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The Humoral Immune Response to Various Domains of Protective Antigen of *Bacillus anthracis* in Cutaneous Anthrax Cases in India

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ABSTRACT

Anthrax, caused by *Bacillus anthracis* is known to occur globally since antiquity. Besides being an important bio-threat agent, it is an important public health importance pathogen also in countries like India. *B. anthracis* secretes three distinct toxins, namely protective antigen (PA), lethal factor (LF) and edema factor (EF). PA is the central moiety of the anthrax toxin complex and therefore has been a molecule of choice for vaccine development. PA has four different domains with different functions. In this study, the major domains of PA were cloned and expressed in bacterial system. The purified recombinant proteins were used to determine the humoral immune response by ELISA using 43 human cutaneous anthrax serum samples. The maximum immunoreactivity was observed with the whole PA protein followed by domain 2, 4 and 1. The study corroborated that in addition to full PA, individual domain 2 and 4 can also be good target for vaccine development as well as for serodiagnostic assays for cutaneous anthrax.

Keywords: Anthrax, *Bacillus anthracis*, domain, protective antigen, vaccine, edema factor

1. INTRODUCTION

Anthrax is caused by *Bacillus anthracis,* a Gram positive, spore forming, non-motile bacterium. Its spores are extremely resistant to natural conditions and can survive for several decades in the environment[1]. Besides a well known bio-threat disease, cutaneous anthrax is a public health disease also in several countries where agriculture is the major source of income[2]. Anthrax is a zoonotic disease, therefore, it is spread either from infected animals to human through contact or using contaminated animal products[3]. Worldwide, approximately 20,000 to 1,00,000 human cases of anthrax occur annually[4]. Mainly, two major factors, i.e. a tripartite toxin and the poly-D-glutamic acid capsule are responsible for the virulence of *B. anthracis*[5]. Human anthrax has three major clinical forms on the basis of route of infection viz. cutaneous (skin), gastrointestinal (ingestion) and pulmonary (through inhalation of spores)[1,6]. The cutaneous route accounts for 95 per cent and inhalational route for 5 per cent anthrax cases reported, while gastrointestinal anthrax is quite rare, mainly due to underreporting of the disease[7]. If not treated well in time, all three forms of anthrax can be fatal. Therefore, early treatment of cutaneous anthrax is usually curative, and early treatment of all forms is important for recovery. Because *B. anthracis* has a high probability for use as an agent in biological terrorism, a lot of research is being carried out on its detection, protection and decontamination[1,8-10].

The anthrax toxin is secreted as three distinct proteins, which are named as protective antigen (PA), lethal factor (LF) and edema factor (EF) and their activities have been well described previously[11,12]. PA is non-toxic and combines with EF and LF to form edema toxin and lethal toxin, respectively. Therefore, being a central component of both the anthrax toxin complexes, PA has been a good target for development of vaccines.

PA is an 83 kD protein which has 4 different domains and each domain has its unique role in toxin function. Domains can exist independently from full protein because these are single-protein subunits and can retain the structural and functional integrity[13]. Domain 1 is made up of amino acid (aa) residues 1 to 258 and contains the furin recognition site RKKR, which is cleaved to release the N- terminal PA20 (aa 1-167) fragement[14]. The remaining portion of domain 1 has interaction site for LF and EF, and two Ca^{++} binding sites[15-17]. Domain 2 (aa 259 to 487) helps in binding of PA63 monomers. Besides, a loop from domain 2 has contact with the host cell receptor binding site[18]. Besides, Domain 2 forms the heptamer of PA63 along with Domain 3 (aa 488 to 595), and in this process, domain 2 becomes buried and inaccessible, along with domain 1, while domain 3 remains accessible with domain 4[17]. This heptameric pore of PA on the cell surface allows LF or EF binding, and subsequently receptor mediated endocytosis of toxin complexes into the cell[19,20]. Domains 4 (aa 596 to 735) is responsible for binding of heptamer to the host cell via cellular receptors[18,21]. As the domains play different roles in toxin function, therefore, there can be different mechanisms of protection because of differences in their immunogenic profiles.

In this study, we have cloned, expressed and purified the various domains of PA. The recombinant proteins were used

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to develop enzyme-linked immunosorbent assay (ELISA) to determine the sero-reactivity of these domain proteins along with full PA with human cutaneous anthrax serum samples.

