Characterization of Ethidium Bromide Resistant Phenotypic Mutants of *Bacillus anthracis*

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**ABSTRACT**

Anthrax is a fatal septicaemic disease affecting a wide range of species, including humans. The disadvantages of live spore veterinary vaccine justify the extensive interest in development of improved safer vaccines against anthrax. In present study, ethidium bromide, the DNA-intercalating agent was used as a mutagenic agent to create various undefined mutations. The mutants so developed were evaluated for various virulence indicators like hydrophobicity, which ranged from 10.53 - 30%, adhesibility, which ranged from 13.11 - 55.37% and antibiotic sensitivity test where all the mutants showed somewhat similar pattern except strain M2, M3 and M7, which were resistant to co-trimoxazole. Remarkable differences observed among the developed mutants of *Bacillus anthracis* clearly indicates strain variability, which might have been occurred because of mutational events.

**Keywords**: Adhesibility, *Bacillus anthracis*, ethidium bromide, hydrophobicity

**Introduction**

*Bacillus anthracis*, the causative agent of anthrax is a Gram-positive, spore-forming bacterium, either aerobic or facultative anaerobic and has been reported as a potential bioterrorism weapon due to its dormant spores that can survive with high stability and low mortality in severe environmental conditions (Wang *et al.*, 2013). Anthrax is primarily a disease of herbivores, but all mammals, including humans, may be affected. The lethality has been attributed to two main aspects of pathogenesis: the activity of the bacterial exotoxins and the remarkable proliferous nature of the bacteria in the host, resulting in massive bacteraemia and consequently generalized systemic failure and death (Chitlaru *et al.*, 2011). The rapid progression of systemic anthrax from nonspecific initial symptoms to death with little opportunity for therapeutic intervention makes the immunoprophylaxis as a most effective defensive strategy against anthrax (Dixon *et al.*, 1999; Inglesby *et al.*, 2002).

A suspension of viable spores from a toxigenic, nonencapsulated strain, commonly used for immunization of livestock today, has an inherent limitation of virulence to small ruminants leading to mortality (Sterne, 1939; Cartwright *et al.*, 1987). Various approaches are being followed continuously for generation of a safe and more efficacious vaccine for the small ruminants, which encompasses the use of recombinant protein based immunogens but only with limited success. Thus, there is need to develop some live attenuated vaccine strain, which can overcome the limitations of live spore vaccine as well as can generate stable and prolonged immunity in these animals. The precise mechanism in this aspect is development of mutants by knockout of virulence genes or by generating some undefined mutations leading to the loss of virulence. A simple, flexible and low-cost molecular manipulation strategy to achieve such mutation in protective antigen (PA) will be highly advantageous in making an anti-anthrax therapeutic cum vaccine agent (Sumithra *et al.*, 2014). Various methods of mutagenesis can be followed and among them, ethidium bromide is well defined mutagenic agent, which may cause various undefined mutations. The mutants so developed are needed to be assessed for the various virulence indicators, which include the study of basic characteristics like hydrophobicity, adhesibility and antibiotic sensitivity.

Bacterial adhesion has been implicated as a possible virulence factor for several pathogenic microorganisms. The role of hydrophobic interactions in the adhesion of
bacteria to the surfaces of inert materials has been addressed in various studies (Marshall, 1985; Rosenberg, and Kjelleberg, 1986; Busscher and Weerkamp, 1987). Established techniques for measuring surface hydrophobicity include adherence to hydrocarbons (Rosenberg, 1984; Beck et al., 1988), hydrophobic interaction chromatography (HIC) (Doyle et al., 1984; Ismaeel et al., 1987), salt aggregation (Takubo et al., 1988) and contact angle measurements (Minagi et al., 1986). Thus, in present study we characterized different mutants developed for relative hydrophobicities by observing bacterial adherence to xylene as well as to inert surface, and also evaluated the antibiogram to access the effect of mutations on sensitivity of these ethidium bromide resistant mutants.

Materials and Methods

Bacteria strain and cultivation

*B. anthracis* strain 34F2 was obtained from Division of Biological Standardization, IVRI, Izatnagar. The freeze dried *B. anthracis* 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 test. Subsequently, single colony was inoculated in nutrient broth and incubated at 37°C for 24 h. Ethidium bromide (50 µg/ml) was serially diluted in 10 ml nutrient broth under the sterile conditions, 100 µl of *B. anthracis* fresh culture was added to each tube and incubated at 37°C for 24 h. Consequently, the highest dilution showing growth was selected and again sub-cultured in nutrient broth containing same concentration of ethidium bromide. The sub-culturing was continued for 10 passages with simultaneous culture on nutrient agar plates and purity of the growth was checked before each sub-culture. After 10 passages plates were observed for phenotypic characteristics and eight mutants were selected, and further characterized for surface hydrophobicity, adhesion to inert surface and antibiotic sensitivity. Presence of plasmid (pXO1) was confirmed by using self designed *pag* gene (encoding PA) specific primers (Frd-5’AAAGCTAGCGTTC CAGACCGTGACAATGA3’, Rev- 5’AAAGGATCCTTATCCTATCTCATAGCCITTTTT A GAA3’).

