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Phylogenetic Analysis of *Bacillus anthracis* Strains Isolated from Clinical and Environmental Samples in Andhra Pradesh

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ABSTRACT

Anthrax, caused by *Bacillus anthracis*, is a severe zoonosis with a great impact on animal and human health. In the present study, 15 out of 16 isolates from clinical ear piece and soil samples (16), 8,6 and one samples isolated respectively from bovine, sheep and goat were confirmed as Bacillus on amplification of rpoB gene, 22 isolates (ear piece13, soil-9) were PCR positive for PA gene of PXO and 10 isolates (ear piece) amplified CAP gene confirming *Bacillus anthracis*. Similarly, all 31 isolates (earpiece-15 and soil-16) were confirmed as *Bacillus anthracis* on amplifying rpoB gene and chromosomal Ba813 gene. On nucleotide analysis with genus specific *rpoB* gene of earpiece and soil isolates shown 99-100% identity, whereas nucleotide analysis with species specific genes; *PA* of PXO1, *CAP* of PXO2, *rpoB* and chromosomal *Ba813* gene shown 98-100% identity with their respective reference strains of Genbank. On phylogenetic analysis, earpiece isolates and soil isolates from endemic districts of Andhra Pradesh shown close evolutionary relationship with each other. However, earpiece isolates of Sr6 (Accession No:MK310254) and N3 shared ancestral relation with global reference strains of USA (Accession No:CP012730) and Japan (Accession no: AP014833) respectively. Similarly soil isolate of VM9 also shared ancestral relation with global reference strain of Japan (Accession no: AP014833). This phylogenetic analysis deciphered that there is no strain variation among isolates of *B. anthracis* collected from different clinical and soil samples of different districts of Andhra Pradesh.

HIGHLIGHTS

- 99 to 100% nucleotide identity with genus specific and 98-100 % with species specific genes.
- Close evolutionary relationship between clinical and environmental isolates.
- No strain variation among isolates.

Keywords: *Bacillus anthracis*, Phylogenetic analysis, Multiple nucleotide alignment, clinical earpiece, Environmental soil

Anthrax is a particularly dangerous zoonotic disease caused by an aerobic gram positive spore forming bacterial species i.e. *Bacillus anthracis*. Genetically *Bacillus anthracis* is closely related to *Bacillus cereus* and *Bacillus thuringiensis* is believed to have diverged from its Bacillus cereus ancestor due to the evolutionary acquisition of two virulence plasmids pXO1 and pXO2 (Keim *et al.*, 2008). The protective antigen (PA) and capsular (CAP) genes located on these plasmids are used as molecular markers for the routine identification of *Bacillus anthracis* from its close relative, *Bacillus cereus* and *Bacillus thuringiensis*. However, occurrence of *Bacillus cereus* and *Bacillus thuringiensis* with either or both of these plasmids pose challenges in unambiguous identification of the species (Maughan *et al.,* 2011).

Analysis of 16SrDNA sequences of *B.anthracis* (Akanza *et al.,* 2020) by canonical single nucleotide polymorphism

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(canSNP) was useful in detection of sublineage of strains isolated during outbreaks from India. Similarly, whole genome SNP analysis (Akiko *et al.,* 2019) of *Bacillus anthracis* genes using DNA libraries were clearly indicated the phylogenetic relationships. Further, it is difficult to differentiate by plasmid analysis because of plasmid transfer among the closest species.

Genes in the plasmid of *Bacillus anthracis* have been successfully expressed in other bacteria (Thwaite *et al.*, 2002) and been reported in other bacillus species (Pannucci *et al.*, 2002). It is important to note that PXO2 can be lost naturally. Due to the natural competence of *Bacillus thuringiensis* and *Bacillus cerus*, the horizontal transfer of plasmids has been reported.

Identification of *Bacillus anthracis* by PCR targeting only toxin encoding genes (Pxo1 or PXO2) may not accurately identify the *Bacillus anthracis*, because of the transfer of plasmid by horizontal gene transfer among closely related species (Kwan *et al.*, 2003). All the *Bacillus anthracis* isolates may not contain the toxin encoding genes and there is a chance of natural loss of plasmids (Kwan *et al.*, 2003; Vahedi *et al.*, 2009). Hence, in the present study, targeting combination of *Bacillus anthracis* specific chromosomal markers (chromosomal *Ba813* & *rpoB* genes) along with toxin encoding genes (PA gene of pXO1 and CAP gene of pXO2) were used for specific identification of *Bacillus anthracis* and to understand the phylogenetic relationship of *Bacillus anthracis* isolates.

