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Molecular characterization and phylogenetic analysis of *Bacillus anthracis* isolates from anthrax outbreaks in Karnataka, India

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Abstract
The study investigated anthrax outbreaks in Karnataka by isolating and characterizing *Bacillus anthracis* from livestock suspected cases across different districts. Ten samples, including blood, spleen, and ear tissues, were collected from animals and processed using standard culture techniques on BHI, PLET, and blood agar to obtain characteristic colonies. Gram staining revealed Gram-positive rod-shaped cells arranged in chains, confirming preliminary identification. Molecular confirmation was achieved through PCR amplification of the pXO2 gene, yielding consistent 846 bp amplicons across all isolates, thereby validating the presence of virulent plasmids. The purified PCR products were sequenced, and phylogenetic analysis was performed using MEGA software with the Neighbor-Joining method and Tamura-Nei model. The results demonstrated that Karnataka isolates clustered distinctly together, forming a clear lineage separate from global reference strains, suggesting localized lineage adaptation and limited gene flow with external populations. Minor intra-cluster variations indicated micro evolutionary changes within the South Indian strains. The study concludes that *Bacillus anthracis* strains circulating in Karnataka exhibit strong genetic relatedness highlighting the importance of continuous molecular surveillance and phylogenetic monitoring to understand transmission patterns and to reform local anthrax control strategies.

Keywords: *Bacillus anthracis*, pXO2, phylogentic analysis, ovine, PCR, Karnataka India

Introduction
Anthrax is known as one of the oldest zoonotic diseases, with records of it going back to ancient civilizations like Egypt and Mesopotamia around 1500 BCE [1]. Descriptions from ancient Greece and Rome also match the signs of anthrax affecting both animals and humans [1, 2]. The bacterium responsible, *Bacillus anthracis*, is a rod-shaped, spore-forming Gram-positive microorganism that mainly infects grazing animals such as cattle, sheep, and goats, though wild animals can be affected as well [3, 4].

For detecting *Bacillus anthracis*, molecular methods like PCR-based assays have proven to be fast and specific [5]. These tests target important genes located on both the chromosome and plasmids, including rpoB, pag (pXO1), and cap (pXO2), with the plasmid genes playing key roles in anthrax's ability to cause disease [6, 7]. However, identifying *B. anthracis* precisely is challenging because it is closely related genetically to species like *B. cereus* and *B. thuringiensis*, which may sometimes carry similar plasmids through horizontal gene transfer [8, 9]. Thus, molecular tests often include chromosomal markers along with plasmid targets to accurately distinguish *B. anthracis*.

Phylogenetic studies have grouped *Bacillus anthracis* into distinct lineages and sub-lineages using genetic markers like multiple-locus variable-number tandem repeats (MLVA) and canonical single nucleotide polymorphisms (canSNPs). These analyses help trace the bacterium's evolutionary background and how it spreads across different regions [10]. Such knowledge is important for understanding anthrax's epidemiology, strain diversity, and how it transmits both globally and within specific locales.

In Karnataka, India, anthrax outbreaks among livestock occur repeatedly. Examining the molecular characteristics of the strains circulating locally provides important insights into how these bacteria are related and how they evolve over time within these endemic areas.

This study focuses on molecularly identifying *Bacillus anthracis* isolates from Karnataka outbreaks by PCR targeting the pXO2 gene and conducting phylogenetic analyses. These local isolates are compared with reference strains from other parts of the world to better understand their genetic relationships and potential transmission routes.

Materials and Methods

Collection of samples from field outbreaks

As part of the field investigations, ten samples comprising blood, spleen, and ear tissues were collected from anthrax-suspected animals across various locations in Karnataka, as detailed in Table 1. Samples were collected in a leak proof zip-lock bag/container, double bagged and labeled clearly, and transported to the laboratory under refrigerated conditions to ensure sample integrity. Once received at the laboratory, the samples were stored at 4 °C. All the diagnostic confirmation tests were achieved using culture, staining, and molecular methods for detection of *Bacillus anthracis*.

