Detection of Virulence-Associated Genes in Clinical Isolates of *Bacillus anthracis* by Multiplex PCR and DNA Probes

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Anthrax is a zoonotic disease caused by *Bacillus anthracis*, and well recognized as a potential agent for bioterrorism. *B. anthracis* can be identified by detecting the virulence factors genes located on two plasmids, pXO1 and pXO2. The aim of the present study was to determine the presence of virulence genes in 27 isolates of *B. anthracis* isolated from clinical and environmental samples. For this purpose, multiplex PCR and DNA probes were designed to detect protective antigen (*pag*), edema factor (*cya*), lethal factor (*lef*), and capsule (*cap*) genes. Our results indicated that all the isolates contained all the above virulence genes, suggesting that the isolates were virulent. To the best our knowledge, this is the first study about the determination of virulence marker genes in clinical and environmental isolates of *B. anthracis* using multiplex PCR and DNA probes in India. We suggest that the above methods can be useful in specific identification of virulent *B. anthracis* in clinical and environmental samples.

Keywords: *Bacillus anthracis*, multiplex PCR, DNA probes, internal amplification control, digoxigenin

Bacillus anthracis, a Gram-positive, nonmotile, facultative anaerobic, and spore-forming rod-shaped bacterium, is a causal agent of anthrax, a serious and often fatal infection in both livestock and humans. Animals are infected by contact with soilborne spores. Humans become infected only incidentally when brought into contact with diseased animals or their waste products. Humans may contract disease *via* three routes: cutaneous, gastrointestinal, and inhalation. In naturally acquired infections, the cutaneous mode of transmission is by far the most common, accounting

for approximately 95% of infection [8]. Death is uncommon in cases of cutaneous anthrax, but not in gastrointestinal or inhalation anthrax [14, 20, 29, 32]. For a long time, this species has attracted attention because of its hardiness, dormancy, and thus its potential use as a biological weapon [15, 16].

Virulent strains of *B. anthracis* have two plasmids: pXO1 (184 kb), which encodes toxins, and pXO2 (95 kb), which encodes capsule [28]. Strains lacking either of the plasmids are of attenuated virulence in most animal hosts. The three genes *pag*, *lef*, and *cya*, which are on pXO1, encode the secreted toxin proteins PA (protective antigen), LF (lethal factor), and EF (edema factor), respectively. The *cap* gene, which encodes a poly-γ-D-glutamic capsule, is on pXO2. The capsule enhances virulence *in vivo* by inhibiting phagocytosis of the organism [28]. DNA homology studies [17] have shown that *B. anthracis* is very closely related to *B. cereus*, *B. thuringiensis*, and *B. mycoides*. The taxonomy of this group is controversial because of many similarities between the nucleotide sequences of 16S rRNA and 23S rRNA genes, and the 16S to 23S intergenic spacer region in the various isolates [1, 5, 11].

Culture represents the "gold standard" for identification of *B. anthracis*. A definitive identification of isolated colonies as *B. anthracis* may take 24–48 h or more and requires specialized testing including direct fluorescent antibody (DFA) staining of capsule and cell wall polysaccharide as well as lysis of colonies by gamma phage. These confirmatory tests are not generally available in most of the clinical microbiology laboratories [25]. Confirmation of a virulent *B. anthracis* is usually carried out by inoculation of the suspected culture in mice or guinea pigs and/or amplification of toxin and capsule genes by PCR [36]. Several conventional PCR assays have been described for the identification of *B. anthracis* [3, 4, 7, 24, 27, 28]. Most of these assays are monoplex PCR, which rely on the amplification of a single target. Moreover, they lack internal amplification control

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(IAC), which has now become almost mandatory in diagnostic PCRs.

However, there are no published data on molecular identification of virulent *B. anthracis* isolated from clinical and environmental samples in India using multiplex PCR (mPCR) and DNA probes. In the present study, the above two techniques were used in order to determine the presence of protective antigen (*pag*), lethal factor (*lef*), edema factor (*cya*), and capsule (*cap*) genes in 27 *B. anthracis* isolates derived from clinical and environmental samples. IAC was incorporated to check the presence of inhibitor, if any, in the PCR mixture.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions:

Bacterial strains used in this study are shown in Table 1. A total of 25 *B. anthracis* clinical isolates (DRDE 1–25) was obtained from Christian Medical College (CMC), Vellore, India, and a total of 2 environmental isolates (DRDE 26–27) were derived from 25 soil samples collected from different anthrax endemic regions of India in a one-year period. Reference strains were obtained from the American Type Culture Collection (ATCC), U.S.A., as well as from the National Center for Industrial Microorganisms (NCIM), Pune, India. *B. anthracis* Sterne strain was obtained from the Institute of Veterinary and Preventive Medicine (IVPM), Vellore, India, and the recombinant

Table 1. Bacterial strains used for the evaluation of specificity of PCR primers.

