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Evaluation of a protective antigen gene based SYBR Green I real time PCR for detection of *Bacillus anthracis* in field samples

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ABSTRACT

In the present study, 27 field materials from different sources were tested in SYBR Green I dye based Real Time PCR optimized using WHO recommended conventional PCR primers targeting *Bacillus anthracis* protective antigen (PA) gene of pX01 plasmid. The assay had detection limit of 0.000002 pg in terms of target DNA quantity and up to 4 copies of pX01 plasmid per reaction, in terms of copy number. Melt curve analysis of PCR products of field samples showed deviation up to 3.2°C in melting temperature from standard anthrax strain suggestive of possible mutation/ mutations in PA gene. Targeted region of PA (596 bp) encompasses domain 2, involved in its binding with lethal factor (LF) and/or edema factor (EF) and mutation in this region may alter the efficacy of PA to traffic toxins inside the cells and results in differed level of virulence of pathogen. The test is useful not only for detection but may be suggestive of mutation also.

Key words: *Bacillus anthracis,* Sequence variation, SYBR Green PCR, Tm variation

Bacillus anthracis is a spore-forming gram-positive bacterium causing the disease anthrax, mainly in grazing animals and occasionally in humans. In naturally acquired infections, cutaneous mode of transmission is the most common due to handling of diseased animals or animal by-products. Death is rare in cases of cutaneous anthrax, but common in gastrointestinal or inhalation anthrax (Klietmann *et al.* 2001). Virulent strains of *B. anthracis* contain two virulence plasmids pX01 and pX02 providing specific targets for rapid detection of pathogen. The 182-kb pX01 plasmid harbors the structural genes for anthrax toxin proteins, namely edema factor (EF), lethal factor (LF), and protective antigen (PA). The 96-kb pX02 plasmid carries 3 genes required for capsule synthesis.

Hardy nature of its spores, its ability to persist in environment for decades and high lethality make it an ideal choice for use as a potent bioweapon. It was used as bioweapon in USA in 2001, where anthrax spores in powder form were deliberately released through postal envelops. In India also several powder filled envelopes were sent to politicians, which were tested in High Security Animal Disease Laboratory, IVRI, Bhopal, Madhya Pradesh by conventional methods and were found to be negative for *B. anthracis*. After these incidences need for a quick, efficient and reliable detection method for anthrax was felt. With the fear of bioterrorism in developing countries like India where grazing animals cow, sheep, goat etc. live in close proximity to the human population, the risk for transmission of pathogen to human increase many folds. Anthrax is endemic in several states of India including Tamil Nadu, Andhra Pradesh, Karnataka, Orissa, Gujrat and Madhya Pradesh, where at least 200 cases of human anthrax have been recorded since the 1950s (Kumar *et al*. 2000). To prevent rapid spread of infection in animal and human populations, rapid identification of the bacilli is stressed. Several conventional techniques are available for identification like isolation and identification on selective media, penicillin resistance, gamma phage sensitivity test etc., each having limitations such as time consumption, presence of antibiotic resistant strains and requirement of vegetative form of bacterium. Several conventional PCR assays have been described for the identification of *B. anthracis* (Beyer *et al*. 1999).

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Recently, real time PCR has gained popularity due to the intrinsic benefits such as quick amplification and simultaneous detection of target nucleic acids, quantitative accuracy, single-copy sensitivity and a high level of specificity, along with other benefits such as low hands on time and reduced chances of cross contamination while handling large number of samples. In the present study, a SYBR Green I based real time PCR using WHO recommended primers, which are intended for conventional PCR targeting the main virulence plasmids, pXO1 and pXO2, was optimized and evaluated for detection of *B. anthracis* in field samples.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Reference *B. anthracis* strain was obtained from Division of Biological Standardization, IVRI, Izatnagar, Bareilly, UP, India. *B. .mycoides* (MTCC 3036)*, B. thuringiensis* (MTCC 6941) and *B. cereus* (MTCC 7166) strains were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. All bacterial strains were grown in nutrient broth containing 2% fetal calf serum at 37°C with shaking at 200 rpm for 16 h.

Samples: A total of 27 field samples including soil (16), tissue (3), blood (7) and bone (1), sent to the laboratory for testing of *B. anthracis* were tested in the study along with the members of *B. cereus* group *(B. mycoides, B. thuringiensis* and *B. cereus*).

