Comparative evaluation of protective antigen produced from
*Bacillus anthracis* & *Escherichia coli*

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**Background & objective:** Anthrax has been reported from almost every country and India is endemic for this disease. There is considerable under reporting of the disease because of lack of microbiological facilities and diagnostic reagents. In India only conventional methods which have limitations, are being used to diagnose the disease. Hence the aim of this study was to isolate and purify protective antigen (PA) using different protocols and to use this PA for detection of anti-PA antibodies from sera samples.

**Methods:** Protective antigen was isolated and purified from the Sterne strain of *Bacillus anthracis*, *B. anthracis* lacking pXO1 and pXO2 transformed with pYS5 (*B. anthracis* pYS5) and recombinant *Escherichia coli* transformed with pQE30 containing PA gene using hydroxyapatite (HA), Q-sepharose fast protein liquid chromatography (FPLC) and nickel-nitritriacetic acid (Ni-NTA) chromatographic methods, respectively. A mixture of PA and edema factor (EF) was injected subcutaneously into rabbits to test the biological activity of the proteins. The immunogenicity of PA was tested by inoculating the protein into rabbits along with adjuvant. Using this PA, 20 bovine sera samples (pre- and post-vaccinated) were tested by Western blotting (WB) for the presence of anti-PA antibodies.

**Results:** The 83 kDa PA protein was obtained from all the bacteria with the yields of 13, 50 and 9.0 mg/l from Sterne *B. anthracis*, *B. anthracis* pYS5 and recombinant *Esch. coli*, respectively. Formation of edematous ulcers at the site of PA+EF injection clearly confirmed the retention of biological activity of the proteins. Of the 10 post-vaccination sera tested, 9 showed clear positive by WB whereas none of the pre-vaccination sera showed the reaction.

**Interpretation & conclusion:** The purified PA preparations obtained in the present study may possibly be utilized for detection of anti-PA antibodies in the sera of anthrax patients for timely diagnosis of the disease and, might also be tested for their efficacy and use as human anthrax vaccine.

**Key words** Anti-PA antibodies - *Bacillus anthracis* - biological activity - immunogenicity - protective antigen (PA)

Protective antigen (PA) of *Bacillus anthracis* is the central moiety of the anthrax toxin complex and it elicits antibody response useful for serodiagnosis of the disease. It is also the main immunogen of the cell-free vaccine against anthrax.

Culture supernatants of *B. anthracis* are the major source for purifying PA for various purposes like human vaccine1,2 and for preparation of disease diagnostic reagents3. The human vaccine licensed in the United States is aluminum hydroxide adsorbed

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111
sterile filtrates of V 770-NP1-R strain of B. anthracis\textsuperscript{4-6}. The United Kingdom vaccine consists of an alum precipitate of sterile culture filtrates of the Sterne strain\textsuperscript{6,7}. Although these vaccines are efficacious, they have certain limitations. The amount of PA varies from lot to lot and they contain traces of edema factor (EF) and lethal factor (LF) that purify along with PA\textsuperscript{7-9}.

Working with B. anthracis culture requires BSL-3 containment facilities that are not readily available in developing countries. Even the Sterne vaccine strain in relatively low doses (10\textsuperscript{3} spores) is lethal for certain inbred mouse lines\textsuperscript{10}. The possible infection by Sterne strain in unusually susceptible human beings cannot entirely be ruled out. In order to avoid handling of toxigenic B. anthracis for PA production, researchers started cloning the PA gene in different expression hosts such as B. subtilis\textsuperscript{11}, Esch. coli\textsuperscript{12,13}, B. anthracis\textsuperscript{14}, etc. Recently a strategy for the rapid purification of recombinant PA under non-denaturing conditions from Esch. coli has been reported\textsuperscript{15}. PA has also been constitutively expressed in Esch. coli DH5 alpha cells and a higher yield of protein obtained using batch culture technique in a fermentor\textsuperscript{16}.