Organism	Strains	PCR detection of			
		pag	*lef*	*cya*	*cap*
Bacillus anthracis clinical isolates	DRDE 1-25	+	+	+	+
B. anthracis environmental isolates	DRDE 26-27	+	+	+	+
B. anthracis Sterne	IVPM	+	+	+	-
B. anthracis pYS5	IGIB	+	-	-	-
B. cereus	ATCC 13061	-	-	-	-
B. cereus	ATCC 11778	-	-	-	-
B. cereus	ATCC 10876	-	-	-	-
B. cereus	ATCC 14579	-	-	-	-
B. cereus	NCIM 2155	-	-	-	-
B. cereus	NCIM 2459	-	-	-	-
B. cereus	NCIM 2458	-	-	-	-
B. cereus	NCIM 2457	-	-	-	-
B. sphaericus	ATCC 4525	-	-	-	-
B. circulans	ATCC 61	-	-	-	-
B. licheniformis	ATCC 12759	-	-	-	-
B. licheniformis	ATCC 14580	-	-	-	-
B. megaterium	ATCC 14581	-	-	-	-
B. megaterium	ATCC 9885	-	-	-	-
B. pumilus	ATCC 14884	-	-	-	-
B. subtilis	ATCC 11774	-	-	-	-
B. subtilis	NCIM 2196	-	-	-	-
B. thuringiensis	ATCC 10792	-	-	-	-
B. thuringienis	NCIM 2159	-	-	-	-
B. thuringienis	NCIM 5019	-	-	-	-
B. thuringienis	NCIM 2130	-	-	-	-
B. thuringienis	NCIM 2979	-	-	-	-
Escherichia coli	ATCC 10536	-	-	-	-
Escherichia coli	ATCC 11775	-	-	-	-
Proteus vulgaris	ATCC 33420	-	-	-	-
Proteus mirabilis	ATCC 12453	-	-	-	-
Pseudomonas aeruginosa	ATCC 10145	-	-	-	-
Staphylococcus aureus	ATCC 51740	-	-	-	-
Shigella flexneri	ATCC 9199	-	-	-	-
Shigella sonnei	ATCC 25931	-	-	-	-
Shigella boydii	ATCC 9207	-	-	-	-
Streptococcus pyogenes	ATCC 12384	-	-	-	-
Yersinia enterocolitica	ATCC 23715	-	-	-	-

+: Positive result, –: negative result.

B. anthracis pYS5 was obtained from Dr. Yogendera Singh, Institute of Genomics and Integrative Biology (IGIB), New Delhi, India. The PA gene was inserted into a shuttle vector, pYS5, and the *B. anthracis* (lacking both pXO1 and pXO2) was transformed with this construct [30]. Isolation and identification of *B. anthracis* were carried out according to the method of Turnbull [36]. Various other *Bacillus* species and other bacterial organisms used in the study were grown overnight at 37°C on brain–heart infusion agar (Difco Laboratories, Detroit, U.S.A.).

Isolation of Genomic DNA from Bacterial Culture

Genomic DNA was isolated from bacterial cultures using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The concentration of extracted DNA was measured using a Smartspec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The purity of genomic DNA used in this study was within the ratio of 1.8 to 2.0 (A_{260}/A_{280}).

Primers and Internal Amplification Control

The sequences of the primers and IAC used in this study are listed in Table 2. Primers were designed using Gene Runner software (Hastings Software, NY, U.S.A.) and synthesized from Operon Biotechnologies, Cologne, Germany. The primers for the *cya*, *pag*, *lef*, and *cap* genes were designed on the basis of the published sequences of *cya* [9], *pag* [38], *lef* [6], and *cap* [23], respectively. IAC was constructed to check the presence of inhibitors in the PCR mixture. The IAC primers used in this reaction had 5' overhanging ends, which were identical to the primers used in mPCR specific for *lef* (Lf-F and Lf-R), whereas 3' ends were complementary to a DNA sequence of pUC 18 (Table 2). The PCR reaction mixture for generation of IAC DNA contained 400 nM of each primer, 0.2 mM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 0.5 U of *Taq* polymerase (MBI Fermentas), and 1.5 mM MgCl₂ in 1× PCR buffer (MBI Fermentas), with 400 pg of template DNA. Reaction parameters included initial denaturation at 94°C for 5 min, 30 cycles of amplification with denaturation at 94°C for 1 min, annealing at 51°C for 1 min, extension at 72°C for 1 min, and final extension of the

incompletely synthesized DNA at 72°C for 5 min in a Bio-Rad myCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.). PCR product was purified using commercially available kit (Qiagen). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and the DNA was stored at -20°C.