DNA isolation: Each field sample (100 mg or 100µl) was inoculated in 5 ml nutrient broth supplemented with 2% FCS and incubated at 37°C for 16 h. Reference strain was cultured from a well isolated colony from sheep blood agar. Five ml of bacterial culture (reference, field sample and member of *B. cereus* group) was used to isolate DNA using DNA purification system as per the protocol described by manufacturer. Finally, DNA was eluted in 200µl of TE buffer and quantified in spectrophotometer.

Oligonucleotide primers used in study: WHO recommended primers (WHO 1998) for detection of *B. anthracis* were used in SYBR Green I based real time PCR assay (Table 1).

Standards for SYBR green assay: Conventional PCR products were generated from reference bacterial DNA template using primers targeting PA and capsule genes. PCR products were cloned into pGEM-T-easy TA cloning vector. Recombinant plasmids purified using DNA purification systems were used as template to evaluate primer sets for real time PCR. PA amplicon was sequenced using automated sequencer.

Assay conditions and primer evaluation: Both the pairs of primers intended for conventional PCR were evaluated in SYBR green based real time PCR. Each 25µl real time PCR reaction mixture contained 1X Brilliant SYBR green PCR master mix, 30 nmoles Rox dye, upstream and downstream primers (ranging 20, 15, 10, 8, 5 pmoles) and 25 ng of template. All reactions were carried out in duplicates in a MX-3000*P* real time PCR machine. No template control (NTC), no primer control (NPC) and buffer controls were also included in the study. Cycling conditions involved initial activation of Surestart *Taq* polymerase, bound with antitaq antibody at 95°C for 10 min, followed by 35 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 30 sec. After completion of 35 cycles, melt curve analysis was carried out by gradual increment of temperature from 50°C to 95°C and melting temperature (Tm) of all amplicons were optically read with an increment of 0.3°C. Ability to generate a specific amplification curve along with a single specific Tm product was considered as the qualifying parameters for selection of primer set. Selected primer set was tested for its specificity using bacterium of related *B. cereus* group *(B. mycoides, B. thuringiensis* and *B. cereus*) and unrelated pig tonsillar DNA.

Plotting standard curve and copy number calculation: Serial ten-fold dilutions ($\log 10^{-1}$ to $\log 10^{-12}$) of recombinant PA gene construct were made in TE ($T_{10}E_1$) buffer to make concentrations ranging from 2×10^4 pg to 2×10^{-7} pg and were used as template to construct standard curve. Copy number was determined by the following formula.

$$\text{Copy number/µl} = \frac{\text{Concentration of plasmid in g/µl} \times 6.023\times 10^{23}}{\text{Construct length (bp)} \times 660}$$

where the construct length is 3621 bp (3025 bp vector size + 596 bp PA amplicon size).

In the standard curve, copy number of each dilution was plotted against Ct value. Limit of detection was determined as the concentration or copy number of the lowest dilution giving Ct value and Tm specific to the expected product.

Field sample testing: Twenty-seven field samples obtained from different sources were tested by the optimized assay using selected primer set. The PCR products were subjected

Table 1. Oligonucleotide primers used for the study

Primer ID	Sequence 5′→3′	Target region	Amplicon size	Reference
PA5	TCCTAACACTAACGAAGTCG	Protective Antigen (pXO1)	596 bp	WHO (1998)
PA8	GAGGTAGAAGGATATACGGT			
CAP F	CTGAGCCATTAATCGATATG	Capsule (pXO2)	846 bp	WHO (1998)
CAP R	TCCCACTTACGTAATCTGAG			

to melt curve analysis using in-built MX3000*P* software to assess their specificity.

RESULTS AND DISCUSSION

Evaluation of primer set for PA: Primer set PA5 and PA8 consistently produced single specific melt peak at 78.8°C even with variable template quantity for the standard template and was devoid of primer dimer (Fig. A). Eight picomoles of primers were found to be the optimum quantity per reaction.

Evaluation of primer set for capsule: Primer set for capsule showed multiple melt peaks and variation in template quantity resulted in shift in melt peak, and hence found unsuitable for use in SYBR green I based real time PCR.

Specificity of selected primer set: Specificity of primer set PA5 and PA8 was determined by evaluating its ability to generate single definitive product in presence of pathogen. Melt curve analysis for *B. anthracis* showed a specific Tm peak with high fluorescence value; whereas with other members of *B. cereus* group i.e., *B. cereus*, *B. mycoides*, *B. thuringiensis* and pig tonsillar DNA, non specific amplicons with imprecise Tm were generated.

