GENETIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF *BACILLUS ANTHRACIS STERNE STRAIN* BY 16S RRNA GENE SEQUENCING

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ABSTRACT: Anthrax, caused by *Bacillus anthracis*, is a fatal disease and all the warm blooded animals are susceptible to this disease. Many livestock populated areas in Pakistan are endemic for Anthrax (Thappa and Karthikeyan 2001), therefore the livestock population is annually vaccinated against Anthrax. Veterinary Research Institute (VRI) has been producing Anthrax Spore Vaccine (ASV) for a couple of decades. The current study was designed for molecular characterization of the microorganism used for vaccine production (*Bacillus anthracis* Sterne strain) and its phylogenetic relationship with related strains and Bacillus species. For this purpose, 16S rRNA gene was amplified and sequenced and was found 100% genetically similar to the reference sequence with respect to 16S rRNA gene. Phylogenetic analysis revealed that sequenced strain showed a close phylogenecity with *Bacillus anthracis* strain 1144 and *Bacillus anthracis* strain V77-NP-1R. Moreover, the organism was found genetically closer to *Bacillus cereus* than to *Bacillus mycoides* and *Bacillus thuringiensis* with respect to 16S rRNA gene sequence.

Key words: Anthrax, *Bacillus anthracis* Sterne strain, 16S rRNA, molecular characterization, sequencing, phylogenetic analysis

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INTRODUCTION

Clinical importance of *Bacillus anthracis* cannot be denied, not only due to the fact that Anthrax, the disease it produces, may affect all the mammals, including human beings; but also due to the incidence related to deliberate intent of dissemination of the disease agent (Mebane *et al*., 2003). B. anthracis is highly homogenous and can therefore be considered a particularly monomorphic species (Helgason *et al*., 2004). As reported by (Hill *et al*., 2004), the *Bacillus anthracis* belongs to the Bacillus cereus group and herbivores are especially susceptible to the disease, owing to its presence in environments, as a soil inhabitant and its resistance to environmental hazards, because of spores formation.

16S ribosomal RNA currently represents the most important target of study in bacterial ecology, though its use for the description of bacterial diversity is limited by the presence of variable copy numbers in bacterial genomes and sequence variation within closely related taxa or within a genome (Vetrovsky and Baldrian, 2013). Although it has been validated that 16S rRNA gene sequencing with a less than 97% of similarity with the closest strain represents a new species, it is not as definitive as DNA-DNA hybridization, however, Bavykin *et al*., 2004 revealed that rRNA and gyrB sequences may be used for discerning B. anthracis from other members of B. cereus group. In the current study, the 16S rRNA gene was targeted as it is considered one of the most common methods to ascertain bacterial taxonomy and phylogeny for the reasons that it is present in all bacteria, its function has not changed over time (Das *et al*., 2014) and, random sequence changes in 16S rRNA gene can be used as a most accurate measure of evolution, to name a few. Moreover, even a similarity score of more than 97% may be due to representation of new specie or due to the clustering within an already defined taxon. Though 16S rRNA sequencing is universally being used for bacterial identification, there is no definite 'threshold value' above which any specimen can be ranked as a new specie. According to Janda *et al*., 2007, there should be a minimum of 99% and ideally 99.5% of sequence similarity with the reference strain as a criterion of specie identification.

Veterinary Research Institute, Lahore has been producing Anthrax Live Spore Vaccine from a long time, and the strain being used for vaccine production, according to departmental records is *Bacillus anthracis* strain 'Sterne'. The microorganism is regularly being tested biochemically and through laboratory animal trials yet there is no documented proof of its being strain 'Sterne'. Therefore the purpose of the current study was; to ascertain the strain of microorganism used for Anthrax vaccine production, to observe any point or frameshift mutation and to conduct phylogenetic analysis of strain under study against various related strains and species.

MATERIALS AND METHODS

Reference Sequence and Primer Designing: National Center for Biotechnology Information **(**NCBI) gene bank was used to search and select the 16S rRNA gene of the selected B. anthracis Sterne strain (Accession:AE017225), B. anthracis strain 1144 (Accession:CP010852) and B. anthracis strain V770-NP-1R (Accession:CP009598). Specific primers for amplification of 1861 bp region of said gene were designed by the software "Primer 3" (http://primer3.ut.ee/). The primers were checked for their specificity on NCBI Primer Blast. Two more forward primers were designed from mid regions of the reference sequence for the purpose of sequencing (Weisburg *et al*., 1991) so that the whole gene region could be covered for sequencing efficiently.

