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APPLICATION OF EXTRACTABLE ANTIGEN 1 (EA1) FOR SPECIFIC DETECTION OF *BACILLUS ANTHRACIS* CELLS

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ABSTRACT

Antibody based assays are very important for early and accurate detection of *Bacillus anthracis* in different sample matrices to initiate effective response and control strategies during biological emergencies and natural outbreaks. To achieve this, murine monoclonal antibodies were generated against Extractable antigen 1 (EA1) of *B. anthracis* which was proven to be present in abundance in vegetative cells and a persistent contaminant on the spores. EA1 protein was cloned as two regions and expressed in *E. coli* host to immunize the BALB/c mice and rabbits. Three monoclonal antibodies (EAC105, EAC108 and EAC202) were very promising, as they were highly reactive to recombinant proteins as well as native antigen and also found to be explicitly specific to the pathogen. The monoclonal antibodies were employed in Western blot, dot ELISA and Sandwich ELISA and also tested onto the artificially contaminated meat and blood samples where they were found capable of reacting with the pathogen alone. The present report illustrates application of monoclonal antibodies based on EA1 for developing simple detection systems for *B. anthracis.*

KEYWORDS: ***Bacillus anthracis*, detection, Extractable antigen 1, monoclonal antibodies, Dot ELISA, sandwich ELISA**

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INTRODUCTION

Bacillus anthracis, a Gram positive, spore forming bacterium causes the lethal anthrax disease in humans and animals. It poses a serious bioterrorist threat problem today because of its capability to cause rapid mortality, morbidity and psychological fear among the masses. The spores easily get aerosolized, stay viable for a longer time in the environment, are difficult to eradicate and infect the host through inhalation, ingestion or through minor injuries on the skin surface. The anthrax scare, especially the anthrax spore release through US mail system during September 2001 attack in USA, reaffirmed the significant impact a systematic bioterrorist attack can cause among the common people. Early and accurate detection of the pathogen in the food, environment and clinical samples can definitely help in mounting an effective response and control strategies during the biological emergencies. *B. anthracis* strains are identified conventionally by their characteristic colony morphology, non-motility, capsule formation, lack of hemolysis in blood agar plates, cell lysis by gamma phage and other routinely used tests such as direct fluorescent antibody (DFA) staining [1]. These time consuming and labor intensive conventional tests are non-confirmative and require to be complemented by PCRs and immunoassays. PCR assays [2, 3, 4] have been found useful for *B. anthracis* detection but are resource and expertise intensive and involve complicated steps such as DNA extraction or isolation of the organism from the substrate. Besides, these techniques are restricted to laboratory use. The antibody based assays which are simple, cost effective and rapid have been developed targeting the vegetative cell components such as poly-γ-d-glutamate capsule (encoded by pXO2 plasmid) [1], protective antigen (PA, encoded by pXO1 plasmid) [5], and spore components [6]. But, recent studies have revealed that horizontal transfer or loss of the plasmids in *B. cereus* group members lead to dramatic alterations in their phenotypes and these results in ambiguous identification of the respective bacterial species [7]. Detection systems, hence, solely relying on plasmid borne genes may find limitations for universal application owing to these possibilities. *Bacillus anthracis* and the related members have a protein layer called S–layer comprising of crystalline arrays made of monomolecular proteinaceous molecules surrounding the cell completely over the cell wall. Extractable antigen1 (EA1), encoded by the *eag* gene located in chromosome, is a major component of S-layer and be the most abundant protein in the vegetative cell form of *B. anthracis* comprising about 15-30% of total cell protein [8,9,10]. Earlier reports have proven that EA1 can be targeted for specific detection of *B. anthracis* [11, 12, 13]. Hence, in the present study we developed simple immunoassays for the detection of *B. anthracis* using specific antibodies targeting EA1 protein. The *eag* gene was cloned as amino and carboxy terminal regions and expressed in *E. coli* host system. The recombinant proteins were used to generate specific murine monoclonal antibodies (mAbs) and rabbit polyclonal antibodies. In this instance, we have generated mAbs that were able to recognize the *B. anthracis* strains with no reactivity towards other members of *B. cereus* group. These mAbs employed to develop simple detection assays for *B. anthracis*. To our knowledge, we are first reporting the utility of mAbs generated against EA1 for direct detection of *B. anthracis* by Dot ELISA and sandwich ELISA assays in food and clinical samples.