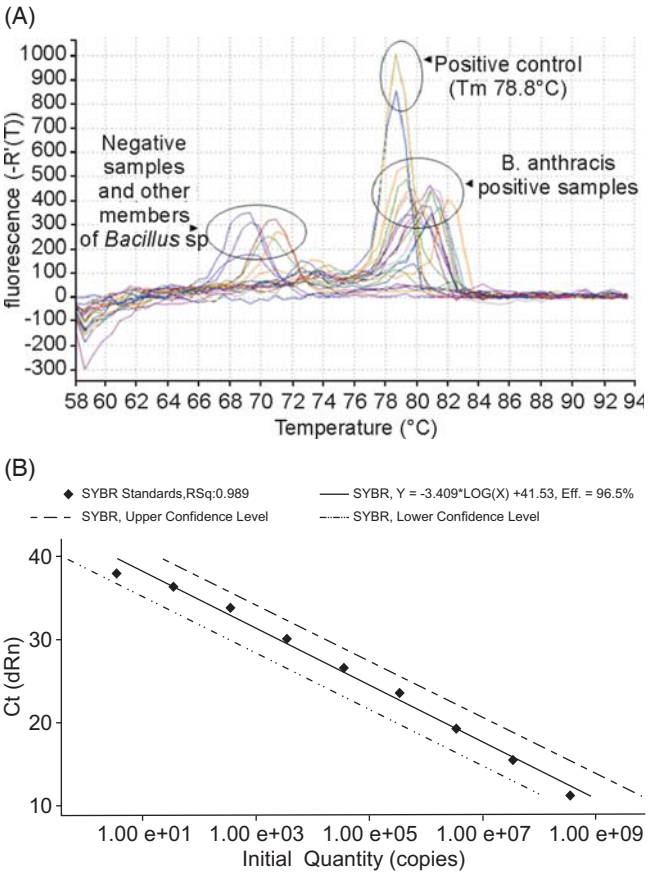

Fig A–B. **A.** Dissociation curve showing the melt peak of PA clone and reference DNA resulted in same amplicon Tm (78.8°C). Field sample representatives showing deviation in melting temperature and positive samples clustered within range of 0.5°C to 3.2°C from the reference, whereas negative samples Tm was clustered between 68°C and 71°C. **B.** Standard curve for PA gene with 95% confidence interval.

Table 2. Limit of detection and copy number

Dilution	Quantity of DNA	Mean Ct value	Standard deviation	Copy number	Tm
$log10^{-1}$	2×10^4 pg	-	-	3.47×10^{10}	-
$log10^{-2}$	2×10^3 pg	-	-	3.47×10^9	-
$log10^{-3}$	2×10^2 pg	11.20	0.26	3.47×10^8	78.8
$log10^{-4}$	2×10^1 pg	15.55	0.15	3.47×10^7	78.8
$log10^{-5}$	2 pg	19.32	0.28	3.47×10^6	78.8
$log10^{-6}$	2×10^{-1} pg	23.66	0.27	3.47×10^5	78.8
$log10^{-7}$	2×10^{-2} pg	26.63	0.44	3.47×10^4	78.8
$log10^{-8}$	2×10^{-3} pg	30.07	0.27	3.47×10^3	78.8
$log10^{-9}$	2×10^{-4} pg	33.82	0.34	3.47×10^2	78.8
$log10^{-10}$	2×10^{-5} pg	36.32	0.4	3.47×10^1	78.8
$log10^{-11}$	2×10^{-6} pg	37.90	0.29	3.47	78.8
$log10^{-12}$	2×10^{-7} pg	-	-	-	-

Standard curve: Initial dilutions of standard template ($log10^{-1}$ and $log10^{-2}$) produced no Ct value, probably due to the presence of excess template. The standard curve for PA gene showed linearity with a slope value of –3.409 and R^2 value 0.989 (Fig. B).

The relationship among quantity of DNA, mean Ct value and copy number at different dilutions of standard clone are given in Table 2. The limit of detection was found to be 2×10^{-6} pg (0.000002 pg). In terms of copy numbers, the detection limit was 3.47 (roughly 4) copies of target DNA.

Detection of B. anthracis *in field samples:* Out of 27 field samples tested by SYBR green based real time PCR, 18 samples had amplification curves with a product Tm values within 0.5°C (sample ID 5130 and 25127) to 3.2°C (sample ID 29150) of the standard and all the products were seen as clear visible band at 596 bp when subjected to agarose gel electrophoresis. The remaining samples either represented no Tm (2146, 5730, 24027) or a small peak [fluorescence value (-R' (T)) less than 200] with a Tm of 70°C (sample 25125, 2135, 2144, 24028, 2137 and 2029) (Fig. A). Tm values of field samples along with the copy number are given in Table 3. No ambiguity in melt peak of PA clone and total DNA of standard strain eliminated the risk of culturing bacteria to serve for positive control.