There is considerable under reporting of the disease largely because of lack of adequate microbiological facilities and test systems for diagnosis of anthrax\textsuperscript{17}. In India only conventional identification methods are being used to diagnose the disease. Serological and molecular diagnostic methods are not in use due to the paucity of standardized reagents and there is no human vaccine in developing countries like India for prevention of the disease.

Therefore, in the present study efforts have been made to evaluate different systems of expression, purification and biological activity of PA from the Sterne strain of B. anthracis and from recombinant strains to achieve better yields and to utilize this purified protein for detection of anti-PA antibodies in bovine serum samples. The studies reported here may help to develop basic reagents for the timely diagnosis of anthrax and also for human vaccine.

Material & Methods

The study was carried out at the Defence Research and Development Establishment (DRDE), Gwalior, MP, India.

Bacterial cultures: Sterne strain of B. anthracis was procured from the Institute of Veterinary and Preventive Medicine, Ranipet, Vellore. Recombinant Esch. coli was obtained from Dr Rakesh Bhatnagar, Jawaharlal Nehru University, New Delhi. The PA gene along with 6X histidine-coding sequence was ligated into pQE30 vector. The SG13009 (pREP4) Esch. coli host cells were transformed with this vector\textsuperscript{18}. B. anthracis pYS5 was obtained from Dr Y. Singh, (Institute of Genomics & Integrative Biology, Delhi). The PA gene was inserted into a shuttle vector, pYS5 and the B. anthracis (lacking both pXO1 and pXO2) was transformed with this construct\textsuperscript{14}.

Sera & animals: Bovine sera samples, before and after vaccination with Sterne spores, were collected from Dr M.K. Lalitha, Christian Medical College & Hospital, Vellore, India. Mice (6-12 wk of age, wt. 15-20 g) and rabbits (1-1.5 kg) were obtained from the animal house of DRDE, Gwalior. All animal experiments were done with the approval of the ethics committee of the institute.

Reagents & chemicals: All media for toxin production were from Difco (USA). Ni-NTA gel was from Qiagen (Germany). Adjuvants and HRP-conjugated secondary antibodies were from Sigma Chemical Co. (USA). All other chemicals were purchased from E. Merck (India) unless indicated otherwise.

Isolation & purification of PA from Sterne strain of B. anthracis: (i) Media — Casaminoacids medium (CA)\textsuperscript{19} and R-medium\textsuperscript{20} were prepared, inoculated and incubated as described. In some experiments, the CA medium was modified by replacing CA either with tryptone or yeast extract (YE). In another modification, charcoal was omitted from the CA medium. Following incubation, the culture media were centrifuged at 6000 g for 30 min in a
refrigerated centrifuge to remove the cells and the supernatants were collected. A protease inhibitor, 1,10-phenanthroline was added to the culture supernatants (CS) to a concentration of 0.2 mM. Further processing of CS for purification of PA was carried out at 4°C.

(ii) Concentration and purification — Concentration and purification of PA from crude CS was carried out either by alum precipitation and citric acid-TCA method or by HA-chromatography. In another procedure, polyethylene glycol (PEG)-6000 was added to CS of R- and CA-media at a final concentration of 10 per cent and shaken till it dissolved. Then the protein was purified by HA-chromatography.

Expression & purification of protective antigen from recombinant Esch. coli: The PA from recombinant Esch. coli was expressed and purified as reported earlier. Briefly, the overnight grown culture of recombinant Esch. coli was inoculated into 500 ml of LB broth containing 100 µg of ampicillin and 25 µg of kanamycin/ml and incubated at 37°C at 200 rpm. When OD reached 0.8, the cells were induced for 5 h with isopropyl-thio-β-D-galactopyranoside (IPTG) at a concentration of 1.5 mM. The PA expressed in Esch. coli was purified by Ni-NTA chromatography under denaturing conditions.

