Introduction

Anthrax is a dreadful fulminate, rather an uncommon fatal bacterial disease, caused by Gram positive bacterium, *Bacillus anthracis*. These bacteria, members of "*Bacillus cereus* group" (Leonard et al., 1998), are known as double edge biological weapon due to their serious disparaging effect on wide range of animals as well as on human being (Pal, 2013). Virulence of *B. anthracis* chiefly relies on a tricomponent toxin and a poly-β-D-glutamic acid capsule, carried by pXO1 and pXO2 plasmid, respectively (Ghosh et al., 2013). The pathological consequences in human being develop as cutaneous anthrax (malignant pustules), pulmonary anthrax (woolsorter’s disease) and gastrointestinal anthrax (splenic fever) after contracted via cutaneous, inhalation or oral route (Dixon et al., 1999). The swift death symptoms and short incubation period provides only diminutive therapeutic options, making its earlier diagnosis and prophylactic immunization of animals and human, the most strategic methods to combat the disease. However, the presently available vaccines due to its non-satisfactory performance make prophylaxis strategy distant from the ideal (Leppla et al., 2002). Thus, to develop a highly efficient anthrax vaccine, it is very essential to understand the protective efficacy and the basis of possible attenuation by characterization of the virulence factors of unstudied *B. anthracis* strains. Recently, Sunita et al. (2015a) made an attempt to determine the production of one virulent factor (extracellular DNAse) by Sterne strain and a virulent strain (IVRI strain) of *B. anthracis* in comparison with other principal bacterial species. Apart from this, description of several *B. anthracis* strains still continues as an untouched focus of investigation in the scientific field. The disease being a human health hazard causing worldwide terror along with its increasing incidence, complex epidemiological cycle and the ability to cause heavy loss to the livestock sector through animal deaths and loss of production makes its characterization a prime need of the hour.

Thus, in the present study, an attempt was made to evaluate the characteristics of unstudied IVRI strain by conventional means following comparison with Sterne strain. Simultaneously, antibiogram was also studied, which showed almost similar pattern for both the strains except co-trimoxazole resistance by IVRI strain. In addition, growth pattern studies were also executed in which faster growth rate and short generation time was shown by the IVRI strain.

Materials and Methods

Bacteria strains

*B. anthracis* IVRI and Sterne (34 F2) strains used in
the study were initially obtained from Bacteriological Laboratory, Mukteshwar in 1975 and since then it is maintained by the Division of Biological Standardization, IVRI, Izatnagar.

Characterization of *B. anthracis*

Morphological, cultural and biochemical characterization of both the strains were carried out as per the WHO guidelines. The freeze dried *B. anthracis* IVRI strain culture was reconstituted in 1 ml sterile physiological saline solution, plated on nutrient agar plates and incubated at 37°C for 24 h. Purity of the growth was checked by Gram’s staining and motility. Further, single colony was inoculated on various media like nutrient broth, blood agar, bicarbonate serum agar and DNase agar with subsequent incubation at 37°C for 48 h. The characteristics of growth produced on each media were observed. Morphological characterization was done microscopically by Gram staining, spore staining and capsule staining. Further, confirmation was done by various biochemical tests like Indole, Citrate, Methyl Red, Nitrate, Catalase, gelatin and Sugar fermentation test and compared with *B. anthracis* Sterne (34 F2) strain.

Growth curve of *B. anthracis*

*B. anthracis* Sterne (34F2) and IVRI strains were streaked on nutrient agar plates and incubated at 37°C for 48 h. Purity of the growth was checked by Gram staining and motility. Subsequently, single colony was inoculated in nutrient broth and incubated at 37°C for 48 h. The OD was taken at 600 nm at 0, 1, 4, 6, 8, 12, 24, 48 and 72 h and a graph was plotted.