MATERIALS AND METHODS

Bacterial Strains, chemicals and media

The *Bacillus* species used in this study (Table 1) were procured from National Collection of Industrial Microorganisms (NCIM), India and American Type Culture Collection (ATCC), USA. The *B. anthracis* isolates were revived from the culture collection facility in Defence Research and Development Establishment (DRDE), Gwalior, India. The *Bacillus* strains were grown in Brain Heart Infusion (BHI) media at 37 ^{O}C and genomic DNA was extracted using DNA

extraction kit (Macherey Nagel, Germany). The *E. coli* strains were propagated in LB broth at 37 ^{O}C supplemented with the appropriate concentration of antibiotics: ampicillin (100 µg ml^{-1}) and kanamycin (40 µg ml^{-1}). All the chemicals and reagents were procured from Sigma-Aldrich, India and bacterial growth media from Himedia, India unless stated otherwise.

Cloning of rea1N and rea1C

In the present study, the amino and carboxy terminal regions *eag* gene of *B. anthracis* were cloned and expressed separately and designated as *EA1N* and *EA1C* respectively. The genes were amplified from *B. anthracis* Sterne strain genomic DNA (gene bank accession no.: AE017225.1) using the forward and reverse primers. The primers for amplifying *ea1N* are as follows:5'- GCA GGT AAA TCA TTC CCA GAC – 3' & 5' - ACC GTG GTT TGA AGA TTT AAT G - 3'; and for *ea1C*: 5'- ACA ATT GGT GTT ACA GGT AAT G - 3' & 5' - TGG GTT ATT AAG AAC GTT CAC - 3'. The plasmids pEA1N and pEA1C were created by ligating the restricted *ea1N* and *ea1C* PCR products into prelinearized pQE 30UA (Qiagen, India) vector following manufacturer's instructions. The recombinant plasmids were transformed into chemically competent *E. coli* M15 cells following the standard transformation procedures as described Sambrook *et al*, 2001[14].

Expression and purification of rEA1N and rEA1C

E. coli M15 cells harboring pEA1N and pEA1C plasmids with proper insert orientation were grown in 5ml LB broth till the absorbance A_{600} reached 0.6. The culture was induced with 0.5, 1.0 and 1.5 mM IPTG for various time intervals. The expression of recombinant proteins was analyzed in 12 % SDS-PAGE gel for optimizing induction time and the IPTG concentration. Recombinant rEA1N and rEA1C proteins were purified from 100 ml *E. coli* M15 cultures induced with 1mM IPTG for 5 h at 37 ^{O}C for optimum protein production. The proteins were purified under denaturing conditions using Ni-NTA agarose following manufacturer's instructions (Qiagen, India). The purification of the recombinant proteins was confirmed by 12% SDS-PAGE. The eluted protein fractions were pooled and dialyzed in PBS followed by quantification using Lowry's method by protein estimation kit (Bangalore Genei, India) as per the manufacturer's instructions.

Generation of monoclonal and polyclonal antibodies against rEA1N and rEA1C

To generate monoclonal antibodies, six week old female BALB/c mice were injected i.m. with 50 µg recombinant proteins emulsified in complete Freund's adjuvant (CFA) (Sigma, India). Three booster s.c. immunizations with 50 µg of protein and equal volumes of incomplete Freund's adjuvant (IFA) (Sigma, India) were given at 10 days interval. Finally, the mice were injected i.p. with 100 µg of recombinant protein dissolved in 1X PBS two days prior to hybridoma. Two days later, mice were bled prior to each immunization and serum antibody titers were estimated by indirect plate ELISA using the rEA1N or rEA1C proteins. The splenocytes were collected 3 days after the last immunization and immortalized by fusion with mouse myeloma SP2/O-Ag14 cells following the protocol established by Kohler and Milstein (1975) [15]. The hybrid cells were screened by indirect plate ELISA using recombinant proteins. The hybridoma cells secreting sensitive antibodies were subcloned by limiting dilution and the secreted antibodies were collected for further purification. Antibodies were precipitated using saturated ammonium sulfate solution and purified by protein-A chromatography. The purified antibodies were dialysed in PBS and assessed by 12% SDS-PAGE. For generating the polysera**,** two white female New Zealand rabbits weighing 1-1.5 kg were immunized s.d. with 100 µg of each protein emulsified with CFA (Sigma, India) at day 0. Subsequent doses prepared with IFA (Sigma, India) were given i.m. at 14, 21 and 28 days following primary injections. Sera were collected from rabbits before each immunization and antibody titer was estimated by indirect plate ELISA using the recombinant proteins. The

hyper immune sera was collected after the last injection and stored at -20°C in aliquots. All the animal experiments were approved by the Institutional Ethical Committee for Animals