Expression & purification of protective antigen from B. anthracis pYSS: The PA from recombinant B. anthracis was expressed and purified using the protocol given by Y. Singh (personal communication). Briefly, a colony of recombinant B. anthracis was inoculated into 100 ml of LB broth containing 100 µg of ampicillin and 25 µg of kanamycin/ml and incubated at 37°C at 200 rpm. When OD reached 0.8, the cells were induced for 5 h with isopropyl-thio-β-D-galactopyranoside (IPTG) at a concentration of 1.5 mM. The PA expressed in B. anthracis was purified by Ni-NTA chromatography under denaturing conditions.

Biological activity of toxins: In order to determine the biological efficacy of the isolated toxins, each purified preparation of PA along with EF was injected in to a rabbit in 1:2 ratio (20 µg of PA and 40 µg of EF) subcutaneously. PA and EF were also injected separately at different sites as controls. Formation of edematous ulcers at the site of injection on the skin of rabbit was considered as having biological efficacy of those PA preparations.

Assay of protective antigen: The antigenicity of purified PA preparations was tested by performing SDS-PAGE and WB. Each purified preparation was mixed with an equal quantity of SDS-lysis buffer and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose (NC) membrane using tris-glycine buffer containing 20 per cent methanol. Transfer was carried out with a constant voltage of 45 V for 1 h in a transblot cell (Hoefer Scientific Instruments, USA). The blots were developed with rabbit polyclonal antisera of PA and goat anti-rabbit polyclonal HRP conjugate.

The antibody response of each purified PA was tested following intramuscular inoculation into mice (2 mice were used for each preparation). The primary dose was prepared with 50 µg of PA along with Freund’s complete adjuvant and subsequent doses with the same amount of protein with Freund’s incomplete adjuvant. Booster doses were given at 14, 21 and 35 days following primary immunization. Seven days after the last booster dose, the sera were harvested from immunized animals and antibody titres were checked by ELISA.

Detection of anti-PA antibodies: Protective antigen at 5 µg/well was loaded onto a SDS-PAGE gel and the proteins were transferred onto NC membrane as described earlier. The membrane was blocked with 5 per cent skimmed milk in PBS overnight at 4°C. The blot was washed with PBS and cut into individual strips. These NC strips were incubated with 1 : 100 dilution of different pre- and post-vaccination bovine sera samples for 1 h at 37°C. After washing thrice with PBST (Tween-20, at a final concentration of 0.05 %), the NC strips were incubated with 1:1000 dilution of anti-bovine polyvalent HRP conjugate for 1 h at 37°C. The NC strips were washed three times, 5 min each, with
PBST and colour reaction was developed with diaminobenzidine (DAB)-H$_2$O$_2$ substrate system.

**Results**

Purification of protective antigen: The protein pellet obtained by alum precipitation of CA medium was dialyzed against distilled water and was purified by citric acid-TCA method. The SDS-PAGE analysis of this protein preparation showed a major 83 kDa PA band along with few other bands (Fig. 1, lanes 3-4). Similar results were obtained when charcoal was omitted from CA medium.

When HA chromatography was used, the proteins were eluted from HA column with a gradient of 0 M and 0.5 M potassium phosphate buffer, pH 7.0. The PA protein eluted at 50-110 mM phosphate gradient. These fractions were pooled and dialyzed. When PEG-6000 was used in the medium, the HA gel was washed thoroughly to remove traces of PEG. After eluting the protein, the PA containing fractions were pooled and dialyzed extensively. The HA purified preparations mainly comprised PA along with very few contaminant low molecular weight proteins as was evident from SDS-PAGE protein profile (Fig.1, lanes 5-7). The amount of PA obtained from the Sterne strain of *B. anthracis* using different media and methods was shown in Table I.

The PA protein present in the culture supernatant following lysis of recombinant *Esch. coli* cells was purified under denaturing conditions by Ni-NTA chromatography. The PA preparations obtained after Ni-NTA chromatography and Q-sepharose column were run on SDS-PAGE to check for the presence and purity of PA (Fig. 2). The comparative yields of PA collected from 3 different bacteria are shown in Table II.

