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Pag gene based phylogenic characterization of *Bacillus anthracis* from sheep

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ABSTRACT

We report the protective antigen gene based molecular epidemiology of a massive anthrax outbreak in sheep that killed over 100 sheep. The outbreak was unique since the use of higher antibiotics immediately after the live anthrax vaccine had facilitated wide spread of the disease and unscientific disposal of carcasses had precipitated the disease magnitude. *B. anthracis* was isolated from ear blood samples collected from dead sheep. The isolates were non-motile, non-hemolytic and were pathogenic to mice. Gene coding for the toxic factor 'protective antigen' was targeted for PCR amplification as per WHO and OIE protocols. Sequencing of nucleotides on the conserved *pag* gene showed that the *B. anthracis* isolated during this study shared a sequence identity of more than 99.9% with *B. anthracis* isolates from different species, including human beings. The phylogeny demonstrated the genetic stability of the immunologically protective *pag* gene across species.

Key words: Anthrax, *pag* gene, PCR, pXO1 plasmid, Sheep

Anthrax caused by *Bacillus anthracis* is an acute bacterial disease primarily of herbivores and is transmissible to humans. Animals become infected mostly by ingesting soil-borne spores while grazing. *B. anthracis* is the only obligate pathogen within the genus *Bacillus.* Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although some are occasionally associated with food poisoning and other clinical manifestations in both humans and animals (Quinn *et al.* 2011, Chandranaik *et al.* 2015).

Pathogenic *B. anthracis* carries two plasmids, the plasmid pX01 with the toxin genes and the plasmid pX02 with the genes for capsule synthesis. The capsule confers resistance to phagocytosis. The anthrax toxin is composed of 3 proteins; lethal factor, edema factor and protective antigen (pag). The increasingly frequent isolation of avirulent *B. anthracis* from humans and animals has made specific detection of *B. anthracis* by PCR as a conclusive diagnostic test for anthrax (Berg *et al.* 2006). No reports are available on genetic similarities or variations between *B. anthracis* isolates from different susceptible species. We report the isolation of *B. anthracis* from a massive anthrax outbreak in sheep at Bellary, Karnataka, India that took toll of over 100 sheep; and its characterization by protective antigen gene based phylogenetic analysis.

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MATERIALS AND METHODS

History of outbreak and collection of samples: Devasamudra village in Bellary district, Karnataka in India, with 4,905 sheep and 865 goats, witnessed a massive anthrax outbreak in sheep. The sheep and goats in the village were previously unvaccinated against anthrax. The first case of anthrax was reported on 24 Aug 2015, and the entire population was vaccinated with live anthrax vaccine on 25 Aug 2015 and booster dose was given to all susceptible animals on 15 Sep 2015. However, in anticipation of further deaths due to the disease, animals were treated with higher antibiotics for more than 15 days, from the day of appearance of the first case of anthrax. Deaths due to the disease continued even after 2 doses of vaccine. By the end of September, the village had lost more than 400 sheep. Blood samples were collected from ear veins of 72 dead sheep for laboratory examination. The carcasses were not opened for post-mortems. The farmers had thrown the carcasses of sheep that died of anthrax, in open fields of grazing and agricultural lands where the village sheep routinely go for grazing.

Growth and characterization of B. anthracis: Thin blood smears made out of the ear blood collected from the carcasses were stained with polychrome methylene blue for initial identification. For cultural examination, the blood

samples were inoculated in culture media as per standard microbiological methods on various agars, including nutrient-agar with 0.7% $NaHCO_3$ under 10% CO_2 and gelatin stab culture.

The *B. anthracis* isolates were characterized by standard microbiological methods (Quinn *et al.* 2011). Motility test was done by microscopic observation of wet mounts of cells grown in soybean casein digest broth. Capsule staining of cells was performed using India ink on cells incubated at 30° and 37°C in heart infusion broth supplemented with 0.8% sodium bicarbonate and 50% horse serum (Chandranaik *et al.* 2015).