The zoonotic nature, potential of *B. anthracis* for use as an agent of bioterrorism and ability to persist in environment for decades necessitates the development of rapid and reliable detection method. Several conventional detection methods are available for detection of the pathogen including selective staining, antibiotic resistance, phage lysis, growth on selective media etc. which encounter problems of high time consumption, lower sensitivity, specificity and reliability. Polymerase chain reaction (PCR) is one of the commonly used methods for nucleic acid amplification of different markers of *B. anthracis* located on pX01 (targeting PA, LF

Table 3. The Tm of field samples in real time PCR and copy number

Sample ID	Material	Tm	Ct value	Copy No.
2139	Soil	81.00	30.19	5.299E+03
2141	Soil	81.00	30.36	4.667E+03
23963	Tissue	81.00	29.21	1.101E+04
2147	Soil	81.00	28.94	1.345E+04
2143	Soil	81.00	27.66	3.504E+04
2138	Soil	79.90	26.26	9.966E+04
24029	Blood	81.60	30.33	4.773E+03
5130	Bone	79.30	25.79	1.415E+05
2140	Soil	79.90	24.77	3.031E+05
2029	Soil	70.50	34.72	–
2136	Soil	81.00	26.44	8.712E+04
25129	Soil	81.60	27.59	3.692E+04
2146	Blood	–	–	–
25127	Blood	79.30	31.71	1.703E+03
25128	Blood	79.90	30.43	4.430E+03
2137	Soil	69.90	33.16	–
29149	Tissue	79.90	35.31	1.159E+02
2148	Soil	81.00	32.71	8.074E+02
29150	Tissue	82.00	32.17	1.208E+03
25126	Blood	80.40	27.71	3.375E+04
5121	Blood	81.00	25.00	2.53e+005
24027	Blood	–	–	–
5730	Soil	–	–	–
24028	Soil	70.50	32.4	–
2144	Soil	69.30	34.4	–
2135	Soil	71.00	33.2	–
25125	Soil	69.30	29.7	–
B. cereus	MTCC 7166	68.70	–	–
B. mycoides	MTCC 3036	–	–	–
B. thuringiensis	MTCC 6941	–	–	–
Pig DNA		69.30	31.9	–
B. anthracis Standard strain		**78.80**	16.60	1.351E+08
Positive control		**78.80**	11.81	4.830E+09
NTC		–	–	–
NPC		–	–	–
Buffer control		–	–	–

or EF genes), pX02 (targeting capsule) and/or genomic markers (targeting BA813, rpoB, gyr, 16S rRNA etc., Beyer *et al*. 1995). WHO has recommended PCR based amplification and confirmation for identification of pXO1 (PA), pXO2 (capsule) and genomic markers (S layer gene). Conventional nucleic acid amplification based methods however require a further confirmation step of electrophoresis. Real-time PCR instruments permit rapid PCR cycling and simultaneous detection of amplification product. Furthermore, PCR and product detection are performed in the same closed PCR tube, which reduces the risk of cross contamination related to the biosafety concerns. There is also an advantage of low hands on time that enables processing of large number of samples, which is extremely important during control programs at time of outbreaks. The anthrax bacterium devoid of both the virulence plasmid is non virulent, hence in the present study, conventional PCR primer sets recommended by WHO recommended targeting pXO1 and pXO2 were evaluated. Out of the two, the primer set targeting capsule was found to be unfit because their product melt curves showed multiple peaks with variable Tm, which varied with template quantity, probably owing to the larger amplicon size and formation of internal melting stretches. The other primer pair targeting PA gene produced desirable single melt peak and all positive samples showed a definitive Tm value, hence SYBR Green I based real time PCR assay for *B. anthracis* was optimized using primers PA5 and PA8. It produced a 596 bp amplicon which encompasses amino acid residues from 215 to 418 that is a part of domain 2 (259aa-487aa) of PA and involved in the binding of LF/EF (Petosa *et al*. 1997). Mispriming in the assay was minimized by using the 2X SYBR green QPCR master mix, containing Surestart Taq DNA polymerase premixed with neutralizing monoclonal antibody to the polymerase that requires hot start for release of the antibody and activation of polymerase and reduce nonspecific amplification. The standard clone of PA gene had the same Tm (78.8°C) as that of reference strain DNA, indicating that the clone is comparable with the whole DNA preparation and suitable as positive control. It eliminates the requirement of whole genomic DNA preparation from reference strain and thus minimizing the risk associated with handling of this pathogenic bacterium. SYBR green I based assay represents a more economical alternative for specific fluorescent probes based assays for screening and quantification of specific target in large number of samples without compromising the sensitivity.