MATERIALS AND METHODS

A total of 81 clinical and soil samples were collected for the study. For genus specific detection 5 amplicons targeting *rpoB* gene were synthesized from 5 ear piece and 4 soil sample isolates. Similarly, for species specific detection, 13 PCR amplicons of PA gene of pXO1 (from 13 ear piece and 9 soil isolates), 10 PCR amplicons of CAP gene of pXO2 (from 10 ear piece isolates), nine PCR amplicons of *rpoB* gene (from 5 ear piece and 4 soil isolates) and 31 PCR amplicons of chromosomal *Ba813* gene (15 ear piece and 16 soil isolates) were synthesized. The PCR amplicons synthesized were sent to Genei private limited, Bangalore for sequencing and detection of *Bacillus anthracis* at genus and species level.

Multiple nucleotide alignment and Phylogenetic analysis

Multiple sequence alignment of nucleotide sequences was carried out using Clustal X 2.1 program to know percent identity and divergence. Phylogenetic analysis of received sequences of isolates were compared with analogous sequences of other *B.anthracis* isolates showing highest percent query coverage retrieved from Genbank, NCBI data base from different geographical regions to know the ancestral relatedness and evolutionary relationship of the isolates (Table 1a-1e).

Table 1a: List of genus specific *rpoB* gene nucleotide sequences retrieved from the Gen Bank for multiple nucleotide alignment and phylogenetic analysis

Sl. No.	Strain	Country	Accession no.	Year
1	SPV 842-15	Brazil	CP019588	2017
2	MCCC 1A02161	China	CP031642	2018
3	Tyrol 4675	Germany	CP018903	2017
4	Shikan-NIID	Japan	AP014833	2015
5	London-499	London	CP029805	2018
6	DL7	Maharastra, India	JN572676	2015
7	Parent-1	USA	CP012730	2016
8	34F2	Uttar Pradesh, India	JQ798178	2016
9	IVRIKOLBAI	West Bengal, India	KT831967	2016

Table 1b: List of species specific *PA* gene nucleotide sequences retrieved from the Gen Bank for multiple nucleotide alignment and phylogenetic analysis

Sl. No.	Strain	Country	Accession no.	Year
1	London499	UK	CP029806	2018
2	SPV 842	Brazil	CP019589	2017
3	Tyrol 4675	Germany	CP018904	2017
4	Shikan-NIID	Japan	AP014834	2015
5	Canadian	USA	CP010321	2016
6	34F2	Uttar Pradesh, India	JQ798178	2016
7	IVRIKOLBAI	West Bengal, India	KT831967	2016

A cladogram, based on partial sequences, was generated using the MEGA 6.0 program (Tamura *et al*., 2013) based on the Kimura-2-parameter model. The sequences were aligned in Clustal W, and phylogenetic trees were

constructed using the Maximum Likelihood method. The robustness of the tree was assessed with 1000 bootstrap replicates and percent identity was performed to identify similarity and differences between analyzed sequences and also between analyzed and reference sequences.

Table 1c: List of species specific *CAP* gene nucleotide sequences retrieved from the Gen Bank for multiple nucleotide alignment and phylogenetic analysis

Sl. No.	Strain	Country	Accession no.	Year
1	London499	UK	CP029807	2018
2	Tyrol 4675	Germany	CP018905	2017
3	Shikan-NIID	Japan	AP014835	2015
5	Canadian	USA	CP010320	2016
6	IVRI-1975	Uttar Pradesh, India	KM019143	2016
7	SVA11	Sweden	CP006744	2016

Table 1d: List of species specific *rpoB* gene nucleotide sequences retrieved from the GenBank for multiple nucleotide alignment and phylogenetic analysis

Sl. No.	Strain	Country	Accession no.	Year
1	SPV 842-15	Brazil	CP019588	2017
2	MCCC 1A02161	China	CP031642	2018
3	Tyrol 4675	Germany	CP018903	2017
4	Shikan-NIID	Japan	AP014833	2015
5	London-499	London	CP029805	2018
6	DL7	Maharastra, India	JN572676	2015
7	Parent-1	USA	CP012730	2016