Table 1. Amplification and sequencing primers specification

Primer	Sequence	Purpose	Annealing (^{0}C)	Product (bp)
B.ant R	GCTAAGGCCCCAAATTGTAT	Amplification	51	
B.antF-1	CGCGATGTTGAACTTTGAAA	Amplification Sequencing	51	1861
B.antF-2	TAATACGTAGGTGGCAAGC	Sequencing	51	1241
B.antF-3	GACTGCCGGTGACAAAC	Sequencing	51	616

DNA extraction: 20 samples of reported *Bacillus anthracis* Sterne strain growth were harvested with normal saline from Raux flasks cultured from different batches of bacterial growth and transferred to eppendorf tubes. Moreover, bacteria were also isolated from the guinea pigs inoculated with vaccinal organism. Two replicas were prepared for each sample. Briefly, all samples were incubated at 45°C for 12 hours with 400 µL of Extraction buffer, 20 µL of 10% Proteinase K, and 20 µL of 10% Lysozyme. DNA was extracted by standard PCI (Phenol-Chloroform-Isoamyl Alcohol) method (Fong *et al*., 2009). Precipitation was done by 100 % Isopropanol followed by purification with 70% and 100 % ethanol.

Amplification: Genomic region of 1861 bp was amplified using primers "B. antF-1 and B. antR" in Bio-Rad thermal cycler. A total of 25 µL PCR mix was prepared and subjected to cycling conditions with initial denaturation of 95^{0}C for 5 min; 30 cycles of denaturation at 95^{0}C for 30 sec, annealing at 51^{0}C for 30 sec and extension at 72^{0}C for 2 min (Table 2). Final extension at 72^{0}C was done for 10 minutes, the as per reported Method by (Ausubel *et al*., 1987).

Table 2. PCR mix preparation (per one reaction, final volume of 25 µl)

Reagents	Volume*
PCR Buffer A (1x)	2.5 µL
dNTPs (5mM)	4.0 µL
PF (10 pM)	0.5 µL
PR (10 pM)	0.5 µL
DNA polymerase (Red Taq)	2U
Water (PCR-grade)	13.7 µL

*1.0 µL of template DNA was added to the above PCR mix to make a final volume of 25 µL.

PCR Product Purification: DNA was subjected to Agarose Gel Electrophoresis using 1 kb DNA marker. Briefly, 0.8 % agarose gel was prepared in 1X TAE buffer and stained with Ethidium Bromide. 2 µL of DNA from each sample loaded on the gel and allowed to run in 1X TAE buffer for 30 minutes. Genomic DNA bands were compared with the specific marker (Sambrook and Russell, 2006).

Sequencing: Amplicon of 1861 bp was subjected to sequencing reaction for two amplification primers "B. ant F-1 and B. ant R" as well as for two additional intragenic forward primers "B. ant F-2 and B. ant F-3". Sequencing reaction was carried out by following Sanger di-deoxy method (Brock *et al*., 1992) using BigDye® Terminator v3.1 Cycle Sequencing Kit. Sequencing reaction was followed by the capillary electrophoresis (Luckey *et al*., 1990) in ABI PRISM 3730xl Genetic Analyzer to achieve the sequencing data.

RESULTS AND DISCUSSION

The amplification product which included the 16S rRNA gene region plus the flanking regions when subjected to electrophoresis against 1 kb DNA ladder appeared to be around 1861 bp which was confirmed by sequencing. The gene of interest in this study, the 16S rRNA gene, had a size of 1510 bp, the rest of it being the flanking region, and was found obligatory as a better primer designing option. The sequence of 16S rRNA gene was submitted to NCBI and the Accession number was SUB863363 B.ant KP942847.

All the sequenced samples of *Bacillus anthracis* Sterne strain (n=20), were lyophilized as separate batches during past 15 years, yielded 100% homology in terms of nucleotide sequence with each other and with the reference strain as well. It was pertinent to mention that the requisite sequence obtained through three forward

primers was also subjected to proof reading due to involvement of a fourth reverse primer.