By comparing the Ct value obtained with a particular concentration of standard DNA in standard curve, the concentration of target in an unknown sample may be extrapolated. Limit of detection was determined as the minimum quantity/copies of DNA which gave products with specific Tm. The minimum detection limit was 0.00002 pg of target DNA, which is greater than detection limit 0.0075 fg, reported previously for anthrax bacterium surrogate

organisms (Saikaly *et al*. 2007). The greater sensitivity may be attributed to the cycler used for real time PCR, cycling conditions, DNA preparation methods and reagents used in the study. In terms of copy number, the limit of detection was 3.47 to 34.7 (roughly 4) copies of target DNA, greater than found for 9.6 copies for PRRSV (Tian *et al*. 2010) and 136 copies of HIV in SYBR Green assay (Mehta *et al*. 2009). Within a single bacterium the copy numbers of bacterial plasmids differ and determine the level of toxicity. It may vary from several to 243 (Coker *et al*. 2003). Thus it is possible to detect the presence of even a single bacterium equivalent to detection of one genome copy of lactococcal bacteriophages from milk and whey (Huong *et al*. 2011). No significant statistical difference among the result from SYBR green assay and Taqman based assay was reported by Medina (2011), while detecting H1N1 NA signatures.

The Tm of amplified product of standard IVRI control was 78.8°C. The nucleotide sequence analysis of PA5/8 amplicon compared with the sequence available in GENBANK and no mutation was found in the targeted region of reference strain. In the present study, 27 field samples were assessed in real time PCR. The Tm of the 18 positive samples differed from standard by 0.5°C to 3.2°C and the products of expected size were visible in agarose gel. The Tm of an amplicon depends on its GC content, length, and sequence characteristics. It can be speculated that despite its asexual mode of reproduction and tendency to form endospores, which protects DNA from altering stresses like phages and/or UV radiations, the DNA sequence, may not be conserved among different strains of *B. anthracis*, as it was previously considered and present data is supported by the results of VNTR analysis also where genotypic variations at 18 different chromosomal loci were characterized in 5 known anthrax vaccine strains (Kuznetsovskii *et al*. 2009). To distinguish the Sterne strain positive control with the wild Italian strain of *B.anthracis*, the shift in Tm was used (Oggoni *et al*. 2002). In other unrelated studies, difference in Tm was used to identify different strains (Norwalk virus; Menton *et al*. 2007). Melt-curve analysis of the PCR products resolved the amplified products into individual peaks depending on their Tm values. Therefore the difference in Tm could probably represent the presence of point mutations. Presence of 5 point mutations in 2294 bp long PA gene have been reported earlier in a study of 26-sample survey in victims of the 1979 Sverdlovsk incident (Price *et al*. 2001).

The region targeted in the PA gene in the present study for diagnosis particularly becomes important as it encompass major part of the domain 2 (amino acid residues 215 to 418) of PA gene. Domain 2 of PA is implicated in membrane insertion and channel formation. Domain 2 has been shown to have a chemotrypsin sensitive region critical in the translocation process of lethal factor and edema factor. Thus, any change in the sequence will have a modulating effect on toxin translocation capacity of PA. It has been shown that, most of the mutations, which may abolish the activity of PA, were also found mainly located in domain 2 (Mourez *et al*. 2003). Some of these mutations have "dominant negative effect", i.e. as little as one mutation per haptamer is sufficient enough to block the whole process of internalization. The nucleotide sequence of standard PA domain 2 was compared with other *B. anthracis* sequences available in GENBANK and no mutation was found to be present in the domain 2 region in any of the strains studied. Though the standard reference strain does not contain any mutation in the PA domain 2, targeted for SYBR green I assay, the presence of mutations in tested field material is suspected due to the shift in Tm of field samples (0.5 – 3.2°C) as determined by the melt curve analysis. However, furthers studies are required to assess the correlation among nucleotide sequence, shift in product Tm and pathogenesis. This may be useful at time of disease outbreak or bioterrorism attacks for quick as well as simultaneous detection and determination of virulence of *B. anthracis* strain within the same closed PCR tube as a part of emergency preparedness for time bound and dependable diagnosis.

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