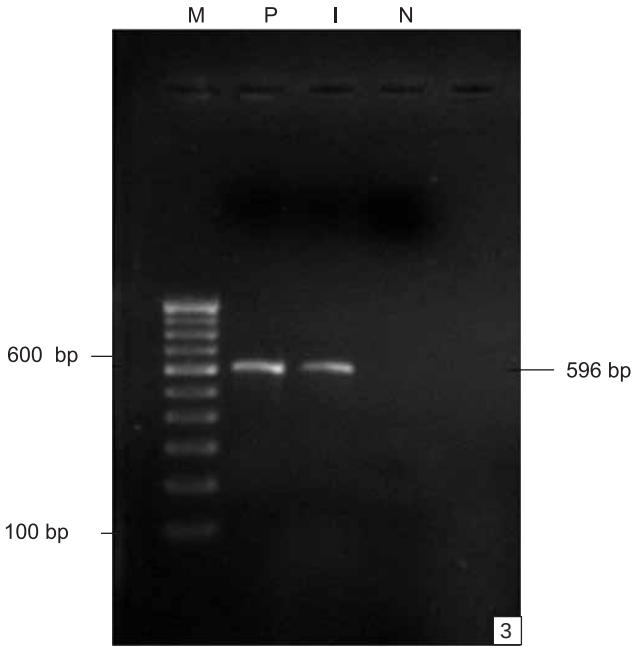
Mice pathogenicity test: Spores for the mouse challenge experiments were produced and inoculated to mice by intraperitoneal route (De *et al.* 2002). The experiments were conducted in accordance with the institutional animal ethics committee regulations. Six-week-old, inbred, Swiss albino mice were housed in cages and provided food and water *ad libitum*.

Polymerase chain reaction: Genomic DNA and plasmid DNA were isolated from the disrupted cells using extraction kits following the protocol provided by the manufacturer. Primer sequences and PCR protocols approved by WHO for amplifying the conserved region of virulent *pag* gene on pXO1 plasmid of *B. anthracis* (Inoue *et al.* 2004, OIE 2008) were used for confirmation and subsequent phylogenetic analysis. The primers for amplification of 596 bp sequences on pXO1 were: Forward primer 5'TCC-TAA-CAC-TAA-CGA-AGT-CG 3'; Reverse primer 5'GAG-GTA-GAA-GGA-TAT-ACG-GT 3'. PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 0.2 mM of each dNTP, 20 pmol of each primer, 2.5 U of Taq DNA polymerase, and approximately 1 ng template DNA in a 100 µl total reaction volume. Template DNA was initially denatured by heating at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 0.5 min, annealing at 55°C for 0.5 min, and primer extension at 72°C for 0.5 min. Incubation for 5 min at 72°C was followed to complete the extension. DNA extracted from *B. anthracis* was included in each set of PCR reactions. PCR amplicons were analyzed by electrophoresis through 2% agarose gel.

Sequencing and phylogenetic analysis: The PCR amplified products were extracted from agarose gel and eluted in 25 µl of nuclease free water using gel extraction kit and subjected for nucleotide sequencing. The nucleotide sequences were aligned with published sequences in GenBank. Phylogenetic tree was constructed and sequence analysis was performed in MEGA version 6.2 software, using neighbour joining method.

RESULTS AND DISCUSSION

The symptoms of sudden death with oozing of unclotted blood from mouth, nostrils, eyes (Fig. 1) and other natural orifices noticed were pathognomonic of anthrax. Absence of rigor mortis was characteristic in all cases. The unscientific disposal of anthrax-infected sheep carcasses on grazing lands (Fig. 2) and agricultural fields, where the sheep in the village routinely graze, had possibly facilitated the rapid spread of the disease, killing more than 100 sheep in the village. Ear blood samples of dead sheep when stained with polychrome methylene blue indicated presence of *B. anthracis* with characteristic McFadyeans staining reaction. Pure cultures of *B. anthracis* were recovered from blood samples. The *B. anthracis* isolates were non-hemolytic and non-motile. The isolates produced similar colony



Figs 1–3. 1. Symptoms of oozing of unclotted blood from mouth, nostrils and eyes. **2.** Remains of unscientifically disposed sheep carcass on grazing land. **3.** PCR yielding specific amplification of 596 bp conserved *pag* gene of *Bacillus anthracis*. Lane M, 100 bp DNA ladder; lane P, positive control; lane 1, *Bacillus anthracis* IAHVB 2016 isolate; lane N, negative control.