Table 1e: List of Chromosomal *Ba813* gene nucleotide sequences retrieved from the Gen Bank for multiple nucleotide alignment and phylogenetic analysis

Sl. No.	Strain	Country	Accession no.	Year
1	SPV 842-15	Brazil	CP019588	2017
2	MCCC 1A02161	China	CP031642	2018
3	Tyrol 4675	Germany	CP018903	2017
4	Shikan-NIID	Japan	AP014833	2015
5	London-499	London	CP029805	2018
6	Parent-1	USA	CP012730	2016

RESULTS AND DISCUSSION

Multiple nucleotide alignment of *Bacillus* isolates recovered from clinical earpiece and environmental soil samples

The results of Multiple nucleotide alignment of genus specific *rpoB* gene of Earpiece (Sr6) and soil isolates (SJ6, VV25) showed a nucleotide identity of 100% with reference gene bank strains of Brazil strain (Accession No: CP019588), Germany strain (Accession No:CP018903), London strain (Accession No: CP029805), USA strain (Accession No: CP012730). Multiple nucleotide alignment of species specific *PA* gene of all the earpiece (13) and soil (9) isolates resulted in a nucleotide identity in the range of 98-99% with reference strains of Brazil (Accession No: CP019589), Germany (Accession No: CP018904), Japan (Accession no: AP014834), London (Accession No: CP029806), USA (Accession No: CP010321), Uttar Pradesh (Accession No: JQ798178) and West Bengal (Accession No: KT831967).

Similarly multiple nucleotide alignment of species specific *CAP* gene of NelloreN3 earpiece isolate showed 100% nucleotide identity with ChittoorI15 earpiece isolate. Multiple nucleotide alignment of species specific *rpoB* gene of NelloreN3 earpiece isolate showed a nucleotide identity with reference sequence of Japan strain (Accession no:AP014835),whereas AnnathapurAS30 showed 100% nucleotide identity with KurnoolKN18 soil isolate.

Multiple alignment of *Ba813* gene of I1, I14, K10, N3, N4, N7, Sr6 earpiece isolates and AS28, KN18, KN21, VG4, VP11, VP12, VV25 soil isolates showed a nucleotide identity of 100% with reference strains of Brazil (Accession No:CP019588), Germany (Accession No: CP018903), London (Accession No: CP029805) and USA (Accession No: CP012730). The results of multiple alignment of nucleotide of all the genes of remaining isolates showed a nucleotide identity in the range of 98-99% with their respective reference strains of Genbank.

Results of Phylogenetic analysis of *Bacillus* isolates recovered from clinical earpiece and environmental soil samples

Phylogenetic analysis of genus specific *rpoB* gene of clinical earpiece isolates, Sr6 isolate showed close

relationship with USA strain (Accession No: CP012730) and remaining other isolates (N3, K12, Ch8 and A5) formed separate clusters with reference strains Brazil strain (Accession No: CP019588), Germany strain (Accession No:CP018903), USA strain (Accession No: CP012730), London strain (Accession No:CP029805), China strain (Accession No:CP031642), Japan strain (Accession no: AP014833). But Sr6, N3, lK12, Ch8 and A5 formed separate clusters with reference Maharastra strain (Accession No: JN572676).Whereas, Sr6, N3, K12, Ch8 and A5 formed separate clusters with each other (Fig. 1).

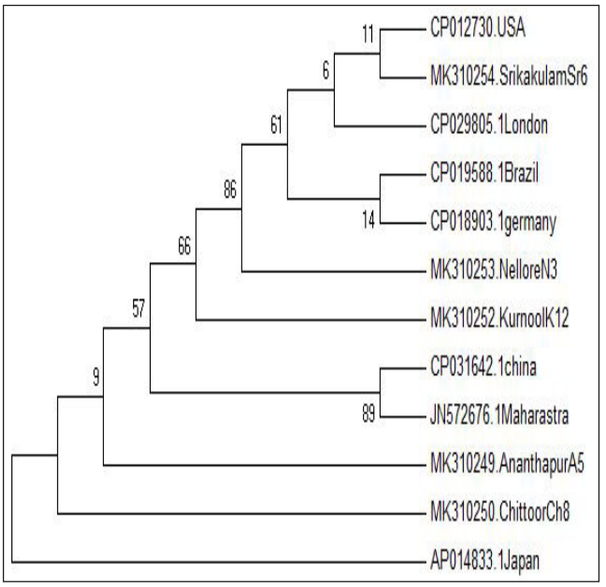

Fig. 1: Phylogenetic analysis of *rpoB* gene (earpiece isolates) of *Bacillus* genus-Andhra Pradesh

