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Novel multiplex-PCR for rapid detection of *Bacillus anthracis* spores present in soils of Sirajganj district in Bangladesh

KHMNH Nazir[1*], J Hassan[1], SMZH Chowdhury[2], MB Rahman[1]

[1]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh; [2]Bangladesh Agricultural Research Council, Farmgate, Dhaka, Bangladesh

Abstract

Bacillus anthracis spores were isolated and detected from soil samples (n=72) using multiplex-PCR method. The bacteria were isolated and primarily identified as *Bacillus anthracis* using selective Polymyxin B - Lysozyme - EDTA - Thallous acetate agar. A multiplex-PCR method targeting three genes; *rpo*B of genome, *pag* of pX01 and *cap* of pX02 was done to confirm the isolated bacteria. Among 72 soil samples, the viable *B. anthracis* spores could be extracted from 14 (19.44%) samples. However, both pX01 and pX02 plasmids were harbored in 5 (6.94%) isolates. On the other hand, pX01 and pX02 was present in 8 (57.14%) and 11 (78.57%) isolates, respectively. This two-step-method was found to be easy, accurate and rapid in identification of *B. anthracis* spores from soil samples and to identify the toxigenic plasmids present in this bacterium.

Key words: Anthrax, *Bacillus anthracis*, spore, multiplex-PCR, soil

 ***Corresponding Author:** nazir@bau.edu.bd

Introduction

Bacillus anthracis, the causal agent of anthrax, is a soil-borne, spore forming bacterium. The bacterium forms a dormant stage called endospore when it comes in contact with unfavorable atmosphere (Dragon and Rennie, 2001). The spores may persist in the environment for several decades and the susceptible animal may be infected after ingestion of the spores through contaminated feed or water (Fasanella *et al.*, 2013). Virulent strain of the bacterium possesses toxin-encoding plasmids; pX01 (181.7 kb) and/or pX02 (96.2 kb) (Vahedi *et al.*, 2009). The pX01 contains three different genes namely protective antigen (*pag*), lethal factor (*lef*), oedema factor (*cya*), whereas pX02 contains capsule (*cap*) gene (Park *et al.*, 2007; Kumar *et al.*, 2012).

Anthrax is found as a common problem of animal and subsequently to human in tropical and sub-tropical countries including Bangladesh (Biswas *et al.*, 2011). In Bangladesh, the disease is considered as an enzootic and the outbreak is mostly occurred in Sirajganj, a north-western district (Ahmed *et al.*, 2010; Biswas *et al.*, 2011; Fasanella *et al.*, 2013; Ahsan *et al.*, 2013). Previously, investigation of anthrax in Bangladesh was limited within surveillance studies during active epizootics (Ahmed *et al.*, 2010; Biswas *et al.*, 2011). Recently, we investigated different environmental parameters (*e.g.*, organic matter, pH, Ca contents of the soil, temperature, and rainfall) and found that some of them are significantly correlated with the repeated anthrax outbreak in Sirajganj, Bangladesh (Ahsan *et al.*, 2013; Hassan *et al.*, 2015a). However, identification of the bacteria was done by a series of complicated and time-consuming traditional bacteriological and biochemical methods. Considering the public health importance, very accurate and quick identification of pathogenic *B. anthracis* is needed. Multiplex-PCR has been used as a useful method for the identification and differentiation of pathogenic *B. anthracis* from other

related pathogenic/nonpathogenic bacilli (Ko *et al.*, 2003; Park *et al.*, 2007; Kumar *et al.*, 2012). In this study, taking an advantage of multiplex-PCR method, we established a two-step-method for an easy, precise and rapid identification of *B. anthracis* spore from soil samples targeting the presence of virulent plasmids (pX01 and/or pX02).