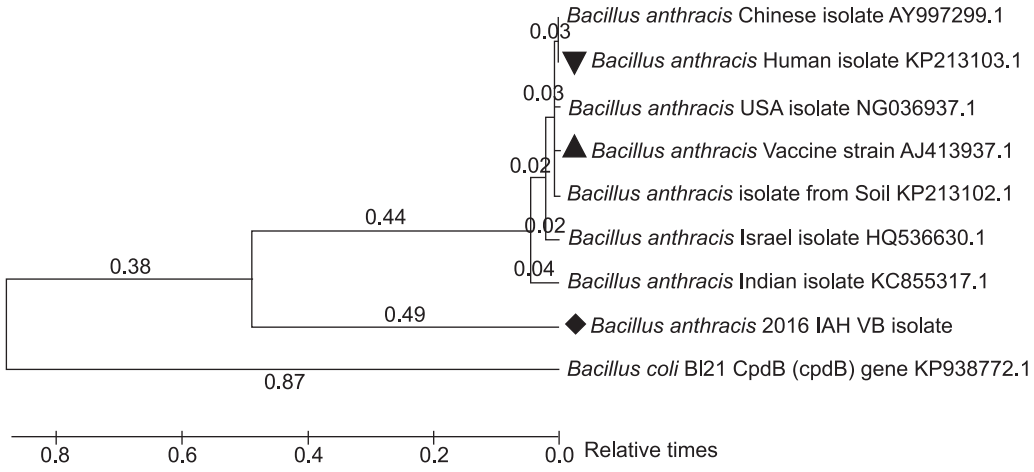


Fig. 4. Phylogenetic analysis of the *pag* gene nucleotides of *Bacillus anthracis.*

morphologies of about 5 mm diameter, flat, dry, grayish with ground-glass appearance and were positive for capsule by India ink staining. The colonies on nutrient agar with 0.7% $NaHCO_3$ under 10% CO_2 appeared mucoid; and the gelatin stab culture showed an inverted fir tree appearance of growth. In mice pathogenicity tests, the clinical isolates induced mortality of mice within 6 h of inoculation.

The PCR used for the detection of conserved *pag* gene sequences on pXO1 plasmid yielded a specific amplification of 596 bp (Fig. 3), in all clinical isolates obtained during this study. The phylogenetic analysis of the *pag* gene nucleotides using MEGA software tool under relative time basis with precision of 0.02, showed that the isolate had the nucleotide sequence identity of more than 99.9% and had sequence divergence of 0.05 with *B. anthracis* isolates from human, cattle and the anthrax vaccine strain deposited in the GenBank (Fig. 4).

All the animals were vaccinated again with a booster dose, with a strict vigilance to avoid antibiotics prior to vaccination and a few days post-vaccination. Steps were taken to scientifically bury the carcasses thrown on the agricultural field and the new deaths occurring due to the disease. Grazing of the sheep was totally banned in areas where the carcasses were thrown. The outbreak was completely stopped and no further deaths were reported.

The symptoms noticed in the dead animals during this study were classical symptoms of anthrax in herbivores and were in accordance with previous descriptions of anthrax in sheep and cattle (Venkatesha *et al.* 2006, Quinn *et al.* 2011). Phenotypic characters exhibited by *B. anthracis* isolates obtained during this study were in concurrence with the findings of Venkatesha *et al.* (2006), Jula *et al.* (2011) and Chandranaik *et al.* (2015). The large scale unscientific disposal of sheep carcasses that died of anthrax, on grazing land had resulted in abundant presence of *B. anthracis* bacteria and/or its spores in the surrounding area. Use of higher antibiotics over a long period of time, concurrent with live vaccine has possibly led to vaccination failure. A combination of high load of pathogenic bacteria on the grazing land and vaccination failure had resulted in wide spread of the disease with continued deaths even after 2 rounds of vaccination.

The PCR results yielding 596 bp specific amplification on the conserved region of *pag* gene was in accordance with protocols approved by WHO and OIE for confirmation of anthrax (Inoue *et al.* 2004, OIE 2008). Strong genetic stability of the immunogenic *pag* gene of the *B. anthracis* isolates from different regions of the world covering different susceptible species including humans was evident through this study. Further, control of a massive anthrax outbreak in sheep using a live vaccine made out of *B. anthracis* isolated from cattle further confirmed the genetic stability of the immunogenic *pag* gene across species, which is evident in the phylogenetic analysis of the current isolate.

Microbial virulence determinants are often under the control of intricate global regulatory networks. In many cases, a number of genes required for virulence are activated by one or more common environmental signals. The virulence of *B. anthracis* is attributed to plasmid encoded virulence factors (Hu *et al.* 2009, Klee *et al.* 2010). The complex toxin consists of three components; protective antigen (PA), edema factor (EF) and lethal factor (LF). Individually, each factor has no toxic activity and they act only in combinations. The protective antigen is very crucial because it is a binding moiety for both EF and LF, and PA is immuno-dominant and produces protective antibodies in the host (Friedlander 2001, Ulrike *et al.* 2006). Considering these factors, we selected to target the *pag* gene for PCR and subsequent phylogeny.

With reports of soil bacteria *Bacillus cereus* acquiring virulent anthrax plasmids by horizontal transfer in contaminated soil (Chandranaik *et al.* 2015), it is important to educate farmers on regular anthrax vaccination and scientific disposal of the anthrax affected carcasses, taking into account not only the classical anthrax caused by *B. anthracis* but also 'anthrax like' disease caused by *B. cereus.*

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