Measurement of the cell surface hydrophobicity

The cell surface hydrophobicity indexes of the *B. anthracis* mutant strains were determined according to technique described by Rosenberg et al. (1980) with slight modification. For this purpose, fresh culture of selected mutants and original Sterne strain culture in nutrient broth were centrifuged for 30 min. Pellet was re-suspended in 1.25 ml sterile normal saline solution and absorbance was taken at 600 nm. Thereafter, ability of all strains to adhere to xylene was determined by adding 0.5 ml xylene to re-suspended pellet followed by vigorous vortexing for 30 sec and left on the bench at room temperature for 20 min to allow the separation of the two phases. The absorbance of aqueous phase present at the bottom of the tube was measured at 600 against PBS. The index of hydrophobicity (HI) was calculated by using following formula:

\[
\text{Hydrophobicity Index} = \frac{\text{OD}_{600} \text{ of control} - \text{OD}_{600} \text{ of test}}{\text{OD}_{600} \text{ of control}}
\]

The hydrophobicity of the bacteria was expressed in percentage of bacteria that adhered to xylene and values are presented in Table 1.

Adhesion of mutants to inert surface

One hundred microlitre, fresh culture of selected mutants and original Sterne strain culture in nutrient broth were added in microtitre plate and OD was taken at 600 nm. Bacteria were allowed to adhere at 37°C for 2 h and the wells were washed carefully with 100 µl of NSS to remove the nonadherent bacteria. Fresh nutrient broth (100 µl) was added to each well and plate was again incubated at 37°C for 4 h to allow the growth of bacteria. After 4 h, OD was taken at 600 nm. The adhesibility of the bacteria was expressed as the percentage of bacteria that adhered to inert surface and values are presented in Table 1.

Antibiotic susceptibility testing

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) discs (Hi-Media, India) were used. Bacterial cultures were prepared in nutrient broth at a density adjusted to a Brown’s opacity tube No. 1, 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 were measured and values are presented in Table 1.

Results

Bacterial strain and cultivation

The freeze dried culture of *B. anthracis* strain 34F2 revived on nutrient agar under aerobic condition showed typical cultural and morphological characteristics. It was further inoculated in nutrient broth and purity was confirmed by smear examination and motility test. After 10 passages, colony size decreased unexpectedly to pin head size and characteristic white, compact, opaque
Table 1. Percent hydrophobicity and adhesibility of various mutants of *B. anthracis* Sterne strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>% hydrophobicity</th>
<th>% adhesibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em> Sterne</td>
<td>20</td>
<td>30.08</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M1</td>
<td>11.11</td>
<td>16.98</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M2</td>
<td>22</td>
<td>38.52</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M3</td>
<td>30</td>
<td>55.37</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M4</td>
<td>10.53</td>
<td>13.11</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M5</td>
<td>21.43</td>
<td>30.50</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M6</td>
<td>21.43</td>
<td>28.44</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M7</td>
<td>20</td>
<td>30.61</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M8</td>
<td>18.18</td>
<td>19.44</td>
</tr>
</tbody>
</table>

Table 2. Susceptibility of *B. anthracis* mutants to selected antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</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>
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<td>24</td>
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<td>30</td>
<td>24</td>
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<td>18</td>
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<td>32</td>
<td>30</td>
<td>32</td>
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<tr>
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<td>22</td>
<td>36</td>
<td>30</td>
<td>22</td>
<td>12</td>
<td>20</td>
<td>24</td>
<td>32</td>
<td>32</td>
<td>32</td>
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<tr>
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<td>28</td>
<td>32</td>
<td>30</td>
<td>22</td>
<td>-</td>
<td>18</td>
<td>24</td>
<td>30</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
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<td>24</td>
<td>28</td>
<td>28</td>
<td>22</td>
<td>-</td>
<td>18</td>
<td>24</td>
<td>34</td>
<td>32</td>
<td>32</td>
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<tr>
<td><em>B. anthracis</em> Sterne M4</td>
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<td>32</td>
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<td>24</td>
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<td>24</td>
<td>32</td>
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<tr>
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<td>30</td>
<td>30</td>
<td>24</td>
<td>-</td>
<td>18</td>
<td>24</td>
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<td>30</td>
<td>32</td>
</tr>
<tr>
<td><em>B. anthracis</em> Sterne M8</td>
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<td>30</td>
<td>30</td>
<td>24</td>
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<td>18</td>
<td>24</td>
<td>36</td>
<td>32</td>
<td>34</td>
</tr>
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</table>

* Zone diameter around disc in millimeter (mm)

Determination of cell surface hydrophobicity

The adhesion of all mutant strains to xylene showed a wide range (10.53-30%), indicating that different strains varied from hydrophilic to hydrophobic (Table 1). The hydrophobicity of Sterne strain was 20% in xylene. Mutant M4 and M1 showed lowest hydrophobicity indexes with 10.53% and 11.11%, respectively; however Mutant M3 showed highest hydrophobicity index of 30%.