Similarly, the phylogenetic analysis of genus specific *rpoB* gene of environmental soil isolates of SJ6, VV25, KN18, AS30 formed separate clusters with reference strains Brazil strain (Accession No: CP019588), Germany strain (Accession No:CP018903), USA strain (Accession No: CP012730), London strain (Accession No: CP029805),China strain (Accession No: CP031642), Japan strain (Accession no: AP014833). Whereas SJ6, VV25, KN18, AS30 formed separate clusters with Maharashtra strain (Accession No: JN572676). SJ6 isolate showed close relationship with VV25 isolate but lKN18 and AS30 formed separate clusters with other isolates (Fig. 2).

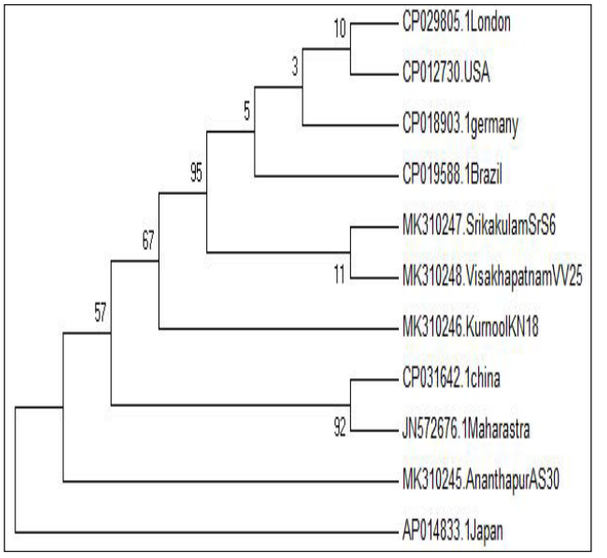

Fig. 2: Phylogenetic analysis of *rpoB* gene (soil isolates) of *Bacillus* genus–Andhra Pradesh

Results of Phylogenetic analysis of *Bacillus anthracis* isolates recovered from clinical earpiece and environmental soil samples

The Phylogenetic analysis of *PA* gene of earpiece isolates of I1, I2, N3, N4, A5, Sr6, N7, Ch8, I9, lK10, Ch11, A13, I15 formed separate clusters with reference strains Brazil strain (Accession No: CP019589), Germany strain (Accession No: CP018904), Japan strain (Accession no:AP014834), London strain (Accession No:CP029806), USA strain (Accession No: CP010321). The isolates I1, I2, N3, N4, A5, Sr6, N7, Ch8, I9, lK10, Ch11, A13,I15 formed separate clusters with reference Uttar Pradesh strain (Accession No: JQ798178) and West Bengal strain (Accession No: KT831967). But I9, I1, Ch8, K10 isolates showed close relationship with Ch11, I15, N7, Sr6 isolates respectively (Fig. 3).

Similarly, the Phylogenetic analysis of PA gene of soil isolates AS33, KN21, AS31, AS38, VM7, AS30, VM9, lKN19, VV25 formed separate clusters reference strains Brazil strain (Accession No: CP019589), Germany strain (Accession No: CP018904), Japan strain (Accession no:AP014834), London strain(Accession No: CP029806),USA strain (Accession No: CP010321) of Gen bank. AS33, KN21, AS31, AS38, VM7, AS30, VM9,

KN19, VV25 formed separate clusters with reference strains i.e. Uttar Pradesh strain (Accession No: JQ798178) and West Bengal strain (Accession No: KT831967). The isolates AS30, VM9 showed close relationship with KN19, VV25 respectively. But AS33, KN21, AS31, AS38, VM7 formed separate clusters with other isolates (Fig. 4).