2. METHODS AND MATERIALS

2.1 Cloning, Expression and Purification of Recombinant PA Domains

The genes for various domains of PA i.e. Domain 1 (PAD1), Domain 2 (PAD2) and Domain 4 (PAD4) were amplified by PCR using the primer given in Table 1. Genomic DNA of *B. anthracis* Sterne was used as DNA template. The PCR products were purified from the agarose gel. The purified PCR products and pET32a+ vector were digested with BamH1 and Xho1 independently. The restricted PCR products and vector were ligated using T4 DNA ligase at 16°C overnight. The ligated plasmid was transformed in the competent cells of *Escherichia coli* BL21 (DE3). The positive transformants were selected on LB agar plates containing ampicillin (100 µg/ml). Further, the transformation was confirmed by the colony PCR using the primers for individual domains. The bacterial cells were grown in Luria-Bertani (LB) broth to an optical density (OD_{600}) of 0.5-0.6 and then induced with 1mM isopropyl-β-D-thiogalactoside at 37 °C for 4 h for expression of recombinant proteins. The cultures pellet was subjected to solubility analysis of the expressed proteins. Recombinant proteins were purified by Ni-NTA columns (Qiagen) as per the standard protocol. The proteins were separated by 12 per cent SDS-PAGE followed by Coomassie blue staining to check the purity. The same set of proteins was transferred from gel to nitrocellulose membrane and probed with anti-his monoclonal antibodies. The concentration of purified proteins was estimated by BCA method after dialysis and passing through Amicon ultra centrifugal filter device (Millipore).

2.2 Serum Samples

A total of 43 serum samples from clinically proved cutaneous anthrax patients from the anthrax endemic area were selected in this study[22,23]. The patients were clinically and epidemiologically well-defined. The serum samples were confirmed for the presence of anti-PA IgG in the previous studies[23].

2.3 ELISA

Maxisorp flat-bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 µl per well of phosphate-buffered saline (PBS), pH 7.4 containing 2 µg/ml of recombinant PA domains as well as full PA protein (PA83) and incubated overnight at 4°C. The recombinant full PA (PA83) was obtained from Alpha Diagnostics International Company, USA. The antigen-coated plates were washed three times with wash buffer (PBS containing 0.1 per cent Tween 20) using ELx 508MS microplate washer (Biotek Instruments Inc, USA). A 300 µl of 3 per cent skim milk in PBS was added in each well as blocking buffer and the plate was incubated for 1 h at 37 °C. The human cutaneous anthrax serum samples were diluted to 1:1000 in PBS containing 1 per cent skim milk, pH 7.4 and 100 µl of each diluted serum was added to each well of ELISA plate. After incubation for 1 h at 37 °C, the plate was washed three times with wash buffer and blotted dry on a paper towel. To detect the bound antibody, 100 µL per well of HRP-conjugated goat anti-human IgG (Sigma- Aldrich) diluted to 1:3000 in PBS containing 1 per cent skimmed milk was added. After incubation for 1 h at 37 °C, the plate was washed again three times with wash buffer and added 100 µL per well of TMB (3,3', 5,5'-tetramethylbenzidine) as substrate (Sigma Aldrich, USA). The colour development was read after 15 min of incubation at room temperature at 630 nm using an ELISA plate reader (BioTek Instruments Inc, USA). The OD values were expressed in ELISA units (EU). All the serum samples were tested in duplicate.

Table 1. List of Oligos used in the study

Domain primer	Oligo sequence	Amplicon size (bp)
PAD1	CGAGGATCCGCAGAAGTTAAACAGGAGAACCG	779
	GATCTCGAGTAAGCTGCCACAAGGGGGTG	
PAD2	CGAGGATCCCACCCCCTTGTGGCAGCTTAT	691
	GATCTCGAGGCGGTAACACTTCACTCCAG	
PAD4	CGAGGATCCTATGATAGAAATAACATAGCAG	414
	GATCTCGAGTCCTATCTCATAGCCTT	

Solid under line indicates the sequence of Bam H1, whereas dotted underline indicates the sequence of Xho1 restriction enzymes

3. STATISTICAL ANALYSIS

Significance of the differences in mean IgG titers using different PA domains as antigens. was tested using a one way analysis of variance (ANOVA). Tukey's multiple comparison tests were applied to analyse pair wise comparison between different groups by using GraphPad version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com) as described previously[22].

