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A Rapid Flow through Membrane Enzyme Linked Immunosorbent Assay for *Bacillus anthracis* using Surface Array Protein as a Biomarker

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ABSTRACT

Anthrax, caused by *Bacillus anthracis* is an important disease of biowarfare and public health importance. It is imperative to develop a simple system which can detect and differentiate *B. anthracis* from other closely related species. The surface array protein (Sap), which is secreted during the early growth phase of bacteria can be an important biomarker for detection of *B. anthracis*. In the present study, we have developed a rapid flow through membrane ELISA for detection of *B. anthracis*. Polyclonal antibodies were used to develop a sandwich plate ELISA, which could detect 3.9 ng/ml of recombinant Sap. *B. anthracis* bacteria grown in culture broth could be detected after 5 h of growth. Finally, a rapid flow through membrane ELISA was developed which can be accomplished just within 2 minutes, instead of 3-4 h as required in sandwich plate ELISA. The results established that the developed flow through membrane ELISA may be used for detection of *B. anthracis.* The proposed method is rapid, safe and user friendly for detection of *B. anthracis* culture.

Keywords: *Bacillus anthracis*; ELISA; Polyclonal antibody; Surface array protein

1. INTRODUCTION

Anthrax, caused by *Bacillus anthracis* is mainly a zoonotic disease of herbivorous mammals, particularly cattle and sheep. However, humans can be infected by coming in contact with infected livestock or by handling infected domestic animals or their products like skin, meat, hides and bones[1,2]. Based on the different routes of infection, anthrax can be cutaneous, gastrointestinal, pulmonary or even injectional[3]. Besides, *B. anthracis* is considered as one of the most potential biological warfare agents[3,4].

B. anthracis belongs to *B. cereus* group (*B. cereus sensu lato*) which comprises of many closely related species: *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. weihenstephanensis*, *B. toyonensis* and *B. anthracis*[5-7]. Genetically, all members of this group are considered as one species[8]. Therefore, a simple system which can detect and differentiate *B. anthracis* from other members of the *Bacillus cereus* group is always desired.

B. anthracis harbours a highly patterned ultra structure layered cell wall which is produced by non-covalent binding of glycoprotein subunits[9,10]. Collectively, these layers are known as the surface layers or S-layers and cover the entire cell surface[11]. Extracellular antigen-1 (EA1) and surface array protein (Sap) are the two major S-layer proteins found in *B. anthracis*[12,13]. Both of these proteins, EA1 (encoded by *eag* gene) and Sap (encoded by *sap* gene) are not synthesised simultaneously during the bacterial growth, as the *sap* represses *eag* transcription and EA1 is an autorepressor[14]. Sap is produced in early logarithmic growth phase, while EA is produced when bacteria reach to stationary phase[15,16]. Various other functions have been reported for Sap such as cell- cell recognition, cell adherence and may be as a virulence factor[17]. *B. anthracis* strains secrete Sap in growth medium. Several other species of *B. cereus* group are also reported to harbour *sap* gene with an amino acid sequence identity of 67 per cent - 74 per cent among different species[18].

Previously, we have shown that Sap may be utilised as a biomarker for the detection of *B. anthracis*[19]. In this report, we have shown it experimentally by developing a flow through sandwich based membrane ELISA for rapid detection of Sap, a biomarker of *B. anthracis* using anti-Sap polyclonal antibodies.

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2. MATERIAL AND METHODS

2.1 Bacterial Species

B. anthracis Sterne, a non-pathogenic but toxigenic strain was obtained from Institute of Veterinary and Preventive Medicine, Ranipet, Vellore, India[20].

2.2 Production of Recombinant Sap (rSap)

An amplicon of 2355 bp of *sap* gene was generated by PCR employing the primers, sap-F (5′-CATGGATCCGCAGGTAAAACATTCCCAG-3′) and sap-R (5′-ATACTCGAGTTTTGTTGCAGGTTTTGCTTCT-3′) as described earlier[19]. After purification, the PCR product was restricted with *Bam*H1 and *Xho*1 restriction enzymes,

cloned in the pET32a+ expression vector (Novagen, USA) and transformed into *E. coli* BL21(DE3). From the clone, Sap protein was produced using shake flask culture as well as bioreactor, and purified by affinity chromatography and diafiltration as described elsewhere [19].

2.3 Production and Purification of Polyclonal Antibodies

Anti-Sap polyclonal antibodies were produced in rabbit (New Zealand white) and mice (BALB/c) as described elsewhere [19]. Briefly, each rabbit (2 Nos) and each mouse (5 Nos) were immunised with 200 µg (subcutaneous) and 25 µg (intramuscular) of rSap, respectively in Freund complete adjuvant. At an interval of two weeks, 3 successive booster doses were administered with Freund incomplete adjuvant. The blood was collected post one week of last booster and the serum titer was estimated employing plate ELISA[19]. The IgG was purified by affinity chromatography using protein A sepharose (Millipore, USA).