**Biological activity:** At the site where a mixture of PA and EF were injected, edematous ulcers were observed on the skin of the rabbit. Ulcers started forming on day 2 and fully developed by day 4. No ulcer was formed where PA and EF were injected separately.

![Fig. 1. SDS-PAGE protein profile of different protective antigen (PA) preparations. lane 1: Molecular weight marker, lane 2: Standard PA, lane 3: Crude culture supernatant, lane 4: Citric acid-trichloro acetic acid (TCA) -purified PA, lane 5: Hydroxyapatite (HA) -purified PA, lane 6: Poly ethylene glycol (PEG) treated HA-purified PA, lane 7: PEG treated HA-purified PA from R-medium.](image-url)
Table I. Yields of protective antigen from Sterne strain of *B. anthracis* using different media and methods

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Medium</th>
<th>Concentrating agent</th>
<th>Purification method</th>
<th>Yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (a)</td>
<td>Casaminoacids medium</td>
<td>Alum</td>
<td>Citric acid-TCA</td>
<td>8.0</td>
</tr>
<tr>
<td>1. (b)</td>
<td>Casaminoacids medium (without charcoal)</td>
<td>Alum</td>
<td>Citric acid-TCA</td>
<td>7.5</td>
</tr>
<tr>
<td>1. (c)</td>
<td>Casaminoacids medium</td>
<td>Hydroxyapatite gel</td>
<td>HA-chromatography</td>
<td>8.0-8.5</td>
</tr>
<tr>
<td>1. (d)</td>
<td>Casaminoacids medium</td>
<td>Hydroxyapatite gel+PEG</td>
<td>HA-chromatography</td>
<td>12-13</td>
</tr>
<tr>
<td>2. (a)</td>
<td>R-medium</td>
<td>Hydroxyapatite gel</td>
<td>HA-chromatography</td>
<td>9.0</td>
</tr>
<tr>
<td>2. (b)</td>
<td>R-medium</td>
<td>Hydroxyapatite gel+PEG</td>
<td>HA-chromatography</td>
<td>13-15</td>
</tr>
<tr>
<td>3.</td>
<td>Tryptone</td>
<td>Alum</td>
<td>Citric acid-TCA</td>
<td>6-7</td>
</tr>
<tr>
<td>4.</td>
<td>Yeast extract</td>
<td>Alum</td>
<td>Citric acid-TCA</td>
<td>5-6</td>
</tr>
</tbody>
</table>

PEG, polyethylene glycol; HA, hydroxyapatite; TCA, trichloroacetic acid

**Antigenicity & antibody response**: The purified PA preparations were tested for their antigenicity by WB. When blots were developed with anti-PA polyclonal antibodies, all PA preparations showed a major 83 kDa PA band along with some low molecular weight degradation products of PA (Fig. 3). The titres of the immunized mice sera were checked by ELISA and reaction was observed up to a dilution of 1:51200.

**Detection of anti-PA antibodies**: Of the 10 post-vaccination bovine sera tested, 9 showed a clear positive reaction on WB whereas none of the pre-vaccination samples showed a reaction (Fig. 4).

Table II. Comparative yields of PA from three PA-producing bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Purification method</th>
<th>Yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterne strain of <em>B. anthracis</em></td>
<td>HA-chromatography</td>
<td>13-15</td>
</tr>
<tr>
<td>Recombinant <em>Esch. coli</em></td>
<td>Ni-NTA chromatography</td>
<td>9-10</td>
</tr>
<tr>
<td><em>B. anthracis</em> pYS5</td>
<td>Q-sepharose FPLC</td>
<td>50</td>
</tr>
</tbody>
</table>