4. RESULTS AND DISCUSSION

4.1 Cloning, Expression and Purification of Domain Proteins

All the genes for different recombinant domains i.e. PAD1, PAD2 and PAD4 were successfully amplified by PCR and digested with BamH1 and Xho1 restriction enzymes. The restricted products were ligated into pre-digested pET32a$^+$ vector and transformed into *E. coli* BL21 (DE3). Transformation of each gene was confirmed by colony PCR (Fig. 1). The recombinant PAD1, PAD2 and PAD4 domain proteins of 46 kDa, 43 kDa and 33 kDa were expressed in BL21 (DE3) at 37 °C. The expression of the desired recombinant protein was tested by inducing the clones with IPTG. The expression profile of recombinant proteins PAD1, PAD2 and PAD4 was analysed by SDS-PAGE for un-induced and IPTG-induced cultures. The 6X-His tag was present at N-terminus for the purification of proteins. Cells lysis under native conditions revealed the association of recombinant PAD1 and PAD4 with the supernatant fraction, demonstrating that the PAD1 and PAD4 proteins were soluble. However, PAD2 was associated with pellet fractions, demonstrating that PAD2 was insoluble. Therefore, the purification of PAD1 and PAD4 proteins was carried out in native

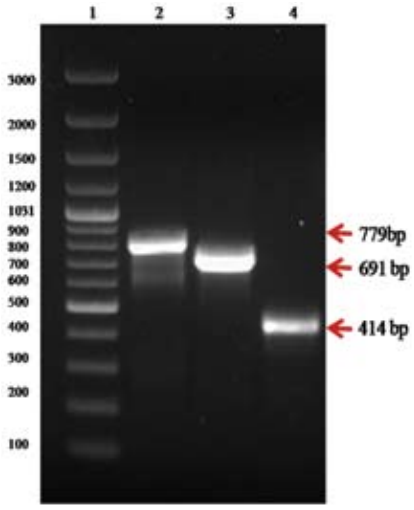

Figure 1. PCR for confirmation of transformation. Lane 1: Standard protein marker, Lane 2: PAD1, Lane 3: PAD2, and Lane 4: PAD4.

conditions, whereas PAD2 protein was solubilised in 8 M urea and purified by Ni-NTA affinity chromatography. The recombinant proteins were tested on SDS-PAGE for purity as shown in Fig. 2(a). The yield of PAD1, PAD2 and PAD4 was 9 mg/L, 16 mg/L, and 18 mg/L under shake flask culture conditions, respectively. The proteins corresponding to their molecular weights were recognised by anti-histidine antibody in a western blot experiment (Fig. 2(b)).

4.2 Determination of Reactivity of Serum with Various PA Antigens

The mean EU values of IgG against various PA antigens have been given in Table 2. The mean EU (overall 95 per cent confidence interval) in tested sera with PA83, PAD1, PAD2 and PAD4 varied between 0.79 to 1.08, 0.46 to 0.59, 0.73 to 0.91 and 0.49 to 0.66 EU, respectively. The mean EU values of PA83 were highest followed by PAD2, PAD4 and PAD1 (Fig. 3). Tukey's multiple comparison tests revealed that there were significant differences between the mean EU values of PA83 with PAD1 and PAD4 (Table 3). However, EU values of PA83 and PAD2 were not significantly different. The mean EU values of PAD2 were also significantly different from the mean EU values of PAD1 and PAD4, whereas the difference in mean EU values of PAD1 and PAD4 was not significant.. The frequency distribution of anti-PA IgG EU for PA83 and different domains is given in Fig. 4.

PA is the major target for development of the diagnostic assays as well as for vaccines and post exposure therapy. It has been established in animal models that the immune response to PA is central to protection against *B. anthracis*[24]. In cutaneous anthrax cases, the immune response against PA is detectable

Table 2. Comparison of anti-PA IgG titer expressed as ELISA units (EU) for various domains.