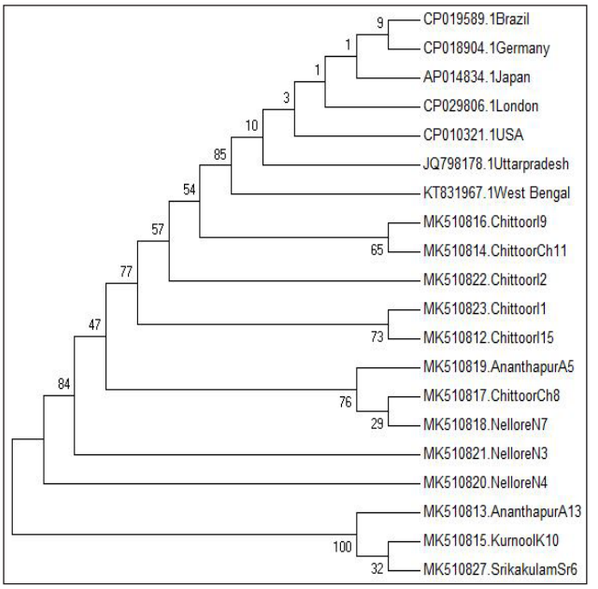

Fig. 3: Phylogenetic analysis of *PA* gene (earpiece isolates) of *Bacillus anthracis* –Andhra Pradesh

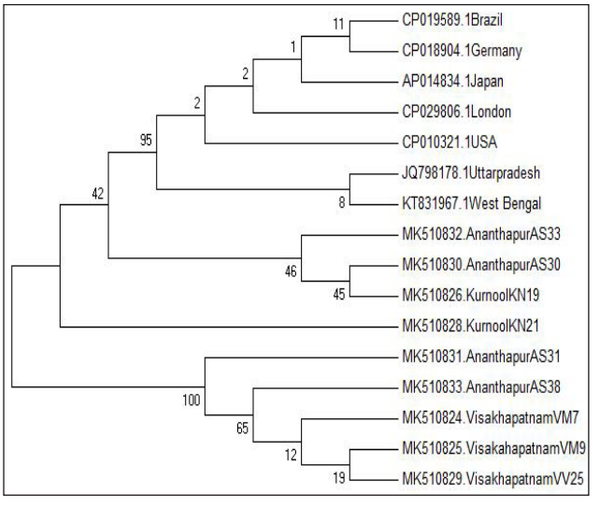

Fig. 4: Phylogenetic analysis of *PA* gene (soil isolates) of *Bacillus anthracis*–Andhra Pradesh

The Phylogenetic analysis of *CAP* gene of earpiece isolates of K10, A5, I2 isolates formed separate clusters with N4, I15 isolates and London (Accession No: CP029807), Germany (Accession No:CP018905), Japan (Accession no: AP014835), Sweden (Accession No: CP006744) reference strains of genbank. Sr6, I1 isolates formed separate clusters with USA (Accession No: CP010320) strain. Sr6, I1 isolates formed separate clusters with Uttar Pradesh (Accession No:KM019143) strain. N3 isolate showed close relationship with Uttar Pradesh strain (Accession No: KM019143). I15, Ch11 showed close relationship with N4, Ch8 isolates and respectively. Sr6, I1 isolates formed separate clusters with Ch8, Ch11, N3 isolates (Fig. 5).

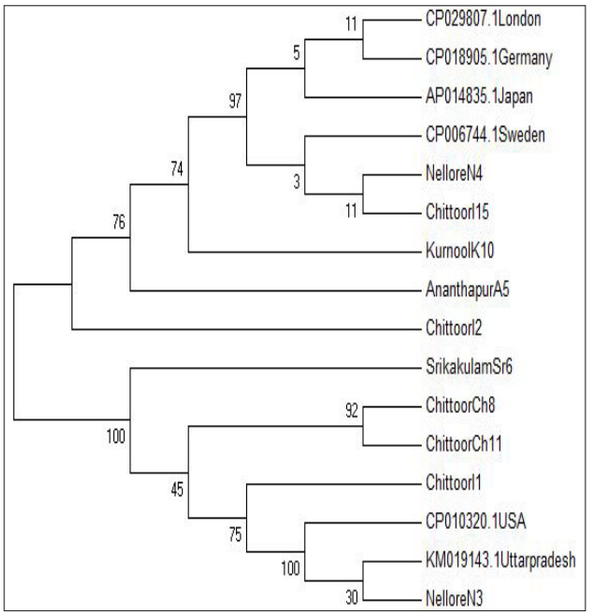

Fig. 5: Phylogenetic analysis of *CAP* gene (earpiece isolates) of *Bacillus anthracis* – Andhra Pradesh