Materials and Methods

To extract the spores, approximately 400-gm of surface soil samples (n=72) were collected from Sirajganj district, Bangladesh (Ahsan *et al.*, 2013). Using the selective Polymyxin B - Lysozyme - EDTA - Thallous acetate (PLET) agar (Sigma-Aldrich, Switzerland), the viable spores were extracted (Dragon and Rennie, 2001; Ahsan *et al.*, 2013). From the *B. anthracis* positive plates, colonies (one colony from each) were taken and cultured overnight at 37^{o}C in liquid culture media. Total DNA was extracted following the procedures of Jula *et al.* (2007) with some modifications. Combination of three primers targeting portions of *rpo*B gene (359-bp, specific for *Bacillus* genus; 208-bp, specific for *Bacillus anthracis*) present in *B. anthracis* chromosome were used in the multiplex-PCR (Ko *et al.*, 2003). In addition, two pairs of primer sets were designed targeting portions of *pag* gene of pX01 (287-bp) and *cap* gene of pX02 (164-bp) by analyzing the nucleotide sequences of these two plasmids collected from GenBank database. The oligonucleotide primers (Table 1) were purchased from the Cosmo Genetech Co., Ltd., South Korea. The multiplex-PCR was performed according to the procedure described by Ko *et al.* (2003) with some modifications. In brief, the PCR assay was performed in a total reaction volume of 25 µl consisting of ca. 50 ng template DNA, 2.5 µl of 10 x PCR buffer, 0.2 mM dNTPs mix (SolGent Co., Ltd.), 20 pmole of each primer; and 1.5 U of SolGent^{TM} *Taq* DNA Polymerase (SolGent Co., Ltd.). Thirty five cycles of amplification were performed in Thermal Cycler (AB Applied Biosystem) following initial denaturation at 95°C for 2 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 45°C for 30 s, and an extension step at 72°C for 30 s, with a final extension at 72°C for 1 min. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels stained with ethidium bromide (0.5 mg/ml). A previously isolated *B. subtilis* isolate (unpublished) was used as a negative control. The purified PCR fragments generated by using specific primers targeting pX01 and pX02 (Table 1) were validated by sequencing using automatic DNA sequencer (ABI 3730XL; Applied Biosystem). The PCR reaction and condition were same as described above.

Results and Discussion

Among the 72 soil samples, viable *B. anthracis* endospores could be extracted from 14 (19.44%) samples. These findings are in support of our previous findings (Ahsan *et al.*, 2013). However, Jule *et al.* (2007) found only 9.1% soil samples were contaminated with *B. anthracis* spore in Iran. This variation may be due to difference in geographical location, soil composition, soil pH, humidity, rainfall etc. (Ahsan *et al.*, 2013). On PLET agar medium, the bacteria form circular, creamy-white colonies with ground glass texture. The PLET medium is a selective medium for *B. anthracis* that inhibits all other contaminants including *B. cereus* (Dragon and Rennie, 2001). However, in our previous study, the bacteria were identified through a series of time-consuming and laborious steps (Jula *et al.*, 2007; Vahedi *et al.*, 2009; Ahsan *et al.*, 2013; Hassan et al., 2015b). Identification of *B. anthracis* by PCR targeting only toxin-encoding genes (pX01 or pX02) may not give accurate results in identification of *B. anthracis* as the plasmid can be transferred among closest species by horizontal gene transfer (Ko *et al.*, 2003). Moreover, all the *B. anthracis* isolates may not contain these toxin-encoding genes and in some cases the plasmids can be lost naturally (Ko *et al.*, 2003; Vahedi *et al.*, 2009). Therefore, combination of *B. anthracis* specific chromosomal marker along with toxin-encoding gene amplification would be an authentic and reliable strategy for the specific detection of this species. Here, an easy, precise and rapid identification of *B. anthracis* spore from soil samples has been established that enabled us to avoid hazards of handling the pathogenic *B. anthracis* during traditional bacteriological techniques. For

this, after screening the spores from soil samples, the bacteria were directly used for an optimized multiplex-PCR. Thus, it is named as a two-step-method.