**LD**<sub>50</sub> of *B. anthracis* spores

Spore suspension was prepared as per the method described by Bincy (2012). The spore suspension was diluted 10 fold up to 10<sup>-10</sup> and starting from highest dilution, 0.1 ml from each dilution of 10<sup>-10</sup>, 10<sup>-9</sup> and 10<sup>-8</sup> was inoculated into nutrient agar plates and incubated at 37°C for 24 h. The dilution showing 30-300 colonies were selected. Average number of colonies per plate was multiplied by the dilution factor to obtain the viable count per ml in the original suspension. A tenfold serial dilution was prepared from this spore suspension and groups of 3 mice were inoculated intraperitoneally with 0.1 ml from each dilution ranging from 10<sup>-2</sup> to 10<sup>-8</sup>. All the inoculated mice were observed for 7 days. The LD<sub>50</sub> was calculated as per the method described by Reed and Muench (1938).

Antibiogram for *B. anthracis*

For the disc diffusion testing, ampicillin (30 µg), doxycycline HCl (30 µg), nalidixic acid (30 µg), kanamycin (30 µg), co-Trimoxazole (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), amoxyclav (30 µg), and tetracycline (30 µg) (Hi Media, India) discs were used. Bacterial cultures were prepared in Nutrient broth at a density adjusted to a Brown’s opacity tube No. 1 (the turbidity standard for the disc diffusion test). The final inoculum was placed on Mueller–Hinton agar plates (Hi Media, India) by spread plate method, discs were positioned at appropriate distances and plates were incubated at 37°C for 24 h. Clear zone around the disc was measured.

Results and Discussion

*B. anthracis*, one of the most highlighted bio-terror agents, has been known since antiquity (Ghosh et al., 2013) for causing solemn devastating disease named anthrax, in mammalian livestock and in humans (Pal, 2013). The disease is still enzootic in several developing countries and responsible for huge economic losses and mortality (Beyer and Turnbull, 2009). Because of its quick onset and rapid progression, early diagnosis and prophylactic immunization of animals and human are the most strategic methods to combat it. The limitations of presently available vaccines for humans and small ruminants (Sterne, 1959; Nass, 1999) generates the vital momentum for the development of a safe and more effective live attenuated vaccine against anthrax and many methods had already been attempted in this field (Little et al., 2004; Yan et al., 2008., Sumithra et al., 2014; Sunita et al., 2015b). However, to develop a highly efficient anthrax vaccine, characterization of the virulence factors of unstudied *B. anthracis* strains is very imperative to understand the protective efficacy and the basis of possible attenuation to devise an efficient therapeutic as well as vaccine candidate. So, in the present study an attempt was made to characterize *B. anthracis* IVRI strain and to find out the variability vis-a-vis 34F2 vaccine strain (Sterne) as type species by morphological, cultural and biochemical means.

Conventional microbiological methods have been considered as the gold standard tests for identification of *B. anthracis* for decades. These are based on morphological features, which are observed after growth and staining of microorganisms with various stains. The present study was mainly planned to characterize the morphology of anthrax bacteria by using Gram’s stain and cultivation in nutrient broth, nutrient agar, DNase agar, blood agar and capsule agar bicarbonate serum agar. Microscopically, *B. anthracis* colony showed central dense and peripheral wavy hair like growth with Medusa head appearance (Fig. 1). Gram’s staining showed both strains to have the typical morphology of *B. anthracis* bacterium, which is a non-motile Gram-
positive rod shaped organism with truncated ends arranged in chains of varying length (Fig. 2) which was in accordance to the observations of other investigators (Koch, 1876; Murshidul, 2012; Dipti et al., 2014). The endospores formed varied in shape from spherical to elliptical with dissimilar location. On staining with 1% polychrome methylene blue, capsule was seen in case of *B. anthracis* IVRI strain, whereas it was absent in case of *B. anthracis* Sterne strain.