**Fig. 2.** SDS-PAGE protein profile of purified protective antigen (PA) preparations from recombinant bacteria. lane 1: Molecular weight marker, lane 2: Nickel-nitrilotriacetic acid (Ni-NTA)-purified PA from recombinant-*Esch. coli*, lane 3: Fast protein liquid chromatography (FPLC)-purified PA from *B. anthracis* pYS5. **Fig. 3.** Western blot reaction of protective antigen (PA) with polyclonal anti sera. lane 1: Citric acid-trichloro acetic acid (TCA)-purified PA from casamino acids medium, lane 2: Hydroxyapatite (HA)-purified PA from CA medium, lane 3: HA-purified PA from R-medium.
Discussion

Anthrax is widely distributed and cases have been reported from almost every country. The actual incidence of anthrax in man and animals is not known accurately in the developing world as a large number of cases are unreported and only a fraction receives medical attention. In India, the states of Andhra Pradesh, Tamil Nadu and Karnataka are confirmed endemic regions with sporadic cases occurring frequently and at times resulting in small outbreaks. Although there are occasional reports of anthrax from other parts of India, there is considerable under reporting of the disease. In India only the conventional identification methods, which are rapid and confirmatory, are not used due to the non-availability of standardized diagnostic reagents. Recently we reported a simple and specific PA-reactive monoclonal antibody dot-ELISA for the confirmatory identification of B. anthracis. In the present work efforts have been made to purify PA and to utilize the same for diagnosis.

Among the strains reported to contain only pXO1, the Sterne strain has been widely used for the production of PA. In the present study also the Sterne strain was used for PA production. Both semi-defined and defined media like CA and R-medium were utilized for in vitro production of PA. In addition, certain modifications were tried in CA medium by replacing CA with either tryptone or yeast extract.

A significant amount of PA undergoes proteolysis resulting in the low level of purity as well as poor yield. Activated charcoal powder in the CA medium acts as a protein adsorbent to minimize the degradation of protein, thereby increasing the yields of PA. In the present study, CA medium with and without charcoal was used for PA production and it was found that the yields were not considerably greater in the medium containing charcoal.

Different systems have been reported for the concentration and purification of protein from dilute culture supernatants. Of them, sintered glass filtration for separation of PA from EF and LF, alum precipitation and citric acid-TCA methods have been employed and the resulting protein has been used for human anthrax vaccine. When these methods were used in the present study, the SDS-PAGE protein profile showed a PA band along with some contaminant proteins (Fig. 1). As TCA has some denaturing effects on proteins leading to low yields of PA, and precipitation with alum is time consuming during which an enormous amount of PA undergoes degradation, researchers started using HA gel for concentration and purification of PA. The HA-purified PA preparations were relatively more pure and have less degradation products than citric acid-TCA-purified ones (Figs 1 and 3). To collect the maximum amount of protein from the culture supernatant, addition of PEG-8000 at 10 per cent was recommended. In the present study higher yields of PA could be obtained when HA chromatography was used with PEG.

The purified protein preparations could retain their biological and functional activities as was evidenced by the appearance of edematous ulcers on the skin of rabbit where PA and EF were injected.

Of the various techniques, ELISA and WB (also called EITB, Electrophoretic Immuno Trans Blot)
have been reported to be more specific and sensitive for detection of anti-PA antibodies in the sera of anthrax patients\textsuperscript{25} and vaccinated individuals. In the present study also anti-PA antibodies were detected by WB.

The present study concludes that among different purification methods of PA, HA chromatography with PEG yields relatively higher amounts of protein from the culture supernatants of the Sterne strain of \textit{B. anthracis}. Maximum yields of PA were obtained from \textit{B. anthracis} pYS5 followed by Sterne strain of \textit{B. anthracis}, and recombinant \textit{Esch. coli}. The purified PA could detect anti-PA antibodies from bovine serum samples. Out of 10 of the post vaccination bovine sera samples tested, 9 showed a clear positive reaction on WB while one sample did not show a positive reaction. This could be because either the animal failed to elicit immune response following vaccination or had antibody levels too low to be detected by WB. The purified PA therefore may possibly be utilized for detection of anti-PA antibodies from sera of anthrax patients for timely management and control of the disease. It might also have potential for use as human anthrax vaccine.

References


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