2.4 Development of sandwich ELISA for detection of *B. anthracis* Sap

Ninety-six-well ELISA plates were coated with 100 µl of purified rabbit anti-Sap IgG (2 µg/ml) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After washing the plates with wash buffer (PBS containing 0.1 per cent Tween 20), to each well 300 µl of 5 per cent skimmed milk protein (SMP) was added. After incubation at 37 °C for 1 h, the plates were washed and added 100 µl of varying concentration of purified rSap (0.09 ng to 200 ng/well), and incubated further for 1 h at 37 °C. Again after washing, 100 µl of mouse anti-Sap IgGs (2 µg/ml) was added to each well and incubated for 1 h at 37 °C. After washing, the specifically bound antibodies were detected using 100 µl/well of rabbit anti-mouse HRP-conjugate (Invitrogen, USA) with 1:4000 dilutions in PBS containing 1 per cent SMP. After incubation at 37 °C for 1 h, the plate was washed. A 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma, USA) substrate solution was then added per well and kept at 37 °C for 10 min. The reaction was stopped with 50 µl of 2.5 N H_2SO_4 and optical density (OD) was read at 450 nm. Each concentration of antigen was tested in triplicate.

To detect native Sap, *B. anthracis* Sterne was inoculated in 5 ml of brain heart infusion broth with shaking at 37 °C. The samples were collected at 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 16 h. The collected samples were heat inactivated (kept in boiling water for 10 min), centrifuged and culture supernatant was used in ELISA. The culture supernatant (100 µl-heat inactivated) was used in place of rSap. There was no effect of heating on Sap degradation or coagulation[19].

2.5 Rapid Flow through Membrane ELISA for Detection of *B. anthracis* Sap

Pre-assembled flow through membrane cassettes (Advanced Microdevices, Ambala) containing a 0.45 µm nitrocellulose (NC) membrane and cellulose based absorbent pads of 0.80 mm thickness were used in the assay. An amount of 0.5 µg and 1.0 µg of anti-Sap rabbit IgG (1 µl) was spotted on the membrane. The same amount of control mouse IgG was spotted as control and left at room temperature for 1 h. The concentration of mouse anti-Sap IgG (0.5 - 2.0 µg) and anti-mouse IgG-HRP conjugate (0.25 - 1.0 µg) were optimised for the assay.

2.6 Standardisation of Assay

For standardisation of assay, first 50 µl of wash buffer (PBS-T) was applied on to the cassette. The cassette was allowed to absorb wash buffer and then 50 µl of sample (Sap containing heat inactivated culture supernatant) was added on the membrane and left for 10-15 sec for absorption. Further, added 50 µl of wash buffer and allowed the cassette to absorb it. Added 50 µl of mouse anti-Sap IgG (200 µg/ml) and allowed it to soak on the membrane (10-15 sec). After adding 50 µl of wash buffer, added 50 µl of anti-mouse HRP conjugate (5 µg/ml) and allowed the membrane to soak it (10-15 sec). Again added 50 µl of wash buffer and allowed the cassette to absorb it. Finally, added 50 µl of TMB liquid substrate on to the membrane. The results were noted within 20-30 sec after addition of TMB.

3. RESULTS AND DISCUSSION

3.1 Production of rSap

Recombinant Sap was produced by cloning and expression of *sap* gene in heterologous host[19]. The recombinant protein of ~110 kDa was purified by immobilised-metal affinity chromatography, diafiltered and used for generation of antibodies.

3.2 Determination of Antibody Titer

Polyclonal antibodies were produced against purified rSap in rabbits and mice. The end point titer of rabbit and mice serum was 1:256000 and 1: 512000, respectively in plate ELISA. Pre-immunisation sera of rabbit and mice were used as negative control in ELISA.

3.3 Sandwich ELISA for Detection of rSap

In sandwich ELISA, rSap concentrations from 200 ng to 0.09 ng per well were tested. The OD values at 200 ng and 100 ng were out of the ELISA reader range. The absorbance at 450 nm vs. rSap concentration (ng/well) profile is shown in Fig. 1. The plate ELISA could detect up to 3.9 ng/ml Sap. PBS was taken as control.

3.4 Sandwich ELISA for Culture Supernatants

For detection of *B. anthracis* based on Sap, the heat treated cultures were tested by ELISA. Recombinant Sap was used as a positive control. The plate ELISA detected the native Sap in heat inactivated culture after 5 h of growth and so on (Fig. 2). Un-inoculated broth was taken as control.