Phylogenetic analysis revealed that the microbe under study lies, as relevant literature emphasizes, in the cluster of *Bacillus cereus* group with closest 16S rRNA sequence affinity (other than *Bacillus anthracis Sterne strain* Accession:AE017225) with B. anthracis strain 1144 (Accession:CP010852) and B. anthracis strain V770-NP-1R (Accession:CP009598). Moreover, the sequenced organism was found genetically close to B. cereus than to B. thuringiensis with respect to 16S rRNA gene. These results were corroborated by the findings of Keim *et al.* (1997) who reported that B. cereus and B. thuringiensis were the closest taxa to B. anthracis, with B. mycoides slightly more distant.

A number of studies were conducted regarding mutations in 16S rRNA gene and its impact on resulting products, on bacterial characteristics and on phylogeny, as it is universally being considered a tool for taxonomic analysis of a wide variety of bacterial specimens. Suzuki *et al*. (1998), Gerrits *et al*. (2006), Finken *et al*. (1993) and Springer *et al*. (2001) in their respective studies on 16S rRNA gene-related drug resistance in *Mycobacterium tuberculosis, Helicobacter pylori, Mycobacterium tuberculosis*, and Mycobacterium spp. mentioned a significant account of mutations in 16S rRNA gene, however, the case may not be consistent with all the bacterial species, as was evident by studies on members of *Bacillus cereus* group. Ivanova *et al.* (2003) in their study on comparative analysis of genome in *Bacillus cereus* and *Bacillus anthracis* found only a couple of differences in the 16S rRNA sequences between two aforementioned species; probably a similar sort of findings urged Helgason *et al.* (2000) to report, on the basis of genetic analysis, *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* as one specie. Our study revealed that there isn't any change of even a single nucleotide in 16S rRNA sequence of the strain under study since its procurement emphasizing the fact that point and/or other sort of mutation(s) was a rare phenomenon with respect to 16S rRNA gene of Bacillus anthracis. This fact had been corroborated by various studies including that of Keim *et al.* (1997) who studied molecular evolution and diversity in *Bacillus anthracis* using AFLP markers and stated that *Bacillus anthracis* might be the most genetically uniform bacterial species known, and also reported a strain of *Bacillus anthracis* that had remained stable over at least 30 years.

The results of the present study revealed that *Bacillus anthracis* and related organisms could be successfully characterized using 16S rRNA gene sequence as there was a minimum chance of mutation in the sequence of said gene. The facts that the sequence under study was found 100 percent similar to that of reference strain and that frequent mutations especially in the 16S rRNA gene region was not the characteristic of *Bacillus anthracis* proposes that the strain used for the production of vaccine against Anthrax need not to be so frequently revitalized as in case of microorganisms depicting frequent point and/or other sort of mutations. Phylogenetic analysis conducted under the current study opened a new area of research for finding ways to synthesize common biologics providing immunogenic results against members of *Bacillus cereus* group by comparative study of immunogenic regions. Similar trials can be undertaken to observe the immunity provided by *Bacillus anthracis* Sterne strain against strains and types of *Bacillus anthracis* prevalent in Pakistan and against the notorious Volum strain as well.