Table 1: Details of sample collected from anthrax suspected cases.

Sl. No.	Sample Number	Sample	Species	Place
1	Ax-1	Ear Piece	Ovine	Ballari
2	Ax-2	Spleen	Ovine	Ballari
3	Ax-3	Ear Piece	Ovine	Ballari
4	Ax-4	Ear Piece	Ovine	Sira Tumakuru
5	Ax-5	Ear Piece	Ovine	Ballari
6	Ax-6	Blood	Ovine	Gadag
7	Ax-7	Ear Piece	Ovine	Sira Tumakuru
8	Ax-8	Ear Piece	Ovine	Tumkur
9	Ax-9	Ear Piece	Ovine	Ballari
10	Ax-10	Ear Piece	Ovine	Ballari

Isolation and identification of *Bacillus anthracis* from tissue samples Isolation of *Bacillus anthracis*

Bacillus anthracis was cultured on both Brain Heart Infusion (BHI) agar and PLET agar. Blood agar served as the diagnostic medium, while PLET agar was the selective medium. A loop full of the sample taken from the ear piece/blood sample was first inoculated onto BHI agar and incubated for 24 hours at 37 °C. Colonies identified as *Bacillus anthracis* by Gram staining were then transferred from BHI agar to PLET agar for pure culture and to reduce contamination.

Identification of *Bacillus anthracis*

Colony morphology

The growth in BHI agar, PLET agar and blood agar was observed for characteristic colony morphology of *Bacillus anthracis* and also its microscopic appearance.

Cellular morphology

Gram's staining was carried out as a standard procedure to observe Gram-positive rods characteristic of *Bacillus anthracis*.

Further, DNA was extracted from the bacterial isolates using the phenol-chloroform method. The extracted DNA was used as the template for polymerase chain reaction (PCR) amplification targeting the pXO1 gene. The PCR amplification was carried out using specific primers designed for the pXO2 gene, and the resulting amplicons were visualized by agarose gel electrophoresis. The PCR product obtained was purified and sent for sequencing.

Table 2: Oligonucleotide primers used for amplification of pXO2 genes of *Bacillus anthracis*

Name of the Primer	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
pXO2 (CAP)	F: CTGAGCCATTAATCGATATG R: TCCCACTTACGTAATCTGAG	846	[11]

Table 3: Thermal cycling conditions amplification for pXO2 (CAP) gene

Primers	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
pXO2 (CAP) F/R	95 °C, 5 min	95 °C, 30 sec	55 °C,	72 °C,	72 °C, 5 min
		Repeated for 30 cycles			

Phylogenetic analysis

PCR amplification products corresponding to the pXO2 gene were purified and sequenced. The obtained nucleotide sequences from nine *Bacillus anthracis* isolates originating from anthrax outbreak samples in different regions of Karnataka were used for phylogenetic analysis. These sequences were compared with reference strains of *Bacillus anthracis* from India and other countries, including isolates from both wild and domestic animals as well as standard reference strains (Table 4). Bacillus cereus sequence was included as the outgroup to root the phylogenetic tree and establish comparative lineage placement. Multiple sequence alignment of the pXO2 gene dataset was carried out using the MUSCLE algorithm integrated within the MEGA software (version 12). A phylogenetic tree was constructed using the Neighbour-Joining method applying the Tamura-Nei model to estimate evolutionary distances among the aligned sequences. Bootstrap analysis with 1000 replicates was performed to assess the confidence levels of the branching patterns obtained in the final phylogenetic tree.

Results

Isolation of *Bacillus anthracis* from animal tissues

Both blood and ear tissue samples were inoculated onto BHI agar, blood agar, and PLET agar for bacterial culture. After overnight incubation on blood agar, large, flat colonies were observed that appeared white to grayish-white in color, with a sticky, ground-glass texture and a distinctive 'medusa head' or 'curled hair' morphology. When grown on PLET agar, *Bacillus anthracis* produced medium-sized, translucent colonies. Similar colony morphology was also observed upon plating the vaccine strain on blood agar.