Multiplex PCR Condition and Analysis of PCR Products

Multiplex PCR was performed in a 25-μl reaction volume containing 1× PCR buffer, 0.2 mM of each dNTP, 2.0 mM MgCl₂, primers (300 nM Ef-F and -R; 400 nM Pa-F and -R; 150 nM Lf-F and -R; Cap-F and -R), 2.0 μl (~500 pg) of template DNA, and 1 U of *Taq* polymerase (MBI Fermentas). Various concentrations of IAC DNA were tried before choosing 10⁴ 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 s, extension at 72°C for 30 s, and final extension of the incompletely synthesized DNA at 72°C for 5 min in a myCycler thermal cycler (Bio-Rad, U.S.A.). The PCR products were analyzed in 2.5% agarose gels containing 0.5 μg of ethidium bromide and subjected to electrophoresis in a 1× TAE buffer.

Determination of Specificity and Sensitivity of mPCR

Specificity of primers was checked employing template DNA from *B. anthracis* as well as other bacterial species as shown in Table 1. In order 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 10⁴ copies of IAC DNA. The PCR reactions were carried out as described above.

DNA Labeling

The 900 bp *cya*, 719 bp *pag*, 373 bp *lef*, and 807 bp *cap* fragments were purified using a PCR Gel extraction kit (Qiagen, Germany) as per the manufacturer's recommendations. The purified amplicons were labeled with digoxigenin-11-dUTP (dig-UTP) by random primed labeling [10], using a DIG DNA labeling and detection kit obtained from Roche Diagnostics, Penzberg, Germany. The reaction

Table 2. Oligonucleotide primers used in this study.

Primer	Primer sequence (5'→3')	Target gene	Amplicon size (bp)
Ef-F	TGCACCTGACCATAGAACGGTATTAG	*cya*	900
Ef-R	AAAGTTCCCTTAGTTGAATCCGGTTTCC		
Pa-F	CAAGTTCCCAGGGGTTACTAGGAT	*pag*	719
Pa-R	GTGTCTTGCCTCTGGTGATACAT		
Lf-F	CCTCAGTAGATAAAAAATCACTACTATC	*lef*	373
Lf-R	AGCAATGAGGTACAAGAAGTATTT		
Cap-F	TAGGAGTTACACTGAGCCTTATTTTTA	*cap*	807
Cap-R	GCACTTAAGTGAATATTCTTATCTGC		
IAC-f	CCTCAGTAGATAAAAAATCACTACTATC TAGCAGAGCGAGGTATGTAGGC	IAC	552
IAC-r	AGCAATGAGGTACAAGAAGTATTT GTAAGCCCTCCCGTATCGTAG		

IAC primers are flanked by Lf primers on 5' ends

mixes contained 500 ng of each denatured amplicon, 2 µl of 10×
hexanucleotide mix, 2 µl of 10× dNTP labeling mix (1 mM dATP,
1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM DIG-11-
dUTP), and 1 µl of Klenow enzyme (2 U) labeling grade. The mixture
was incubated overnight at 37°C.

Dot-Blot Hybridization and Detection

DNA samples of *B. anthracis* isolates were applied to 4 sets of
Hybond N+ membrane (Amersham, IL, U.S.A.), presoaked in 10×
SSC using HYBRI-DOT manifold (Life Technologies, MD, U.S.A.).
The spotted DNA was denatured in denaturation buffer (1.5 M NaCl
and 0.5 M NaOH) for 5 min at 37°C and then neutralized in neutralization
buffer (1.5 M NaCl and 0.5 M Tris-Cl, pH 7.5) for 5 min at 37°C
followed by washing in 1× SSC buffer for 5 min. The membranes
were exposed to 1,200 mJ of UV light in a UV-Stratalinker 2400
(Stratagene, CA, U.S.A.) to cross-link DNA to the membrane.
Membranes were prehybridized for 2 h at 51°C in 5× SSC, 0.5%
blocking agent (Roche), 0.1% sodium lauryl sarcosine, and 0.2%
SDS. The denatured probe was then added to the prehybridization
solution and hybridization carried out for 12 h followed by washing,
according to the manufacturer's recommendations. The bound probe
was detected immunologically following the protocols supplied with
the DIG DNA labeling and detection kit (Roche, Germany).