As *B. anthracis* is a facultative anaerobe, it can multiply readily in a variety of common laboratory media using multiple sugars and amino acids as the carbon sources (Puziss and Wright, 1959; Charlton et al., 2007). This study also provided the evidence that, in nutrient broth both the strains of *B. anthracis* grow as planktonic cell and produce long interwoven chains appearing like cotton wool. On stationary incubation, pellicle formation was seen without turbidity. Pellicle formation by Sterne strain was slow and easy to break apart but that produced by the IVRI strain was faster in growth and difficult to break apart. On nutrient agar, both the strains produced flat or slightly convex colonies with irregular edges, varying from two to several millimeters in diameter. Sterne strain produced colonies smaller in size, whereas IVRI strain produced comparatively large, rough colony with frosted glass appearance and curled edges. Tenacity of IVRI strain was comparatively higher than the Sterne strain and it adhered firmly to the agar surface. Specific differences in the degree of tenacity have been associated with genotypic groups (Smith et al., 2000). On blood agar, the colonies formed were larger in size when compared to that in nutrient agar and the IVRI strain produced larger colonies with a glossy glass appearance when compared to that formed by Sterne strain. Hemolysis was absent in both the strains. However, weak hemolysis occurred in older cultures of *B. anthracis* IVRI strain after 96 h. The growth characteristics on DNase test agar (DTA) were same as that in nutrient agar. But in case of DTA once the HCl had been applied, the test had to be read
Table 1. Growth curve of *B. anthracis* sterne 34F2 and IVRI strain

<table>
<thead>
<tr>
<th>Strains</th>
<th>Optical Density at 600 nm at different time interval (H)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>B. anthracis</em> IVRI strain</td>
<td>-</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne 34F2</td>
<td>-</td>
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</tbody>
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Table 2. Antibiogram of *Bacillus anthracis* Sterne 34F2 and IVRI strains

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Strain</th>
<th>AMP</th>
<th>DO</th>
<th>NX</th>
<th>K</th>
<th>COT</th>
<th>CTR</th>
<th>C</th>
<th>TE</th>
<th>AMC</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em> IVRI strain</td>
<td>24</td>
<td>30</td>
<td>34</td>
<td>20</td>
<td>-</td>
<td>16</td>
<td>24</td>
<td>34</td>
<td>34</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne 34F2 strain</td>
<td>24</td>
<td>30</td>
<td>34</td>
<td>24</td>
<td>18</td>
<td>18</td>
<td>24</td>
<td>32</td>
<td>32</td>
<td>30</td>
<td></td>
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</tbody>
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* Zone diameter around disc in millimeter (mm); *AMP- Ampicillin, DO - Doxycycline HCl, NX - Nalidixic acid, K - Kanamycin, COT- Co-Trimoxazole, CTR- Ceftriaxone, C- Chloramphenicol, TE- Tetracycline, AMC- Amoxyclyav, CIP- Ciprofloxacn

Fig. 3. S-shaped growth curve of *Bacillus anthracis*

and could not be continued by re-incubation (Langlois et al., 1998). On DTA containing 0.005% methyl green (DMG) both strains were incapable to grow because of the inhibitory nature of methyl green dye. Whereas on DNase test agar containing 0.005% toluidine blue (DTB) both strains produced a bright pink zone surrounding the colony on a royal blue background. *B. anthracis* Sterne strain produced characteristic growth after 48 h whereas as IVRI strain grew within 24 h and produced a larger zone than the Sterne strain indicating higher production of extracellular DNase, thus revealing its more virulent nature (Sunita et al., 2015a). Both the strains were unable to grow on MacConkey agar due to the inhibitory effect of bile salt. On Bicarbonate serum agar *B. anthracis* Sterne strain produced smooth, round, small and non-mucoid colonies because of absence of capsule where as IVRI strain produced large, round and mucoid colonies when incubated at 37°C with 10-15% CO₂. The “CO₂/ bicarbonate effect” on toxin and capsule synthesis is long known (Puziss and Wright, 1954; Thorne, 1993). Virulent strains of *B. anthracis* are invariably capsulated in-vivo but form capsules in-vitro only under special conditions which includes incubation in air with added CO₂, (Ivbnovics, 1937) and the customary use of nutrient agar containing either horse serum (Sterne, 1987) or bicarbonate (Thorne, 1956; Meynell and Meynell, 1964; Green et al., 1985). In present study, *B. anthracis* IVRI strain also produced mucoid colonies indicating more virulence than the reference strain.