The Phylogenetic analysis of species specific *rpoB* gene of earpiece isolates of NelloreN3 isolate showed close relationship with Japan strain (Accession No: AP014833). Ch8, Sr6, rA5, K12 isolates formed separate clusters with Brazil strain (Accession No:CP019588), Germany strain (Accession No: CP018903), London strain (Accession No: CP029805), USA strain (Accession No: CP012730), Japan strain (Accession No: AP014833) China strain (Accession No: CP031642). N3, Ch8, Sr6, A5, K12 isolates formed separate clusters with Uttar Pradesh strain (Accession

No: JQ798178), West Bengal strain (KT831967) and Maharashtra strain (Accession No: JN572676) (Fig. 6).

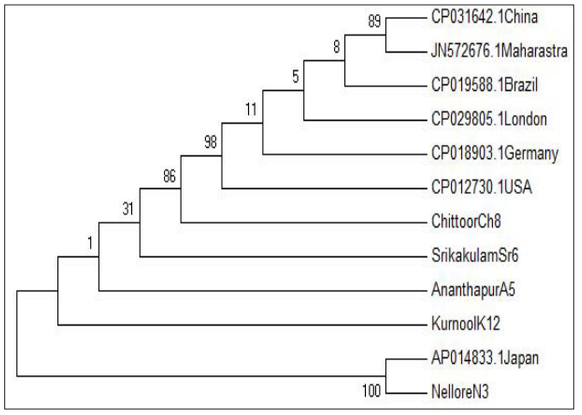

Fig. 6: Phylogenetic analysis of *rpoB* gene (earpiece isolates) of *Bacillus anthracis*–Andhra Pradesh

Similarly, the phylogenetic analysis of species specific *rpoB* gene of soil isolates, AS30, SJ6, KN18, VV25 formed separate clusters with Brazil strain (Accession No: CP019588), Germany strain (Accession No: CP018903), London strain (Accession No: CP029805), USA strain (Accession No: CP012730), Japan strain (Accession No: AP014833) China strain (Accession No: CP031642). AS30, SJ6, KN18, VV25 isolates formed separate clusters Uttar Pradesh strain (Accession No: JQ798178), West Bengal strain (KT831967) and Maharashtra strain (Accession No: JN572676). Whereas, AS30, SJ6 isolates showed close relationship with KN18; VV25 isolates respectively (Fig. 7).

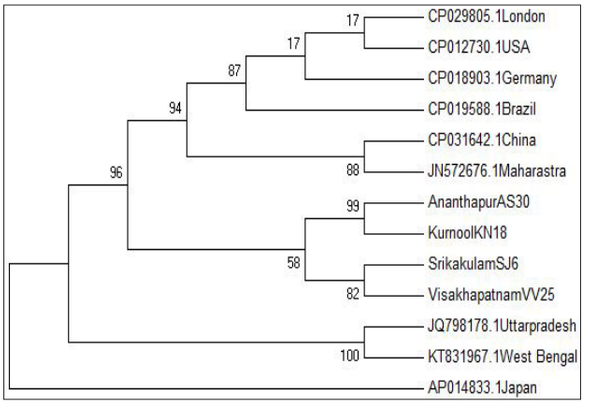

Fig. 7: Phylogenetic analysis of *rpoB* gene (soil isolates) of *Bacillus anthracis*–Andhra Pradesh

Results of Phylogenetic analysis of chromosomal *Bacillus anthracis* isolates recovered from clinical earpiece and environmental soil samples

The Phylogenetic analysis of chromosomal *Ba813* gene of earpiece isolates, K12, I2, I15, Ch11 formed separate clusters with Japan strain (Accession No: AP014833). N3, K10, I14, I1, Sr6 formed separate clusters with London strain (Accession No: CP029805), China strain (Accession No:CP031642), Brazil strain (Accession No: CP019588), Germany strain (Accession No: CP018903). But N4, A5, A13 isolates showed close relationship with N7, Ch8, I9 respectively. The isolates K12, I2, I15, Ch11 formed separate clusters with A5, Ch8, A13, I9 isolates. Whereas N3, K10, I14, I1, Sr6 formed separate clusters with N4, N7 (Fig. 8).