Determination of Specificity and Sensitivity of DNA Probes

Specificity of DNA probes was checked employing template DNA
from *B. anthracis* as well as other bacterial species as shown in
Table 1. In order to investigate the sensitivity of DNA probes, serial
10-fold dilutions of the genomic DNA samples were prepared from
B. anthracis cell suspension. A 2.0-µl aliquot of each dilution was
applied to Hybond N+ membrane. The membrane was probed and
developed as per the procedure described above.

RESULTS AND DISCUSSION

Anthrax is known to occur globally but is extremely rare in
the industrialized world. Naturally occurring anthrax can
be found in agricultural regions throughout the world [35,
39]. It has been estimated that as many as 20,000–100,000
human cases of anthrax occur annually, mostly in the
underdeveloped regions of the world, where livestock is
not vaccinated [26, 35, 39]. The actual incidence of anthrax
in India is not accurately known, mostly owing to under-
reporting [34]. Many regions in India are still enzootic for
animal anthrax but this is less frequent in northern India,
and sporadic cases of human anthrax have been reported
especially from southern India [21, 34].

Multiplex PCR

In the present study, a multiplex PCR assay was developed
to determine the presence of virulence-associated genes in
a total of 27 isolates of *B. anthracis* derived from clinical
and environmental samples over a period of 3–4 years.
The assay targeted the amplification of four virulent genes,
pag, *cya*, *lef*, and *cap*, that encode for the synthesis of
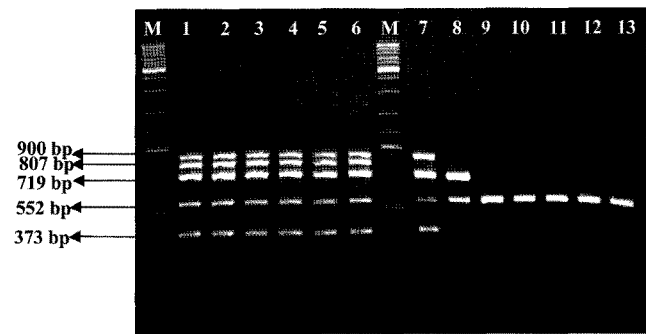protective antigen, edema factor, lethal factor, and capsular

Fig. 1. Coamplification of IAC (552 bp) and DNA from *B. anthracis* isolates by multiplex PCR.
Lane M, GeneRuler DNA Ladder (MBI Fermentas); lanes 1–6, *B. anthracis* DRDE isolates; lane 7, *B. anthracis* Sterne; lane 8, recombinant *B. anthracis* pYS5; lane 9, *B. cereus* ATCC 13061; lane 10, *B. licheniformis* ATCC 12759; lane 11, *B. megaterium* ATCC 14581; lane 12, *B. thuringiensis* ATCC 10792; lane 13, *B. sphaericus* ATCC 4525.

antigens, respectively. The amplification of multiple rather
than a single *B. anthracis* DNA targets provides an increased
assurance of specificity [31]. In order to verify and evaluate
the specificity of the primers used in this study, PCR was
performed with DNA templates prepared from different
bacterial type strains including *B. anthracis* strains, as
shown in Table 1. The primers Ef-F and -R, Pa-F and -R,
Lf-F and -R, and Cap-F and -R produced an amplicon of
900 bp, 719 bp, 373 bp, and 807 bp for *cya*, *pag*, *lef*, 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 virulence-associated genes in 27 isolates of *B. anthracis*
along with other bacterial species. All the 27 isolates of *B.
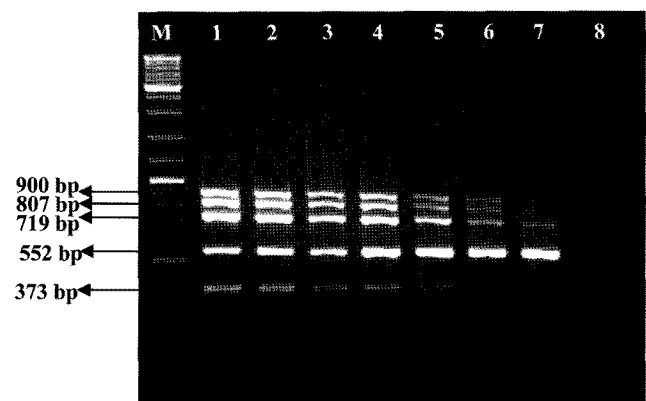anthracis* used in this study showed the specific amplifications

