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Correspondence

A multiplex PCR assay for the simultaneous identification of virulent & avirulent *Bacillus anthracis* targeting genes of plasmids & chromosomal DNA

Sir,

Bacillus anthracis, the aetiological agent of anthrax, is responsible for a serious and often fatal disease of mammalian livestock and humans[1]. Its spore-forming capability and highly pathogenic nature have made it one of the most effective bioterrorism agents. Animals are infected by contact with soil borne spores. Humans become infected only incidentally when brought into contact with diseased animals or their waste products.

Identification of *B. anthracis* has traditionally been determined by using phenotypic differences between *B. anthracis* and the rest of the *B. cereus* group (*i.e.*, lack of motility and haemolysis, susceptibility to penicillin, typical colony morphology, and susceptibility to lysis by gamma phage); however, these methods are slow and require at least 24 h for completion. The main characteristic used to distinguish *B. anthracis* from closely related soil-borne *Bacillus* is the presence of two virulent plasmids pXO1 and pXO2. For *B. anthracis*, the main targets for development of such assays, primarily PCR-based, have been and continue to be genes encoding its virulence factors: a tripartite exotoxin and an antiphagocytic capsule[2-5]. Plasmid-located virulence genes seem to be restricted to *B. anthracis*, giving the plasmid-based assays a high degree of specificity[6]. However, strains of *B. anthracis* that lack these plasmids have also been reported[2,7]. Further, the pX02 plasmid has been transferred into other bacillus species, and genes from the pX01 plasmid have been successfully expressed in other bacteria[8]. Consequently, an assay focused on a specific stable chromosomal target for detection of avirulent and plasmid cured *B. anthracis*, as well as those that potentially could have been genetically engineered, is essential. The amplification of multiple rather than a single *B. anthracis* DNA targets provides an increased assurance of specificity[9]. Several chromosomal markers were tested for *B. anthracis* detection, such as the *vrrA* gene[10], Ba813 marker[11]. Most of these assays are monoplex PCR which rely on the amplification of a single target. Moreover, these lack internal amplification control (IAC), which has now become almost mandatory in diagnostic PCRs.

In the present study, multiplex PCR (mPCR) was used to determine the presence of four plasmids genes *viz.*, protective antigen (*pag*), lethal factor (*lef*), oedema factor (*cya*), capsule (*cap*) for the differentiation of virulent and avirulent strains of *B. anthracis*. Three chromosomal markers which include S-layer (*sap*), gyrase B (*gyrB*) and Ba813 marker have been targeted to confirm the *B. anthracis* species. IAC was incorporated to check the presence of inhibitor, if any, in the PCR mixture. We have also successfully evaluated the mPCR employing 25 *B. anthracis* known clinical isolates obtained from Christian Medical College (CMC), Vellore, and two isolates from 25 soil samples derived by us from different anthrax endemic regions in 2009 [12]. This study was aimed at the genotype determination targeting virulence and chromosomal marker genes in clinical and environmental isolates of *B. anthracis* using multiplex PCR. The study was conducted in Microbiology Division of Defense Research and Development Establishment, Gwalior, India.

The primers for *cya* and Ba813[7]; *cap* and *sap*[13]; *gyrB*[14]; *pag*, *lef*, and IAC[12] were used in the present study. IAC was constructed as per the method described earlier[14]. Multiplex PCR was performed in a 25-µl reaction volume containing 1× PCR buffer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, primers (100nM protective antigen (Pa)-F and-R; 200 nM S-layer-F and –R; edema factor (Ef)-F and-R; lethal factor (Lf)-F and-R; 300 nM R1 and -R2; 350 nM BA1 and -BA2r; 500 nM Cap-F and -R;), 2.0 µl (~500 pg) of template DNA, and 1 U of

Taq polymerase. Various concentrations of IAC DNA were tried before choosing 10^4 copies per reaction. Amplification consisted of initial denaturation at 94 °C for 4 min, 30 cycles of amplification with denaturation at 94 °C for 1 min, annealing at 51 °C for 40 sec, extension at 72 °C for 30 sec, and final extension of the incompletely synthesized DNA at 72 °C for 5 min in a Bio-Rad myCycler thermal cycler (Bio-Rad Laboratories, USA). The PCR products were analyzed in 2.5 per cent agarose gels containing 0.5 µg/ml of ethidium bromide and subjected to electrophoresis in a 1× Tris base acetic acid, EDTA (TAE) buffer.

To verify and evaluate the specificity of the primers used, PCR was performed employing template DNA prepared from *B. anthracis* as well as different bacterial strains (*Source*: MTCC, Chandigarh and DRDE, Gwalior) namely, *B. cereus* (n=8), *B. thuringienis* (n=5), *B. licheniformis* (n=3), *B. megaterium* (n=2), *B. subtilis* (n=2), *B. sphaericus, B. circulans*, *B. pumilus*, *Escherichia coli*, *Proteus vulgaris*, *P. mirabilis*, *Shigella flexneri* , *S. sonnei*, *S. boydii*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Streptococcus pyogenes* and *Yersinia enterocolitica.* The primers produced amplicons of 719, 639, 929, 373, 152, 245 and 846 bp for *pag*, *sap*, *cya*, *lef*, Ba813, *gyrB* and *cap* genes, respectively. The primers did not amplify any product from any bacterial species other than *B. anthracis*. Moreover, PCR was performed to determine the presence of all the 7 genes in 27 isolates of *B. anthracis*. *B. anthracis* Sterne strain as well as recombinant *B. anthracis* pYS5 was also checked by mPCR. All the 27 isolates of *B. anthracis* used in this study showed the specific amplifications of all the seven genes (Fig.). No amplification of the *cap* gene was observed in the Sterne strain of *B. anthracis*, which lacks the plasmid pXO2 (Fig.). There was no amplification of the *cap*, *lef*, and *cya* genes, but the amplification of *pag*, *sap*, Ba813, and *gyrB* in recombinant *B. anthracis* pYS5 was evident (Fig.), which was originally deficient of both pXO1 and pXO2 plasmids, but has the *pag* gene inserted into a shuttle vector, pYS5[15]. This shows that mPCR targeting both plasmid and chromosomal genes could unambiguously identify virulent and avirulent strains of *B. anthracis* and can easily distinguished 'anthrax like' strains from other *B. cereus* group bacteria.