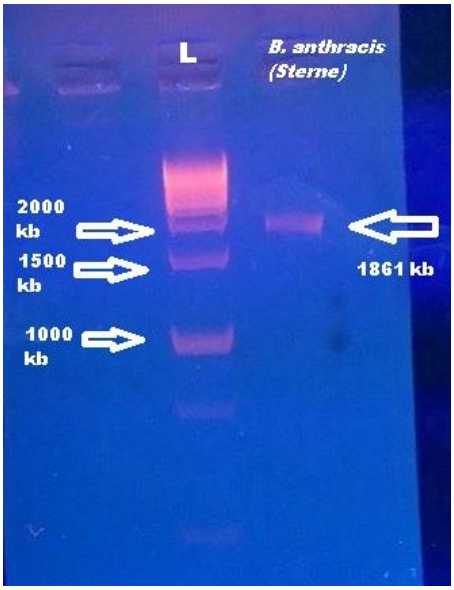

Fig-1: Gel result of amplified product against DNA Ladder

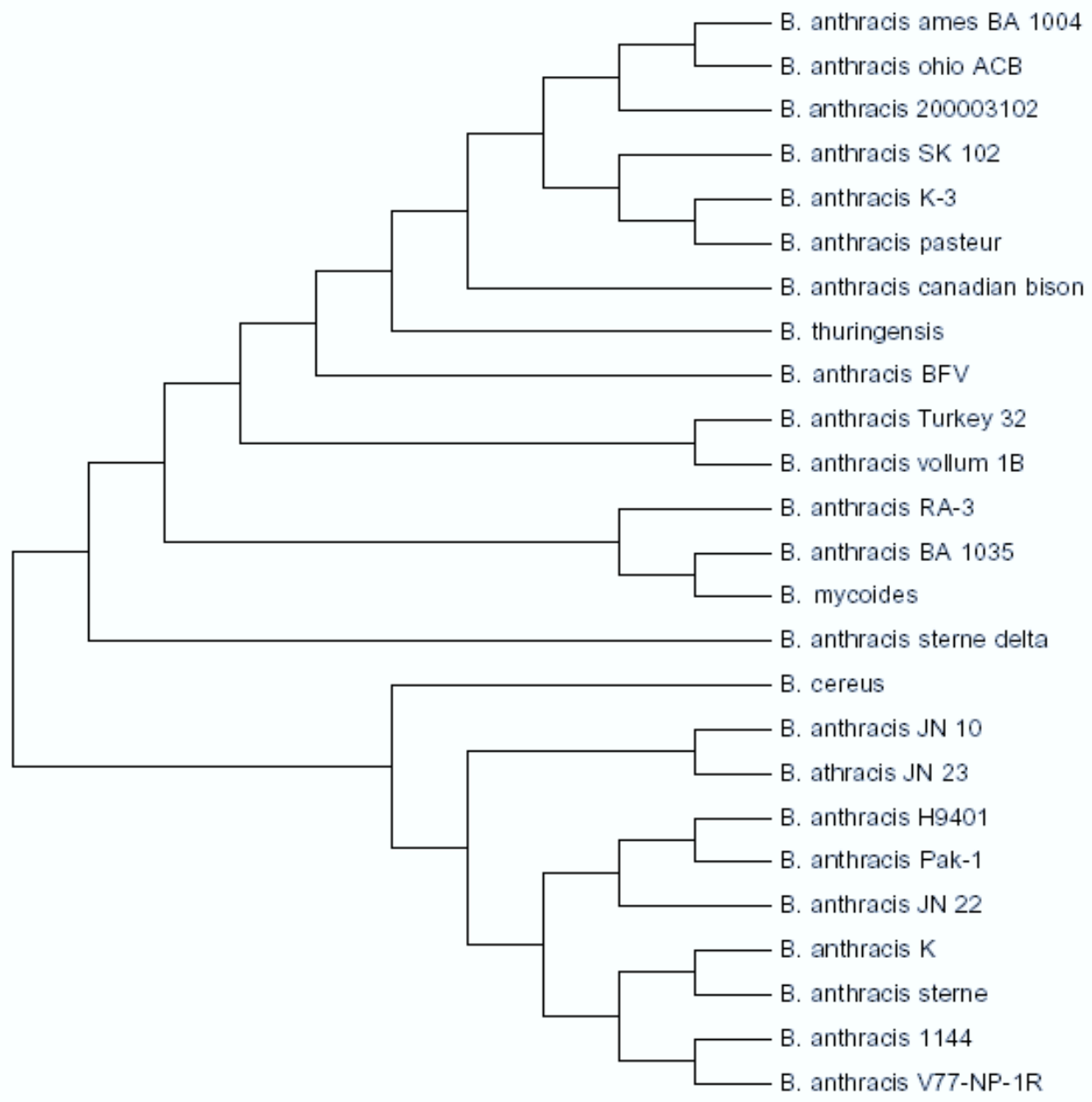

Fig-2: Phylogenetic tree (by Maximum likelihood method) of *Bacillus cereus* group w.r.t 16S rRNA gene sequence

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