Immunoassays of mAbs for their specificity

Indirect plate ELISA

Microtiter plates (Nunc, USA) were coated with 1 µg of rEA1N or rEA1C per well in 50 mM carbonate – bicarbonate (CB) buffer (pH 9.6) at 4 ^{O}C overnight. The unbound sites were blocked with 3% BSA in 1X PBS for 1 h at room temperature. The plates were washed briefly with PBST (1X PBS with 0.05% Tween 20) and 100 µl of a 1:1000 diluti on of mouse anti-EA1N or anti-EA1C sera or mAbs was added to the appropriate well. The plates were incubated at 37 ^{O}C for 30 min, washed thrice with PBST and incubated with 100 µl of goat anti-mouse polyvalent Igs conjugated with horse radish peroxidase (HRP) enzyme for 30 min at 37 ^{O}C at 1:1000 dilution. The secondary antibodies were detected with o-phenylenediamine in citrate phosphate buffer (pH 5.6) containing 0.004% H_2O_2. The plates were kept in room temperature for colour development. The chromogenic reaction was stopped by adding 20 µl of 2.5 M H_2SO_4 to each well. The absorbance readings were taken at 490 nm.

Western blot

The serum collected from mice after the final booster was checked for their reactivity against native protein from *B. anthracis* Sterne strain on Western blot. Similarly, the various mAbs generated were also employed in Western blot to evaluate their reactivity with various Bacillus and non-Bacillus species. The bacterial cells grown overnight in BHI broth at 37 ^{O}C were pelleted and SDS-PAGE was performed in 10% SDS-PAGE as described by Sambrook *et al* (2001). The Proteins were electroblotted onto nitrocellulose membrane and membrane was blocked using 5% defatted milk in 1X PBS for 2 hours at 37 ^{O}C and probed with mouse anti-rEA1C or rEA1N sera at 1:1,000 dilution or mAbs and maintained at room temperature for 30 min. The membrane was washed thrice with PBST and incubated with goat anti-mouse polyvalent Ig conjugated with HRP (1:1,000) for 30 min at room temperature. After washing in PBST, the blots were developed with 0.004% H_2O_2 and 3, 3', 5, 5'– Diamino benzidine tetrahydrochloride in 1X PBS.

Dot ELISA

The bacterial cultures were grown overnight in BHI broth. 1ml of cells were centrifuged and resuspended in 100 µl of 10 mM CB buffer containing 0.01% thimerosal. Dot ELISA was performed by spotting 10 µl of bacterial cell suspension directly onto the nitrocellulose membrane and the cells were fixed by air drying at 45 ^{O}C for 15 min. The membrane was then processed as described in the western blotting section. To determine the sensitivity of mAb EAC108 in dot ELISA, *B. anthracis* Sterne strain culture grown overnight was serially diluted in CB buffer ranging from 10^8 cfu ml^{-1} to 10^0 cfu ml^{-1}. Then the diluted samples were tested by Dot ELISA.

Sandwich ELISA

Microtiter plates (Nunc, USA) were coated with 100 µl of 10 µg ml^{-1}of anti-EA1N or anti-EA1C rabbit antibodies dissolved in 50 mM CB buffer and stored at 4 ^{O}C overnight. The wells were washed and blocked with 3% BSA in 1X PBS for 1 h at room temperature. 100 µl of vegetative cells of various *Bacillus* species suspended in concentrations ranging from 10^8 to 10^0 cfu ml^{-1} were added to appropriate wells and incubated for 30 min at 37 ^{O}C. After a brief washing with PBST, 100 µl of 10 µg ml^{-1}of mAb (EAC108 or EAC105 or EAC202) in 1:1000 dilution was added to the appropriate wells as revealing antibodies. The plates were incubated for 30 min at room temperature after which the wells were washed with PBST thrice. Secondary antibody incubation and chromogenic development was performed as described in the indirect plate ELISA section. The absorbance readings were taken at 490 nm. The cut off value was defined as twice the mean absorbance of negative control.