Fig. 2. Sensitivity test of multiplex PCR performed with primers
Ef-F and -R, Pa-F and -R, Lf-F and -R, Cap-F and -R, and the
purified DNA of *B. anthracis* isolate (DRDE-4) as well as IAC
DNA.
The PCR products were electrophoresed in a 2.5% agarose gel. Lane M,
GeneRuler DNA Ladder (MBI Fermentas); lanes 1 through 7, purified
genomic DNA serially diluted 10-fold from 1 µg to 1 pg; lane 8, sterilized
deionized water (negative control).

of all the four genes (Fig. 1), which shows that all the isolates were virulent. No amplification of the *cap* gene was observed in the Sterne strain of *B. anthracis*, which lacks the plasmid pXO2 (Fig. 1). There was no amplification of the *cap*, *lef*, and *cya* genes, except *pag*, in recombinant *B. anthracis* pYS5 (Fig. 1), which was originally deficient of both pXO1 and pXO2 plasmids, but has the *pag* gene inserted into a shuttle vector, pYS5. This shows that the mPCR targeting genes of pXO1 and pXO2 plasmids reported here can correctly identify virulent *B. anthracis*. 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 [12, 22]. The IAC was co-amplified with target DNA and showed an amplification of 552 bp (Fig. 1) in all the DNA samples isolated from bacterial cultures, as shown in Table 1. The detection limit of mPCR was 10 pg with genomic DNA (Fig. 2).

DNA Probes

Digoxigenin-11-dUTP-labeled DNA probes were made in the present study with the idea to be used as another tool for molecular identification. Radioactively labeled nucleic acid probes have been in use for a number of years for detecting and distinguishing bacteria [18]. Digoxigenin labeling and detection was developed because of the inherent problems of short shelf life of the isotopes and
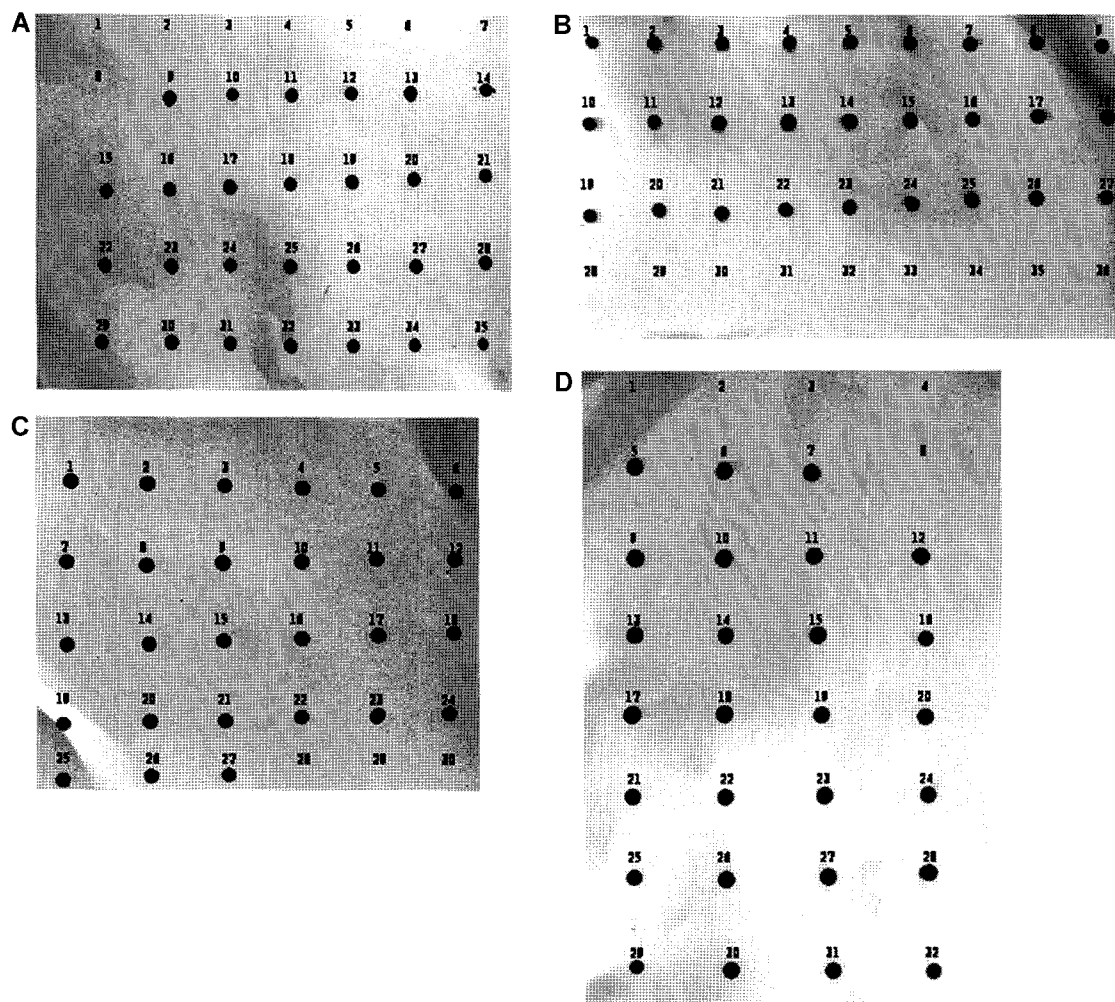