Table 1. Oligonucleotide sequences used in the multiplex-PCR

Name	Sequence (5´-3´)	Target gene	Expected size	Reference
Ba-SF	TTCGTCCTGTTATTGCAG	*rpo*B of genomic DNA	359-bp and 208-bp	Ko *et al.* (2003)
BA-RF	GACGATCATYTWGGAAACCG			
BA-RR	GGNGTYTCRATYGGACACAT			
Pag-F	GAAGAGTGAGGGTGGATACA	*pag* of pX01	164-bp	This study
Pag-R	CATATCCGGTTTAGTCGTTT			
Cap-F	GTACTTAGAACTTTGTGGTATG	*cap* of pX02	287-bp	This study
Cap-R	TTTCGTCTCATTCTACCTCAC			

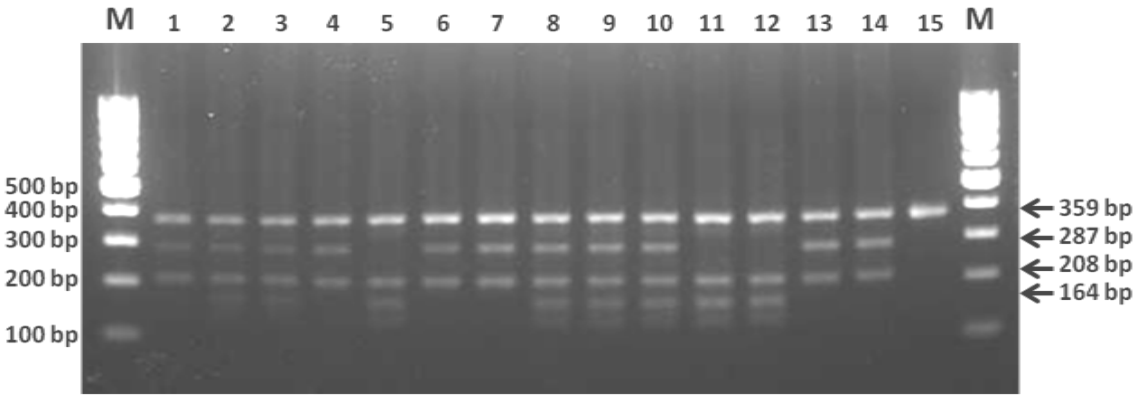

Figure 1. Agarose gel electrophoresis of multiplex-PCR products of *B. anthracis* isolates. M = 100-bp DNA ladder; Lanes 1, 4, 6, 7, 13 and 14 indicate the isolates as *B. anthracis* containing pX02 plasmid. Lanes - 2, 3, 8, 9 and 10 indicate *B. anthracis* containing both pX01 and pX02 plasmids. Lanes- 5, 11 and 12 indicated the bacteria as *B. anthracis* harboring pX01 plasmid. Lane-15 is the negative control having no plasmid but confirmed to be the genus *Bacillus*.

The multiplex-PCR method targeting *rpo*B gene of *B. anthracis* genome has been assessed to distinguish the bacteria from a number of related strains including *B. cereus* and *B. thuringiensis* which are considered as the closest neighbors in phylogenetic analysis (Ko *et al.*, 2003). The same primer pairs (Table 1) were used in our experiment. In addition, we designed and used the primer-sets specific for pX01 and pX02 plasmids indicating that our strategy expanded the previous method. In our study, 100% isolates grown on PLET agar were confirmed as *B. anthracis* (Figure 1). However, variation was found in the presence of toxin-encoding plasmids (Vahedi *et al.*, 2009). Only 5 (6.94%) isolates contained both pX01 and pX02 plasmids. Six isolates were found to be positive for only pX02 and three were positive for only pX01. On overall evaluation, 8 (57.14%) harbored pX01 plasmid, and the capsule encoding pX02 plasmid was identified in 11 (78.57%) isolates (Figure 1). For final validation of our newly developed two-step-method, all the isolates were re-identified as expected by traditional bacteriological and biochemical methods.

Conclusion

The newly established two-step-method was found sensitive to identify virulent strains of *B. anthracis*. The findings of this study can be used as an easy,

precise and rapid screening of *B. anthracis* from soil and other environmental samples.

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