Table 4: Reference Strains of pXO1 used in Phylogenetic Tree Construction

Sl. No.	Accession No.	Source/Host/Strain	Location
1.	KU726964.1	Blood	Bangladesh
2.	AB125962.1	-	Japan
3.	NZ_CP167926.1	Elephant	India
4.	NZ_JAINUB010000143.1	Bovine	India
5.	KT995459.1	Bovine	Bnagladesh
6.	CP047133.1	Bovine	Germany
7.	CP029325.1	Bovine	Switzerland
8.	CP135589.1	Soil	China
9.	AP018445.1	Hippopotamus	Zambia
10.	CP170739.1	-	Austria
11.	CP140729.1	Soil	Austria
12.	KM019143.1	Elephant	India
13.	KP759891.1	Soil	Vietnam
14.	DQ517347.1	-	USA
15.	MK310255.1	Ovine	India
16.	KP759892.1	Homo Sapiens	Vietnam
17.	MT709158.1	Soil	India
18.	HQ536626.1	Str Isr1980	-
19.	CP029807.1	Animal Hide	London
20.	HQ536629.1	Str. Isr1980b	-
21.	CP009695.1	Bovine	France
22.	CP009475.1	Feline Pasteur Strain	
23.	CP009329.1	Homo Sapiens	South Africa
24.	CP076206.1	Str. 3-Izslt	Italy
25.	CP060196.1	Bovine Blood	Kanchipuram India
26.	CP002093.1	Strain H9401	-
27.	NC007323.3	Ames Ancestor	-
28.	AP019733.1	Bone Powder	Japan
29.	Qy324142.1	*B. cereus*	out group

Identification by colony morphology

Upon Gram staining of the cultures obtained from the samples, all the preparations revealed the presence of Gram-positive, rod-shaped organisms arranged in short chains. When observed under 1000X magnification, the cells displayed a characteristic appearance resembling railway carriages or "box car" formations. These morphological features were consistent across all examined cultures, confirming the typical microscopic characteristics of *Bacillus anthracis* isolates.

Identification of *Bacillus anthracis* isolates by PCR amplification of the pXO2 gene

All ten isolates yielded amplified products of 846 bp of the CAP (pXO2) gene (Plate 1), indicating the presence of the pXO2 plasmid in these samples. However, both the negative control (Staphylococcus aureus) and the no-template control showed no amplification. The positive control a pathogenic field isolate previously confirmed by whole genome sequencing at SRDDL, Bengaluru also produced the expected 846 bp amplicon