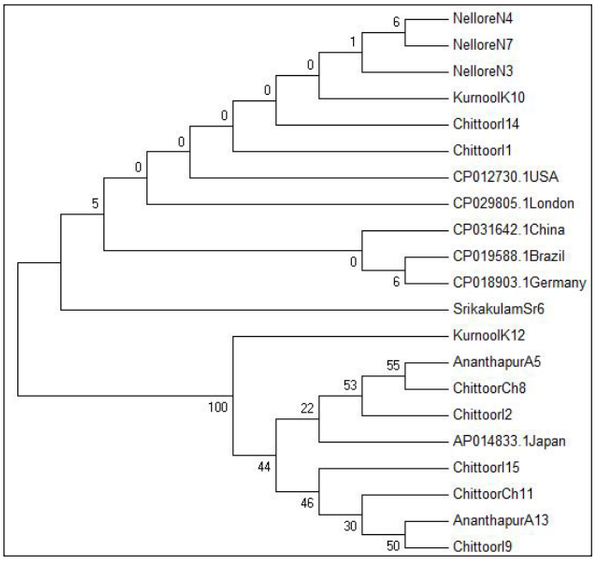

Fig. 8: Phylogenetic analysis of chromosomal *Ba813* gene (earpiece isolates) of *Bacillus anthracis*–Andhra Pradesh

Similarly, the phylogenetic analysis of Chromosomal *Ba813* gene of soil isolates of VM9 showed close relationship with Japan strain (Accession No: AP014833). AS33, KN19, VM7, AS31 formed separate clusters with Japan strain (Accession No: AP014833). lKN21, VG4, VP11 formed separate clusters with USA strain (Accession No: CP012730), London strain (Accession No:CP029805), Germany strain (Accession No:CP018903),Brazil strain (Accession No:CP019588), China strain (Accession No:CP031642). AS28, VP12, AS30, AS38 isolates showed

close relationship with KN18, VV25, AS42, SJ6 isolates respectively. AS33, KN19, VM7, AS31 formed separate clusters with VM9, AS30, AS42, AS38, and SJ6 isolates. lKN21, VG4, VP11 formed separate clusters with AS28, lKN18, VP12, VV25 (Fig. 9).

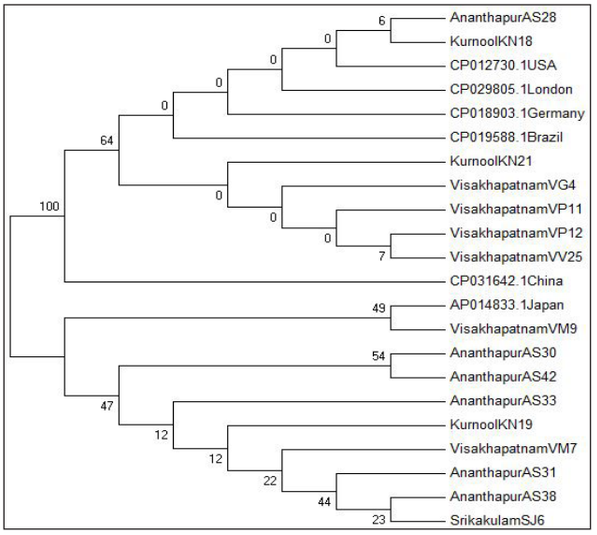

Fig. 9: Phylogenetic analysis of chromosomal *Ba813* gene (soil isolates) of *Bacillus anthracis* – Andhra Pradesh

On Phylogenetic analysis, genus specific *rpoB* gene of Sr6 earpiece and SJ6 soil isolates showed close relationship with reference strain of USA(Accession No: CP012730) and VV25 soil isolate respectively. Phylogenetic analysis of species specific *PA* gene of I9, I11, Ch8, K10 earpiece isolates showed close relationship with Ch11, I15,N7 and Sr6 earpiece isolates respectively whereas AS30, KN19 soil isolates showed close relation with KN19 and VV25 respectively. Phylogenetic analysis of chromosomal *Ba813* gene of N4, A5, and A13 isolates are closely related to N7, Ch8 and I9 earpiece isolates respectively whereas AS30, AS31, AS42 soil isolates are closely related to KN18, VV25 and SJ6 soil isolates respectively. The results of phylogenetic analysis of all the genes of remaining isolates formed separate clusters with respective reference strains of genbank.