Spiking Studies

The spiking studies were conducted using the *B. anthracis* Sterne strain to evaluate the sensitivity of EAC108 mAb in the presence of interfering agents. The study was performed as follows. The human blood was collected from a local hospital and the meat was collected from local market. These samples were confirmed to be *B. anthracis* negative by culturing the samples before inoculation. *B. anthracis* Sterne strain was cultured for 16 h at 37 ^{O}C in BHI broth. 1 gm of meat was mixed in 9 ml saline or PBS and homogenized thoroughly. The mixture was evenly dispensed into several test tubes. The culture was serially diluted in sterile saline or PBS in the concentrations ranging between 10^{8} to 10^{0} cfu ml^{-1}. 1 ml of inoculum from each diluent was added to tubes containing blood or of meat homogenate and incubated at 37 ^{O}C for 1 h. 100 µl of sample from each tube was added to microtiter plates precoated with 100 µl of 10 µg ml^{-1} anti rEA1C rabbit sera and sandwich ELISA was performed as mentioned above. Simultaneously, 100 µl of the samples were plated on BHI agar for total cell count. The uninoculated blood or meat homogenate was taken as negative control.

Statistical Data

All the data in this study were represented as the mean absorbance ± SD. All the graphical illustrations were constructed using MS Excel worksheet 2007.

RESULTS

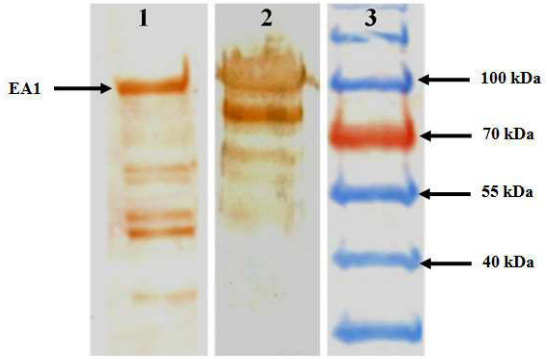
Protein expression and purification studies

SDS PAGE analysis of 1 mM IPTG induced *E. coli* M15-pEA1N and *E. coli* M15-pEA1C cells revealed recombinant proteins of 46 kDa and 32 kDa, respectively. The recombinant proteins were found to be localized in the insoluble fraction as inclusion bodies. About 2.1 mg of rEA1N and 1.83 mg of rEA1C were purified from 100 ml *E. coli* M15 culture broth using 1 ml bed volume of Ni-NTA agarose column.

Generation of monoclonal antibodies

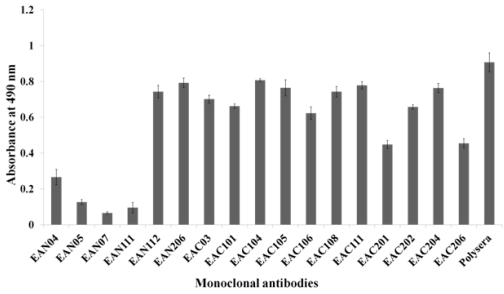
After the third booster the mice immunized with rEA1N and rEA1C showed antibody titer of 1:64,000 and 1:32,000, respectively. Polyclonal antisera against both the recombinant proteins showed reactivity with native antigen prepared from *B. anthracis* Sterne strain (fig. 1). Hybridomas obtained after fusion were screened by indirect ELISA for reactivity with their respective proteins. A total of 06 and 11 hybridomas showing reactivity with rEA1N and rEA1C respectively were stabilized after successive cloning (table 2, fig. 2).

Figure 1
Western blot analysis of antisera generated against rEA1N and rEA1C



Lane 1: Anti-rEA1C sera reacting with Bacillus anthracis Sterne strain cell lysate;
Lane 2: Anti-rEA1N sera reacting with Bacillus anthracis Sterne strain cell lysate;
Lane 3: Prestained protein ladder (Fermentas, Thermoscientific, USA).

Figure 2
Indirect ELISA to demonstrate binding affinities of mAbs to B. anthracis recombinant proteins rEA1N and rEA1C.



The bars represent the mean of duplicate samples ± SD.