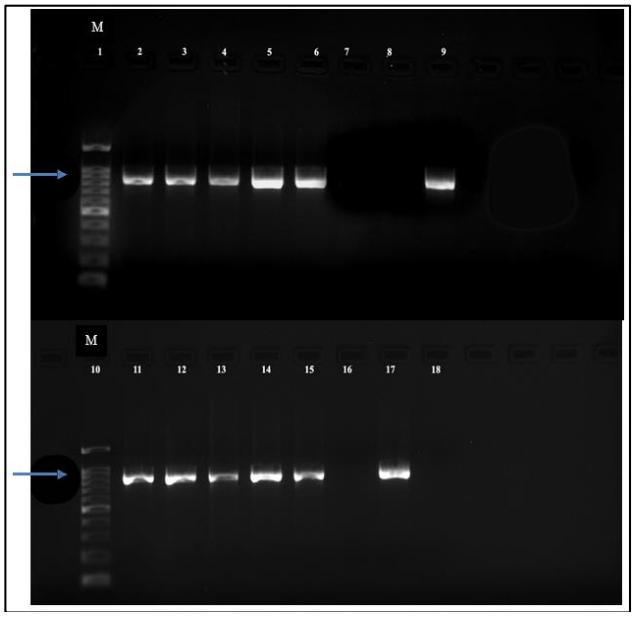
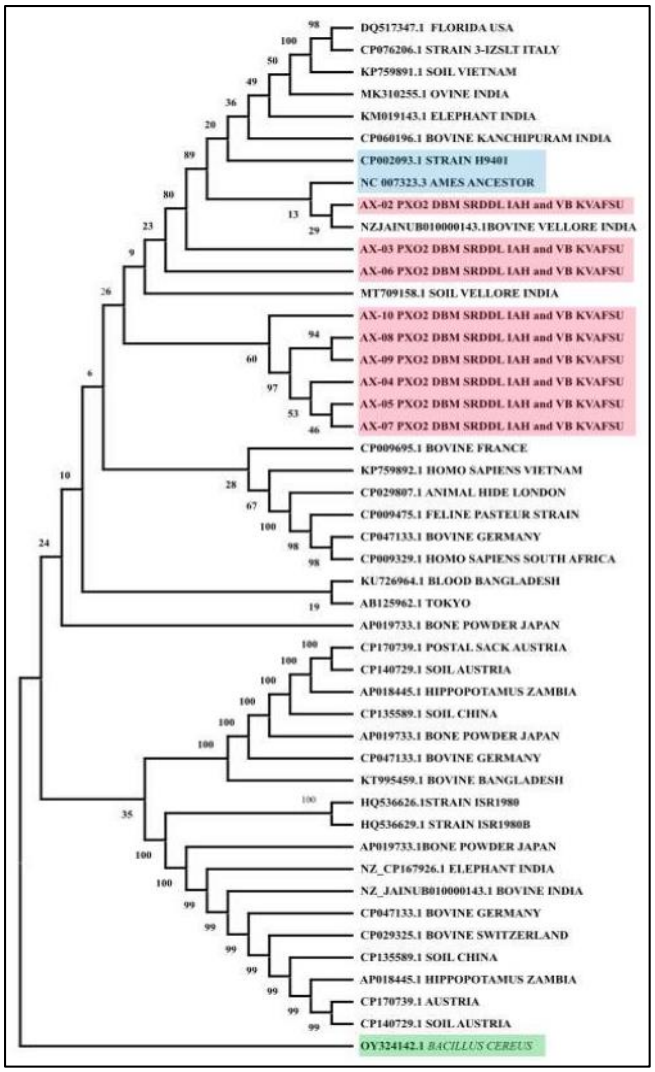


Plate 1: PCR amplification of pXO2 gene (846 bp) of *Bacillus anthracis* field isolates
Lane M : 100bp DNA ladder
L2-L6 and L11-L15: *Bacillus anthracis* (AX-1 to AX-10) L7 and L16: S. aureus-Negative control
L8 and L18: No template control (NTC)
L9 and L17: *Bacillus anthracis* (SRDDL)-Positive control

Phylogenetic Analysis of pXO2 gene sequences

The pXO2 sequence based phylogeny tree showed the Karnataka sequences cluster predominantly together, indicating strong genetic relationship among South Indian strains.



Phylogenetic tree for pXO2 showing the query sequence (red), standard sequence (blue), and out group (green) along with other reference sequences

Discussion

The phylogenetic analysis of the pXO2 gene revealed that the isolates from Karnataka predominantly clustered together, indicating a strong genetic relatedness among the South Indian strains. Minor subdivisions within this cluster suggested possible ongoing diversification or the emergence of sub-lineages influenced by local evolutionary factors or population dynamics. The Odisha isolates clustered closely with Chinese strains in the pXO2 tree, supporting the existence of a close genetic relationship between these geographic regions. The pXO2 phylogenetic tree also included isolates from Europe and Africa, which formed distinct clades without intermixing with the Indian isolates. This spatial separation indicated limited recent genetic exchange between Indian and non-Indian populations, consistent with the geographic structuring of *Bacillus anthracis* reported by Van Ert *et al.* [10]. Standard reference strains clustered distinctly apart from the field isolates, confirming their genetic divergence and strengthening the overall phylogenetic framework. Similar observations were reported by Chaitra Bai [12]