	PA83	PAD1	PAD2	PAD4
Total number of values	43	43	43	43
Number of excluded values	0	0	0	0
Number of binned values	43	43	43	43
Minimum	0.44	0.176	0.272	0.197
25% Percentile	0.5845	0.329	0.583	0.3615
Median	0.825	0.509	0.822	0.4995
75% Percentile	1.107	0.6665	1.004	0.7135
Maximum	2.274	1.042	1.661	1.2925
Mean	0.942227	0.531593	0.820849	0.57943
Std. Deviation	0.463014	0.215787	0.29089	0.278358
Std. Error of Mean	0.070609	0.0329072	0.0443603	0.0424492
Lower 95% CI of mean	0.799732	0.465184	0.731326	0.493764
Upper 95% CI of mean	1.08472	0.598002	0.910371	0.665096

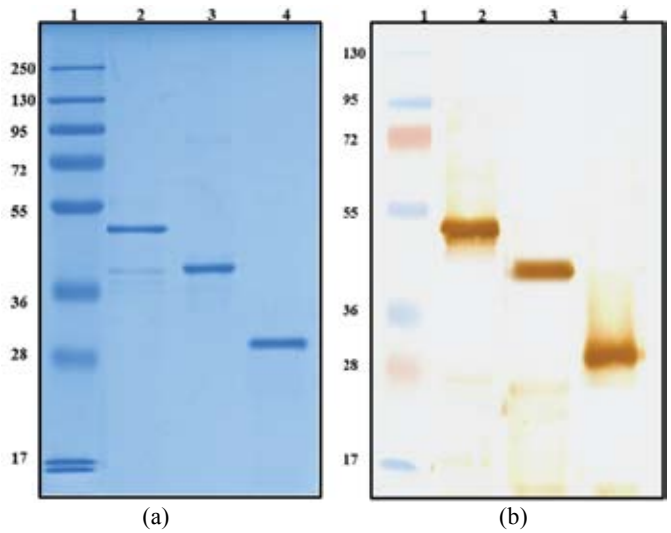

Figure 2. (a) SDS-PAGE Profile of Ni-NTA purified recombinant domain proteins and (b) Western blot with anti-His antibodies. Lane 1: Standard protein marker, Lane 2: PAD1, Lane 3: PAD2 and Lane 4: PAD4.

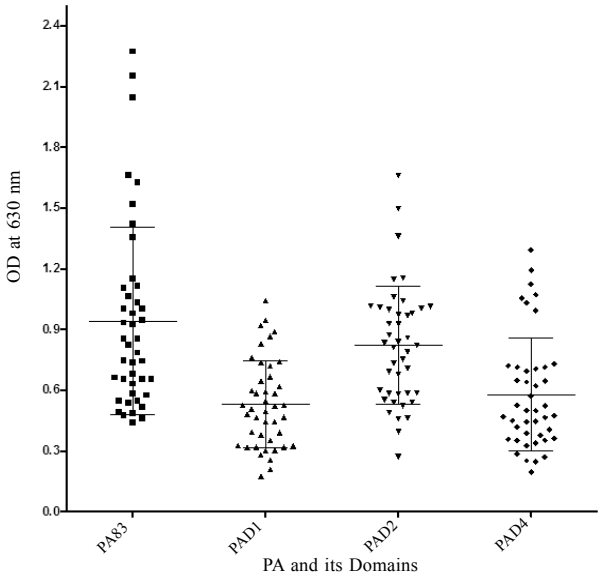

Figure 3. IgG responses to anthrax PA domain protein in cutaneous anthrax serum samples (diluted 1:1000) obtained from human.

Table 3. Multiple comparison tests for significant differences between the mean EU values of various domains

Tukey's multiple comparisons test	Mean Diff.	95 per cent CI of diff.	Significant?	Summary
PA83 vs. PAD1	0.4106	0.2286 to 0.5926	Yes	****
PA83 vs. PAD2	0.1214	-0.06062 to 0.3034	No	ns
PA83 vs. PAD4	0.3628	0.1808 to 0.5448	Yes	****
PAD1 vs. PAD2	-0.2893	-0.4713 to -0.1073	Yes	***
PAD1 vs. PAD4	-0.04784	-0.2298 to 0.1342	No	ns
PAD2 vs. PAD4	0.2414	0.05942 to 0.4234	Yes	**