3.5 Flow through Membrane ELISA

The pre-assembled flow through membrane kits were coated with different amount of rabbit anti-Sap IgG antibodies. Likewise, different amount of secondary antibodies (mouse anti-Sap IgGs) and anti-mouse HRP conjugated antibodies were also evaluated. Finally, 1 µg of primary antibody (rabbit anti-Sap IgG), 1 µg of secondary antibody (Mouse anti-Sap IgG)

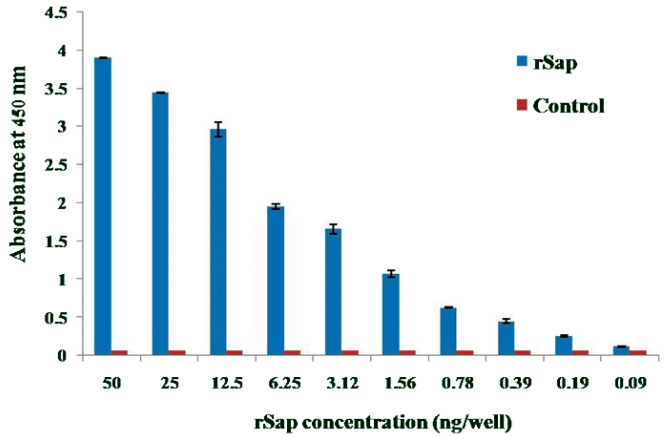

Figure 1. Detection sensitivity of recombinant Sap in ELISA.

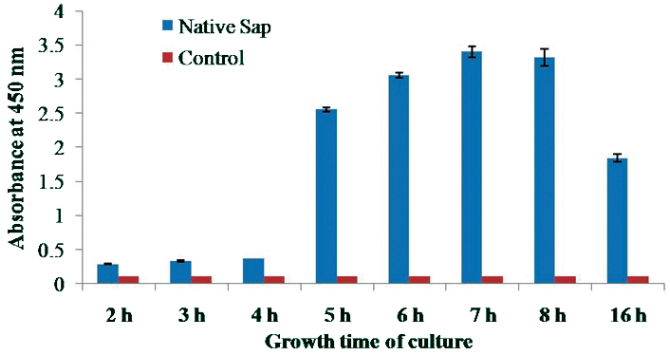

Figure 2. Detection of native Sap in *B. anthracis* Sterne broth at different time intervals.

and 25 ng of revealing antibody (anti-mouse HRP conjugated antibodies) were found to be the best for the assay.

Appearance of a dark blue colour test spot indicated the presence of Sap in the culture. Sap of the culture reacted with anti-Sap rabbit IgG pre-coated on the cassette. Further, anti-Sap mouse IgG reacted with the Sap adhered on the cassette with rabbit anti-Sap IgG. The conjugated secondary antibody i.e. HRP conjugated anti-mouse IgG yielded blue color after reacting with mouse IgG on addition of TMB as substrate. Dark blue colour appeared on control spot also after addition of TMB as pre-coated mouse IgG on the cassette reacted with anti-mouse IgG-HRP conjugate. In the negative test, we got only one dark blue spot (Fig. 3(a)), whereas in positive test we got two spots (Fig. 3(b)). In the absence of any dark blue color spot, the test is not accepted. In the flow through cassette, the excess sample or reagents move vertically down to the absorbent pad. The assay is completed in less than 2 min. However, enrichment time is extra than the assay time.

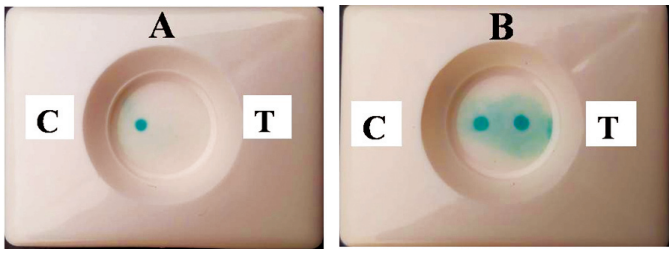

Figure 3. Flow through membrane ELISA: C, Control spot; T, Test spot. (a) Negative sample, (b)Positive sample.

For *B. anthracis*, which is an important biothreat agent a simple and fast detection method is required. Conventionally, *B. anthracis* is confirmed by culture followed by Gram or capsule staining[21]. There are several serodiagnostic assays for anthrax infection in humans[22-25]. However, sophisticated tools and techniques are required for its detection from the environmental or clinical samples[20,26,27]. Sap is considered an important biomarker of *B. anthracis*[19]. In the present study, anti-Sap antibodies were used for detection of *B. anthracis* by a rapid membrane ELISA. The flow through membrane ELISA is a rapid test as it takes only 2 minutes instead of more than 3 h in sandwich plate ELISA. However, presence of sap gene and its production has been reported in some other pathogenic species of *Bacillus* like *B. cereus* G9241[28] and *B. thuringiensis*[18]. Though, the amino acid sequence identity among different species of *B. cereus* group varied from 67 per cent - 74 per cent[18]. Hence, there may be some cross reactivity with any of *B. cereus* group member, which we have not studied. Yet, this method is effective for rapid and safe screening of bacterial isolates as the culture is heat treated to inactivate all the viable bacteria before testing on the flow through membrane ELISA. Thus, Sap is an important detection biomarker for *B. anthracis* and the present assay can be used for rapid detection of *B. anthracis* bacterial isolates after culturing in broth.

ETHICS APPROVAL

The study was approved by Institutional Biosafety Committee of Defence Research and Development Establishment, DRDO, Ministry of Defence, Government of India wide protocol no: IBSC/12/BT/AKG/22.

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