size ladder (Fermentase); 3, negative control (double distilled water); 4,PCR products of spore suspension of 1/32 dilution after disruption; 5, PCR products of1/64 dilution spore suspension after disruption; 6,PCR products of 1/128 dilution spore suspension after disruption; 7, PCR products of 1/256 dilution spore suspension after disruption; 8, PCR products of 1/512dilution spore suspension after disruption.

Fig. 4. sensitivity of multiplex PCR for diluted sample of disrupted spores.

decontamination, we need a rapid and sensitive methods to confirm the *B.anthracis* spores within a few hours.

For disruption of B.anthracis spores several physical and chemical ultrasonic disruptor, and thermal treatment methods like freeze and thaw, hot detergent treatment and bead mill homogenization have been used (Chandler *et al.*, 2001; Doebler *et al.*, 2009; Kuske *et al.*, 1998; Popham & Setlow, 1993; Vandeventer *et al.*, 2011; Wickus *et al.*, 1972). Physical disruption methods are preferred, as most chemical agents inhibits the PCR process, requiring removal in subsequent, additional steps. Low energy bead beating, which increase process complexity and potentially introduce PCR inhibitors (Wickus *et al.*, 1972).

Bead mill homogenization method increase rapidity (60 seconds) and efficacy in disrupting spores (Doebler *et al.*, 2009; Kuske *et al.*, 1998). The common methods that used for DNA extraction are multistep included sample, enzymes, and buffers introduction to various mixing, incubation, and tube transfer steps. Also several buffers like TENS 2X (Tens 1X is 50 mM Tris HCL [pH 8.0], 20 mM EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate [SDS]) are used in NA extraction from spores. Hence this step is omitted (Kuske *et al.*, 1998; Warriner & Waites, 1999). In the Bead mill homogenization method used in this work there is no need to such manipulations (Doebler *et al.*, 2009; Warriner & Waites, 1999). Likewise, other studies have confirmed the ability of the BioSpec Mini-Bead Beater to disrupt bacillus spores (Jones *et al.*, 2005). For production of spore In this study specific media (Ammonium salt sugar) was used that contained Ca and Mn ions that are important factors in the structure of spore. With extended by incubation period of few days most of vegetative bacteria convert to spore so purity of spore is higher in this situation. We only used deionized water for the spore suspension and homogenization that reduce the time and costs. To remove extracellular DNA and DNA contamination the spore suspension was diluted before homogenization. Multiplex PCR products, were used for disrupting spores. With traditional methods, confirmation of *B.anthracis* spores can require several days. To convert *B.anthracis* spores to vegetative cells is time consuming. However, gaining access to these nucleic acids involves complex protocols that are typically performed in laboratories using many steps to lyse the cells and purify the nucleic acid. By disrupting the spores directly, to obtain bacterial genome, much less time is required (Ellerbrok *et al.*, 2002). In order to do not use chemical material, preserve the environment, protect of users in the laboratory and to avoid any harmful toxic chemicals, need to develop rapid methods and physical method for disrupting the spores and spore suspension purification methods. In this study we omitted the step of purifying of extracting DNA that is time consuming in previous methods.

CONCLUSIONS

We report here a simple method for rapid detection of *B.anthracis* spores. According to article one of the convention biological weapons, Each State Party to this convention undertakes never in any circumstances to develop, produce, stockpile or otherwise acquire or retain(1); microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes; (2) weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict." *B. anthracis* is a potent biological agent listed in the annex to the biological agent list and has been used as bioterrorism agents in several occasions because it has the characteristic of resistance to environmental factors due its spore formation. Due legal, ethical and biosafety restrictions we used non pathogenic vaccine strain

(Strain Sterne) to produce pure spores. This strain is absolutely similar to pathogenic strain and only lacks one virulent plasmid. Optimization results showed that with use of Mini bead beater-8 for disruption of spore we were able to rapidly detect *B. anthracis* spore by specific primers in multiplex PCR reaction. The sensitivity of reaction is comparable to other detection methods filed. This method is simple, reliable and rapid for filed detection of biological samples suspected to *B. anthracis* spore in bioterrorist incident or any suspected outbreak of anthrax in animal population.

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