Fig. 3. Identification of *B. anthracis* by DNA probes.
A. Reactivity of dig-labeled *pag* probe. Spot 1, *B. cereus* ATCC 13061; 2, *B. licheniformis* ATCC 12759; 3, *B. megaterium* ATCC 14581; 4, *B. thuringiensis* ATCC 10792; 5, *B. sphaericus* ATCC 4525; 6, *B. circulans* ATCC 61; 7, *B. pumilus* ATCC 14884; 8, *B. subtilis* ATCC 11774; 9–35, *B. anthracis* isolates DRDE 1–27. B. Reactivity of dig-labeled *cya* probe. Spots 1–27, *B. anthracis* isolates DRDE 1–27; 28, *B. cereus* ATCC 13061; 29, *B. licheniformis* ATCC 12759; 30, *B. megaterium* ATCC 14581; 31, *B. thuringiensis* ATCC 10792; 32, *B. sphaericus* ATCC 4525; 33, *B. circulans* ATCC 61; 34, *B. pumilus* ATCC 14884; 35, *B. subtilis* ATCC 11774; 36, *Escherichia coli* ATCC 10536. C. Reactivity of dig-labeled *lef* probe. Spots 1–27, *B. anthracis* isolates DRDE 1–27; 28, *B. cereus* ATCC 13061; 29, *B. licheniformis* ATCC 12759; 30, *B. megaterium* ATCC 14581. D. Reactivity of dig-labeled *cap* probe. Spot 1, *B. cereus* ATCC 13061; 2, *B. licheniformis* ATCC 12759; 3, *B. megaterium* ATCC 14581; 4, *B. thuringiensis* ATCC 10792; 5-7 and 9–32, *B. anthracis* isolates DRDE 1–27, 8, *B. anthracis* Sterne.

safety problems associated with radioactive labeling and detection [2, 19, 33, 37]. Digoxigenin-labeled *cap, lef, cya,* and *pag* probes were used to determine the presence of virulent genes in all the 27 isolates of *B. anthracis* along with other bacterial species, as reported in Table 1. All the *B. anthracis* isolates showed reaction with the probes used (Fig. 3A, 3B, and 3C) except the *B. anthracis* Sterne strain, which did not show any signal for the *cap* gene (Fig. 3D) because of the absence of plasmid pXO2. No cross-reaction was observed with the other organisms listed (Fig. 3A, 3B, 3C, and 3D). The sensitivity of DNA probes was checked and it was found to be 300 pg. The results of DNA probes corroborated the findings of mPCR. DNA probes would be useful for the detection of *B. anthracis* in environmental samples such as dust, soil, water, sewage sludge, or feedstuffs in which there are large numbers of the closely related *B. cereus* organisms [13].

In conclusion, our results indicate that all the 27 isolates of *B. anthracis* from clinical and environmental samples contained all the virulence-associated genes and that all are virulent. Multiplex PCR and DNA probes can be used as a tool for the early identification of *B. anthracis* isolates as compared with the conventional methods that generally take 3–6 days. Both methodologies will contribute a lot in reducing the risk of laboratory-acquired infection and should be of great help as a routine test for confirmation of *B. anthracis* in clinical and environmental samples.

Acknowledgments

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