Table 1
List of Bacillus strains used in this study and tested with mAb EAC108

Organism	Western blot	Dot ELISA	Sandwich ELISA
Bacillus anthracis Sterne strain	+	+	+
Bacillus anthracis isolates (31)	+	+	+
Bacillus cereus strains (15)	-	-	-
Bacillus thuringiensis strains (17)	-	-	-
Bacillus pasteurii NCIM 2477	-	-	-
Bacillus circulans NCIM 5048	-	-	-
Bacillus subtilis NCIM 2124	-	-	-
Bacillus mesentericus NCIM 2019	-	-	-
Bacillus licheniformis NCIM 2044	-	-	-
Bacillus licheniformis NCIM 2051	-	-	-
Bacillus licheniformis NCIM 2059	-	-	-
Bacillus firmus NCIM 2264	-	-	-
Bacillus brevis NCIM 2216	-	-	-
Bacillus laterosporus NCIM 2465	-	-	-
Bacillus pumilus NCIM 2108	-	-	-
Escherichia coli ATCC 10536	-	-	-
Salmonella typhimurium ATCC 14028	-	-	-
Clostridium perfringens ATCC 13124	-	-	-

Specificity of monoclonal antibodies

The monoclonal antibodies were initially evaluated to determine their reactivity with *Bacillus* species by Western blot. The monoclonal antibodies reacted with *B. anthracis* strains used in this study (table 1) and a band near 94 kDa could be seen in Western blot. Three anti-EA1C mAbs viz., EAC202, EAC108 and EAC105 exhibited specific reactivity with *B. anthracis.* and EAC108 was used in later studies. The specificity of mAb EAC108 was further examined by Western blot using an extended array of bacterial strains listed in table1. When tested on Dot ELISA, the mAb was able to react with all the *B. anthracis* isolates but not with the other *Bacillus* species. Dot ELISA results for all the strains were found to be consistent with results of Western blot (table 1). Therefore, mAb EAC108 was further employed to determine its specificity in Sandwich ELISA. The mAb retained its specificity in sandwich ELISA and the reactivity with *B. anthracis* strains was far higher in comparison to other *Bacillus* species.

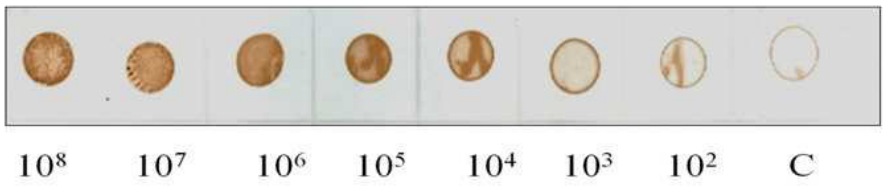
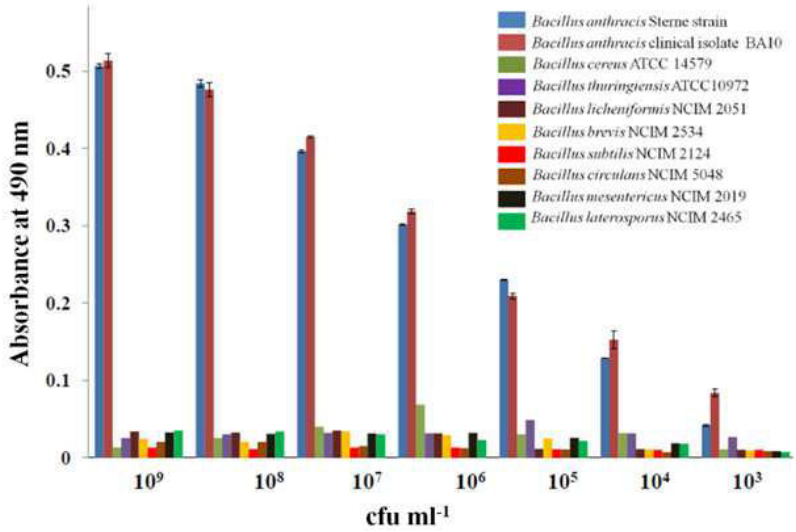


Figure 3
The sensitivity of dot ELISA using mAb EAC108 for Bacillus anthracis Sterne strain.