Determination of cell adhesion to inert surface

The strength of attachment of different mutant strains is shown in Table 1. The number of bacteria of the mutant strains that adhered to the microtitre plate was ranged from 13.11-55.37%. Adhesion to microtitre plate appeared to be strain specific and was not related to polymer composition. Sterne strain showed 30.08% adherence, Strain M4 and M1 showed lowest adherence 13.11% and...
16.98%, respectively, where as M3 showed highest adherence 55.37%, which can be correlated with hydrophobicity indexes of these strains. Statistically, Hydrophobicity (%) and Adhesibility(%) are positively correlated (p<0.01) with correlation coefficient of 0.94.

Antibiotic sensitivity test

The results of antibiotic sensitivity test are presented in Table 2. All the mutants showed somewhat similar pattern for all the antibiotics with highest susceptibility for doxycycline HCl, followed by tetracycline, ciprofloxacin and amoxyclav, and however strain M2, M3 and M7 showed resistance for co-trimoxazole.

Discussion

*B. anthracis* is a spore-forming, Gram-positive bacterium that causes a highly fatal septicemia disease of various animals (Twenhafel, 2010) leading to mortality and huge economic losses in various developing countries (Beyer and Turnbull, 2009). The limitations of presently available vaccines for small ruminants (Sterne, 1939; Sterne, 1959; Ivins and Welkos, 1988; Nass, 1999; Welkos et al., 2001; Little et al., 2006; Turnbull and Cosivi, 2008) generates the vital momentum for the development of a safe and more effective live attenuated vaccine against anthrax. Crameri et al. (1986) used ethidium bromide, DNA-intercalating agent to eliminate plasmid DNA from Streptomyces and also reported other mutational events associated with chromosome changes which occurred at high frequency resulting into phenotypic changes such as loss of enzyme activity, antibiotic resistance or ability to sporulate. In the present study, we used ethidium bromide as mutagenic agent which may produce various undefined mutations. After 10 passages colony size was decreased unexpectedly to smaller size with more compact arrangement of organisms in colony formation is indicative of mutational changes occurred in chromosomal DNA.

The hydrophobicity and charge surface of microorganisms affect their capacity to adhere to the surface (Hood and Zottola, 1995; Hood, 1996) which influences bacterial adhesion and proliferation. Vacheethasanee et al. (1998) showed that more hydrophobic *S. epidermidis* adhered to a greater extent than the less hydrophobic *S. epidermidis*. Hydrophobicity can be evaluated semi-quantitatively by bacterial adhesion to hydrocarbon (BATH), hydrophobic interaction chromatography and contact angle (van Loosdrecht et al., 1987; Pelletier et al., 1997). Oliveira et al. (2001) reported that, the surface charges and hydrophobicities of bacteria can vary between species, serotypes or strains, and can change with variation in growth conditions, physiological states of cells, and composition of suspension media. In this study, dramatic differences were observed in hydrophobicity between the mutants of *B. anthracis* which clearly indicates strain variability which might have been occurred because of mutational events.

Growth conditions such as culture medium, pH, salt concentration, organic compounds, time and temperature contact, agitation, substrate hydrophobicity, electric charge and its microtopography play an important role in bacterial adherence (Jeong and Frank, 1994; Zottola and Sasahara, 1994; Smoot and Pierson, 1998). The correlation between cell surface hydrophobicity and bacterial adhesion has been found by some authors (Dickson and Kooitmaaria, 1989; Benito et al., 1997), which can corroborated with present study as we found correlation coefficient of 0.94 between hydrophobicity and adhesibility.

The effect of mutagensis on drug resistance was observed by disc diffusion method of antibiotic sensitivity test. Three mutants M2, M3 and M7 showed resistance to co-trimoxazole indicating increase in virulence. As M1 and M4 showed lowest hydrophobicity, adhesibility and susceptivity to antibiotics, there may be chances of possible useful attenuations in these strains, and can be used as future vaccine candidates. The present study is only a preliminary effort to observe the mutagenic efficiency of ethidium bromide to create an attenuated strain of *B. anthracis*, and needs further intensive research.

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References