Conclusion

This study highlights the pronounced genetic relatedness and spatial structuring of *Bacillus anthracis* strains in Karnataka, distinct from those found in other Indian regions and abroad. The local clustering of isolates in pXO2 gene-based trees points to limited genetic exchange with outside populations. These insights provide valuable context for anthrax surveillance, emphasizing the importance of region specific control strategies and continued monitoring of emerging sub-lineages to address evolving threats

Summary

This research investigated anthrax outbreaks in Karnataka by collecting ten animal samples suspected of *Bacillus anthracis* infection from various locations throughout the region. Further isolation and identification of the bacterium from the collected samples was done by culturing, Grams staining, PCR-based detection, and subsequent phylogenetic analysis targeting the pXO2 plasmid genes. Culturing techniques, along with molecular confirmation identified *Bacillus anthracis* in all samples, with distinctive colony morphology and Gram-positive, rod-shaped cellular appearance was observed. PCR amplification of the pXO2 gene confirmed plasmid presence in all isolates, supporting the cultural techniques. Phylogenetic analysis, utilizing MEGA software, revealed that the Karnataka isolates formed a distinct genetic cluster apart from reference strains from various other regions, including East Asia, Africa, and Europe. Within this cluster, minor subdivisions suggest ongoing local evolutionary changes or diversification, potentially influenced by environmental or population dynamics unique to South India.

References

1. Alam ME, Kamal MM, Rahman M, Kabir A, Islam MS, *et al.* Review of anthrax: A disease of farm animals. Journal of Advanced Veterinary and Animal Research. 2022;9(2):323-332.
2. Goel AK. Anthrax: A disease of biowarfare and public health importance. World Journal of Clinical Cases. 2015;3(1):20-33.
3. World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), World Organisation for Animal Health (OIE). Anthrax in Humans and Animals. 4th ed. Geneva: WHO; 2008. p. 1-219.
4. Jones SD. Death in a Small Package: A Short History of Anthrax. Baltimore (MD): Johns Hopkins University Press; 2010. p. 1-288.
5. Ochai SO, Hassim A, Dekker EH, Magome T, Lekota KE, Makgabo SM, *et al.* Comparing microbiological and molecular diagnostic tools for the surveillance of anthrax. PLOS Neglected Tropical Diseases. 2024;18(11):e0012122.
6. Keim P, Pearson T, Okinaka RT. Evolution of *Bacillus anthracis*, causative agent of anthrax. In: Evolutionary Biology of Bacterial and Fungal Pathogens. Washington (DC): ASM Press; 2007. p. 523-533.
7. Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carmagnola L, Brusetti L, *et al.* Strategy for identification of *Bacillus cereus* and *Bacillus*

thuringiensis strains closely related to *Bacillus anthracis*. Applied and Environmental Microbiology. 2006;72(2):1295-1301.
8. Thwaite JE, Baillie LW, Carter NM, Stephenson K, Rees M, Harwood CR, *et al.* Optimization of the cell wall microenvironment allows increased production of recombinant *Bacillus anthracis* protective antigen from *Bacillus subtilis*. Applied and Environmental Microbiology. 2002;68(1):227-234.
9. Logan AN. *Bacillus* and recently derived genera. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of Clinical Microbiology. 7th ed. Washington (DC): ASM Press; 1999. p. 357-369.
10. Van Ert MN, Easterday WR, Huynh LY, Okinaka RT, Hugh-Jones ME, Ravel J, *et al.* Global genetic population structure of *Bacillus anthracis*. PLoS One. 2007;2(5):e461.
11. World Organisation for Animal Health (WOAH). Anthrax. In: WOAH Terrestrial Manual. Chapter 3.1.1. Paris: WOAH; 2023. p. 1-24.
12. Chaitra Bai KT. Molecular Epidemiology of *Bacillus anthracis* Isolates in Ruminants of Karnataka [Master's thesis]. Bidar: Department of Veterinary Microbiology, Karnataka Veterinary, Animal and Fisheries Sciences University; 2024. p. 91-92.