Sensitivity of Immunoassays

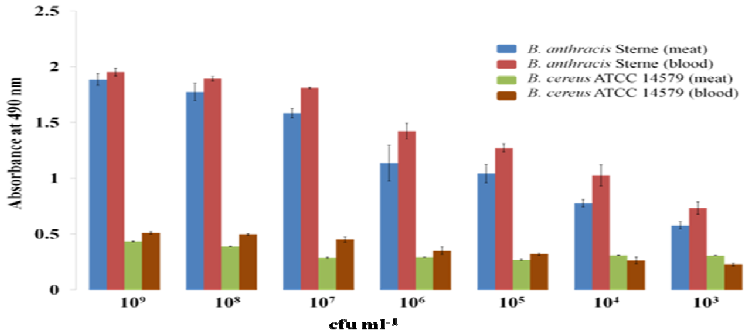
Sensitivity of dot ELISA was evaluated by using *B. anthracis* Sterne strain bacterial cell dilutions ranging from 10^8 to 10^2 cells coated onto the nitrocellulose membrane. Distinct dots could be detected upto 10^3 bacterial cells and the dilutions less than 10^3 cells could not produce intense dots (fig. 3). Therefore, in Dot ELISA, upto 10^3 cells could be detected. The sandwich ELISA was established using rabbit anti-EA1C hyperimmune sera as capturing antibody and mAb EAC108 as revealing antibody. Here, we have used 10 µl of 1/1000th dilution of 1:64,000 rabbit polysera. The lower detection limit was determined as 10^4 bacterial cells by culture dilution assay (fig.4). The cut-off value was determined as twice the mean of negative control and the bacterial dilutions showing OD values lesser than twice the cut-off value were considered negative. We found that, in the blood samples and/or meat samples artificially contaminated with bacterial cell dilutions, the pathogen was detectable upto 10^4 cells by sandwich ELISA (fig.5). In samples where the bacterial load is below detection levels, an enrichment step in BHI broth can be included to increase the cell load to detectable levels. Even though both the methods are simple, dot ELISA needs the cells to be suspended in CB buffer to coat onto the nitrocellulose membrane whereas in case of sandwich ELISA, the cells can be directly detected irrespective of source of the pathogen.

Figure 4
The sensitivity of sandwich ELISA of mAb EAC108 against vegetative forms of various Bacillus species.



The bars represent the average of absorbance value ± SD.

Figure 5
The sensitivity of sandwich ELISA of mAb EAC108 in artificially spiked meat and blood samples



The bars represent the average of absorbance value ± SD.

DISCUSSION

EA1 protein has been proven to be highly antigenic protein present in vegetative cells and spore preparations of *B. anthracis* [8, 16]. Mesnage *et al* (1997) reported that EA1 was the only S-layer protein recognized by sera from animals injected with Sterne derivatives even though Sap was also present which has about 66% identity with the EA1, suggesting that EA1 is the immunogenic protein present in the S-layer *in vivo.* EA1-like proteins are distributed in related bacterial species. These proteins have higher sequence identity confined to the amino terminal region where SLH motifs are located. These are associated with anchoring the S-layer to cell envelope while the carboxy terminal region is exposed to the exterior of the cell. Therefore, the amino and carboxy terminal regions of EA1 were cloned and expressed separately to generate monoclonal antibodies and examined for their utility in specific detection of *B. anthracis*. The mAbs EAC108, EAC105 and EAC202 specifically reacted with *B. anthracis* in western blot and no cross reactivity was observed even with *B. cereus* group that are closely related to *B. anthracis* and genetically almost identical to the pathogen [7]. Further simple dot ELISA and sandwich ELISA methods were developed using mAb EAC108 for the easy detection of *B. anthracis* from blood and meat samples. The mAb based dot-ELISA immunoassay standardized earlier by us employed anti-PA mAbs for detecting *B. anthracis* by targeting the PA elaborated into casamino acids medium [5]. This required culturing the *B. anthracis* isolates in suitable media or germination of spores followed by culturing to secrete detectable concentration of PA before performing the dot ELISA. In contrast, the sandwich ELISA standardized in the present study allows direct detection of *B. anthracis* cells from sample enrichments from blood and food samples. Similarly, dot ELISA also allows detection of the pathogen from the culture isolated from food or clinical samples. EA1 has been reported to be present on spore surface as a contaminant and the mAbs generated against EA1 antigen were able to detect the vegetative cells and spores simultaneously [12, 13, 16, 17]. Hence, the mAbs described in the present study may have applications in detecting the spores as well. Though the earlier reports have explored its possibility as a detection marker for *B. anthracis* [11, 12, 13], little work has been carried out on development of simple assays for cost effective detection systems. Therefore, in the present work, we focused on developing simple and cost effective assays which help in routine investigation of *B. anthracis* in fields and laboratories especially in developing countries where cost is still a main setback.

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