after 10 to 11 days of onset of symptoms and remains detectable after 8 month to 16 month of exposure[25]. PA has four domains and each domain plays a critical role in toxin action. Therefore, in this study, antibody responses for individual domain antigen, with human anthrax infected sera were evaluated separately by enzyme linked immunosorbent assays (ELISAs). Each domain has specific role in the intoxication process of anthrax toxin[26]. The study revealed that there is great variation in the immune response of individual domain. Among the domains, the highest immune response was found against domain 2 (PAD2), which is necessary for membrane insertion and heptamerisation of PA. Next higher response was found against domain 4 (PAD4), which has overall more exposure during heptamerisation , and more specifically, the accessibility of an exposed loop region (703–722)[27]. Besides, it binds to the host receptor also. However, A83 exhibited the highest immune response showing that all four domains are required for the maximum protection. Previously, the immunogenicity of different domains has been found variable in within and between genetically different strains of mice[28]. However, in mouse model, domain 1 elicited the highest immune response, whereas domain 2 or 4 were significantly less effective in eliciting the higher antibody titre[28]. Because the host genetics plays a very important role in antibody response, therefore domains could elicit different immune response in mouse and human.

The immunoreactivity of human cutaneous anthrax serum samples with individual PA domains showed that besides full PA protein (PA83), individual domain 2 and 4 can also be good target for development of vaccine by generating the chimeric protein with other relevant proteins. Besides, these domains can be used for development of serodiagnostic assays.

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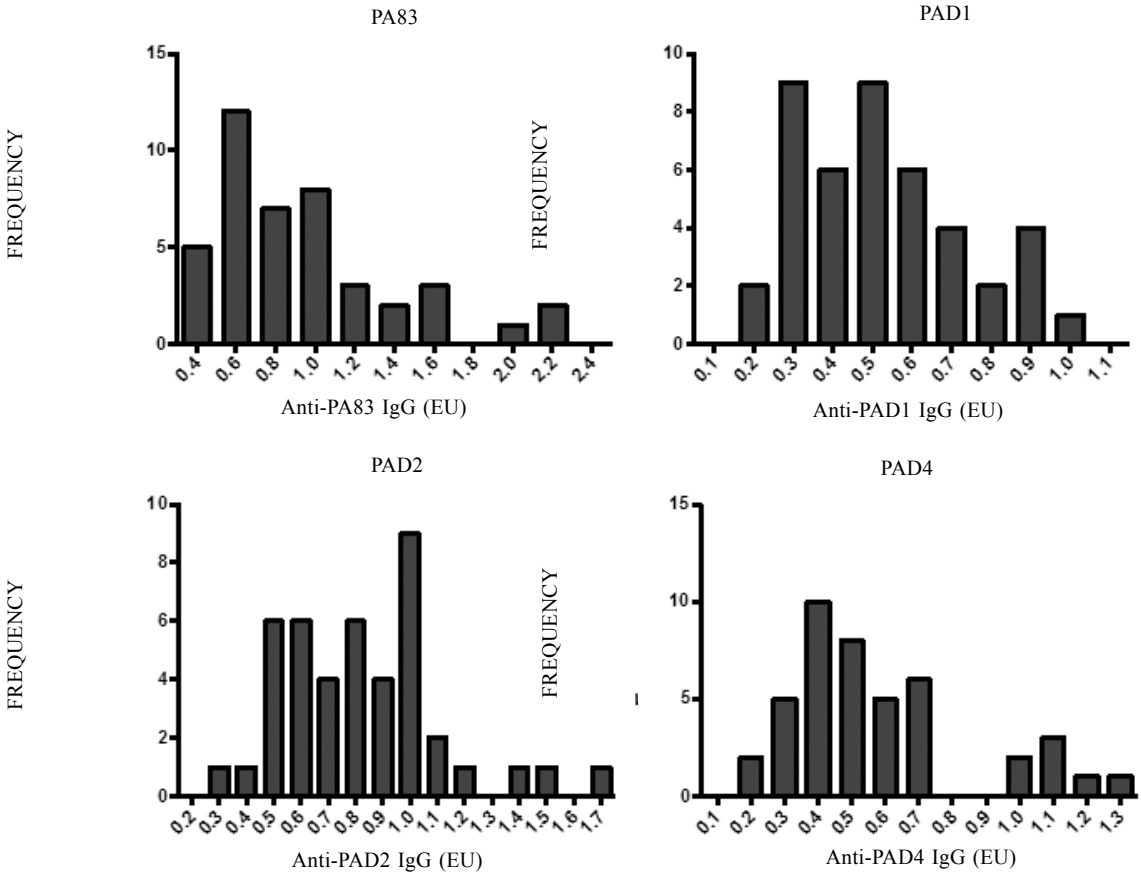

Figure 4. Frequency distribution of the anti-PA IgG titers (EU) for different domain proteins.

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