To investigate the sensitivity of mPCR detection, serial 10-fold dilutions of the genomic DNA samples were prepared from *B. anthracis* cell suspension. An aliquot of 2.0 µl of each dilution was added to five separate PCR tubes in the presence of 10^4 copies of IAC DNA. The PCR reactions were carried out as described

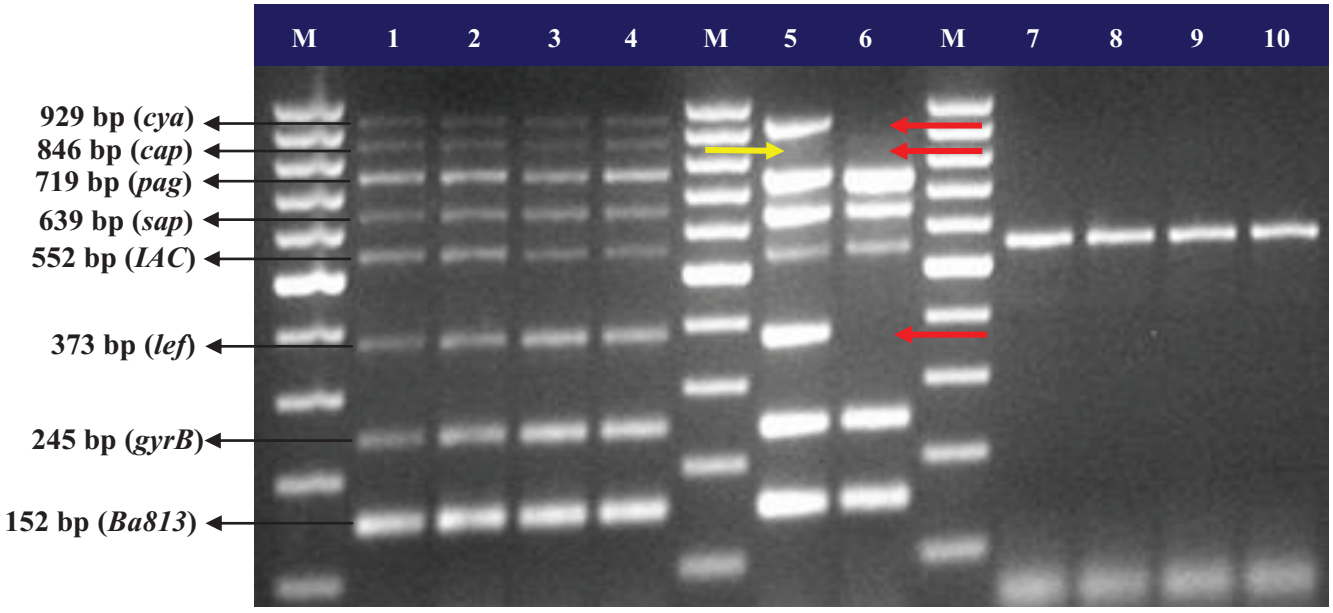

Fig. Multiplex PCR showing amplification of DNA from *B. anthracis* isolates and IAC (552 bp). Lane M, Molecular weight marker (GeneRuler DNA Ladder-MBI Fermentas); lanes 1-4, *B. anthracis* DRDE isolates; lane 5, *B. anthracis* Sterne strain (lacking *cap* gene indicated by yellow arrow); lane 6, Recombinant *B. anthracis* pYS5 (lacking *cap*, *lef*, and *cya* gene indicated by red arrows); lane 7, *B. cereus* ATCC 13061; lane 8, *B. thuringiensis* ATCC 10792; lane 9, *B. licheniformis* ATCC 12759; lane 10, *B. megaterium* ATCC 14581.

above. The detection limit of mPCR was 1 pg with genomic DNA. The method is rapid, taking less than 2.5 h after picking up an isolated colony from an agar plate. IAC was included in the mPCR to increase the confidence of the assay by pinpointing false-negatives that may be the result of assay failure, and/or reaction inhibition[5,14,16]. The IAC was co-amplified with target DNA and showed an amplification of 552 bp in all the DNA samples isolated from bacterial cultures.

This study demonstrates that the mPCR assay with an IAC is simple and easy to perform and represents a highly specific approach for the identification of *B. anthracis*. All the 27 isolates of *B. anthracis* from clinical and environmental samples contained all the 7 genes. Finally, this method will contribute significantly in reducing the risk of laboratory acquired infection and the multiplex format of the PCR assay will reduce reagent cost and staff time making this technique a very useful tool for confirmation of *B. anthracis*.

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