During the study, all the 31 isolates earpiece (15) and soil (16) were positive for bacillus genus with genus specific primers on PCR.Out of 15 earpiece isolates,only 13 isolates for plasmid encoding *PA* gene and 10 isolates for *CAP* gene were positive on PCR confirming these isolates were *B.anthracis*. However,none of the isolates from soil were positive for *CAP* gene, but only 9 isolates of 16 soil isolates were positive for *PA* gene confirming *B. anthracis*. But all the 31 (earpiece-15, soil-16) were confirmed as *B. anthracis* with species specific *rpoB* and chromosomal Ba813 genes.

Identification of *B.anthracis* by PCR targeting only toxin encoding genes (pXO1 or pXO2) may not accurately identify the *B. anthracis*, because of the transfer of plasmid by horizontal gene transfer among closely related species (Kwan *et al*., 2003). All the *B. anthracis* isolates may not contain the toxin encoding genes and there is a chance of natural loss of plasmids (Ko *et al.*, 2003; Vahedi *et al.*, 2009). Hence, in the present study, combination of *B.anthracis* specific chromosomal markers(chromosomal *Ba813* and *rpoB*) along with toxin encoding genes(*PA* of pXO1 and *CAP* gene of PXO2) targeting PCR's were performed for specific identification of *B.anthracis*. A total of 81 PCR amplicons targeting (Table 2) genus specific *rpoB* gene (9); species specific genes*, PA* gene of pXO1 (22),*CAP* gene of pXO2(10), *rpoB* gene(9) and chromosomal *Ba813* gene (31) from outbreak/endemic areas were sent to Genei Pvt, Ltd., Bangalore for sequencing. The sequences received were analyzed through NCBI blast tool to compare nucleotide percentage identity and ancestral relatedness of isolates with references sequences available in the gene bank database.

Table 2: Number of PCR amplicons sent for sequencing

Sl. No.	Targeting genes		Earpiece	Soil	Total
1	Genus specific gene	*rpoB* gene for Bacillus	5	4	9
2	Species specific genes	*PA* gene of pXO1	13	9	22
		CAP gene of pXO2	10	0	10
		rpoB gene of *B.anthracis*	5	4	9
3	Chromosomal specific gene	*Ba813* gene	15	16	31
	Grand total		**48**	**33**	**81**

In the present study, all the isolates of clinical earpiece and environmental soil were identified as bacillus and shown identity of 99-100% with reference sequences deposited in the gene bank whereas all the *Bacillus anthracis* isolates of clinical earpiece and environmental soil samples

were shown 98-100% identity with gene bank reference sequences. Hence, the present molecular characterization studies revealed that 99% of the isolates yielded as Bacillus with genus specific primers and 98% of the isolates as *B. anthracis* with species specific primers.

Further, phylogenetic analysis of *Bacillus anthracis* isolates recovered from earpiece and soil in endemic regions of Andhra Pradesh shown close evolutionary relationship with each other but earpiece isolates of Srikakulam (Accession No: MK310254) and Nellore shared ancestral relation with global reference strains of USA (Accession No: CP012730) and Japan(Accession no: AP014833) respectively. Similarly soil isolate of Visakhapatnam also shared ancestral relation with global reference strain of Japan (Accession no: AP014833). This phylogenetic analysis deciphered that there is no strain variation among isolates of *B.anthracis* identified in endemic regions from different districts of Andhra Pradesh.

CONCLUSION

The study revealed that *B. anthracis* from all the endemic districts of Andhra Pradesh are capable of producing fresh outbreaks even though they lack genes encoding major virulence producing Plasmids. However, nature of *B. anthracis* has to be studied further to know its changing mechanisms in producing fresh out breaks at intervals even after annual vaccinations at endemic areas of Andhra Pradesh.

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