To confirm the pathogen identity of the suspected isolates, further characterization by biochemical or molecular techniques must be carried out (Irenge and Gala, 2012). Biochemical tests are the pattern recognition systems based on metabolic characteristics of isolates (Bailie et al., 1995). In present study, both strains of *B. anthracis* conferred same biochemical results viz. Citrate-ve, Indole -ve, catalase +ve, nitrate +ve, Gelatin liquefation +ve with variable carbohydrate fermentation test results (positive for D-glucose, maltose, sucrose and trehalose whereas negative for lactose, L-rhamnose, L-arabinose, cellobios, D-mannitol, salicin, glycerol, inulin, raffinose and sorbitol) depending on the sugar used for test which are in consonance with the findings of Jula et al. (2011). But these assays are difficult to use as its utility is limited by quality and completeness of available databases, which requires indeed requires a regular update to maintain the value of the tool (Irenge and Gala, 2012).

Looking into little information available regarding the growth behavior, including generation time and lag time of *B. anthracis*, an attempt was also made to draw definite growth pattern of Sterne and IVRI strain. Both the strains attained stationary phase after 12 h (Table 1) whereas after 72 h bacteria were observed to attain the decline phase. Lag period for Sterne strain was found slightly longer than IVRI strain and generation time of virulent IVRI strain reported here was shorter than for Sterne strain. The course was most conveniently represented in graphical form as S-shaped growth curve (Fig. 3). Siano et al. (2007) also compared generation and
lag times of virulent B. anthracis strains with avirulent Sterne strain and ended into same conclusion that virulent and avirulent strain along with B. cereus have similar growth curve but avirulent Sterne strain possessed slightly longer generation and lag time when compared to virulent B. anthracis strains. To evaluate pathogenicity of B. anthracis IVRI strain lethal dose was calculated by injecting mice intraperitoneally with spores and vegetative cells. It was found that, LD50 of B. anthracis IVRI strain spores was 10^-6.75 while it was 10^-6.33 for Sterne strain 34 F2.

Prevention of anthrax infection relies mainly on series of vaccinations and prolonged antibiotic treatment. Owing to limited global availability of the anthrax vaccine, most treatment strategies utilize antibiotics. The drugs of choice for post-exposure prophylaxis include: penicillin G, amoxicillin, doxycycline, and ciprofloxacin or ofloxacin given for 60 days or more (Inglesby et al., 2002; Athamna et al., 2004). In present study, antibiogram was determined for IVRI and Sterne strain of B. anthracis with an aim to elucidate pattern of resistance to antibacterial agents belonging to various classes, in particular to those used for treatment of anthrax. It was found that both Sterne and IVRI strain showed same results with highest susceptibility for Ciprofloxacin followed by Tetracycline, Amoxyclav and Nalidixic acid (Table 2). However, IVRI strain showed resistance for Co-Trimoxazole to which the Sterne strain was susceptible. Dipti et al. (2014) also found sensitivity to penicillin, streptomycin, amoxicillin and kanamycin. The same findings were found by Doganay et al., 1991; Odendaal et al., 1991; Mohammed et al., 2002; Cavrollo et al., 2002 and Athamna et al., 2004.

The present study is only a preliminary effort to study the morphological and cultural difference between the two strains and to find out the genetic variability, advanced molecular techniques should be used for further intensive research.

Acknowledgement

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