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Heterologous Expression of Staphylococcal Enterotoxin B and Its Suitability for Development of Field Based Diagnostic Test System

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Abstract

Staphylococcal Enterotoxin B (SEB) is one of the potent enterotoxins produced by certain strains of *Staphylococcus aureus* bacteria. In the present context of its use as bio-terror agent, it is imperative to develop simple to use and rapid detection systems as well as therapeutic vaccines to detect SEB and protect against SEB intoxication. In the present study, recombinant staphylococcal enterotoxin B, produced as a NH_2-terminal histidine hexamer fusion protein, expressed in heterologous expression system, was tested for its diagnostic potentials. His6-tagged SEB was immunogenic for IgG in mice and rabbits, either singly or in combination with adjuvant. Antigenic authenticity of the recombinant SEB to native SEB was confirmed by antibody-based capture detection assays across different host species. Using recombinant SEB as bound target, a sandwich ELISA was developed and utilized for detection of SEB *in vitro* and *in vivo*. The ELISA described here can be a useful tool for detection of SEB intoxication as well as infection due to SEB producing *S. aureus* bacteria. Data indicated potentials of recombinant SEB in diagnosis and vaccine development.

Keywords: Fusion protein; Recombinant protein; Staphylococcal enterotoxin B; *Staphylococcus aureus*; Superantigen

Abbreviations: His6: Hexahistidine; HRP: Horseradish Peroxidase; IgG: Immunoglobulin G; IPTG: Isopropyl β-D-Thiogalactoside; LB: Luria-Bertani; LPS: lipopolysaccharide; Ni-NTA: Ni^{2+}-nitrilotriacetate; nSEB: Native Staphylococcal Enterotoxin B; rSEB: Recombinant Staphylococcal Enterotoxin B; SEA-D: Staphylococcal Enterotoxin A-D; SET-RPLA: Staphylococcal Enterotoxins-Rapid Passive Latex Agglutination; TSST: Toxic Shock Syndrome Toxin.

Introduction

Staphylococcus aureus is an important bacterial pathogen capable of causing a wide variety of infections in humans and animals [1,2]. It produces a spectrum of extracellular protein toxins and virulence factors viz., enterotoxins, proteinases, pore-forming hemolysins and leucocidin etc. which contribute to the pathogenicity of the organism in the host [3-6].

The Staphylococcal Enterotoxins (SEs), being the emetic toxins, are the recognized agents of the staphylococcal food poisoning syndrome and may be involved in other type of infections with sequelae of shock in humans and animals [4-8]. SEs act as pyrogens and Superantigens (SAgs) that stimulate non-specific T-cell proliferation by binding with high affinity to Major Histocompatibility Complex class II (MHC II) molecules on antigen-presenting cells. This binding exerts strong mitogenic activity on T-cells; thus, triggering an excessive TH1-cytokine response, characterized by IL-2, IFN-γ, and TNF-β production, leading to toxic shock [9-11].

Among all SEs, Staphylococcal Enterotoxin B (SEB) is the known weaponized staphylococcal enterotoxin, which could be used to sabotage food or water and is potentially most dangerous when delivered as an aerosol. It is a well characterized 28 kDa protein and is one of the most potent mitogens serving as a super antigen [12-14]. Inhaled at high doses, SEB may be fatal; whereas, in low doses, it can incapacitate individuals for a long time. Estimates predict an effective attack using SEB could incapacitate approximately 80 percent of the exposed individuals for up to two weeks. The airborne, waterborne and foodborne use of SEB and its potential to induce fatal respiratory distress syndrome followed by toxic shock were the significant reasons for listing it as a Category B agent of bioterrorism [15-17].

Keeping in view the putative significance of SEB as a potent bioterror agent and in pursuance towards proper control of outbreak situation, particularly in the biological warfare and bioterror threat scenarios; it is highly imperative to look for simple to use, reliable, rapid, and low-cost detection systems, as well as therapeutic strategies to prevent public health hazards caused due to SEB intoxication.

Towards diagnosis of staphylococcal infections, conventional culture based methods used for isolation and identification of pathogens is laborious, time-consuming, and bears low sensitivity. To overcome the drawbacks of conventional identification methods, immunological and molecular methods have been described. Among molecular methods, PCR is one of the most common nucleic acid based tests used for detection of enterotoxigenic *Staphylococci*. Loop mediated isothermal amplification (LAMP) and real time LAMP assays have been developed and found to be sensitive and specific as well as less time consuming than PCR [18-20]. However, mere presence of an enterotoxin gene in isolated staphylococcus bacteria does not ensure the production of biologically active toxin.

More advanced detection methods, viz., Dissociation-Enhanced Lanthanide Fluoroimmunoassay (DELFIA) Time-Resolved Fluorometry (TRF) assay system, surface plasmon resonance, microarray immunosensor and mass spectrometric methods [21-26] have been described for the detection of SEB in a variety of biological and environmental matrices. However, despite the elegance and high sensitivity, these tests may not be well suited for detection of SEB in field conditions because of their sophisticated instrumentation, lengthy protocols, and high cost. Furthermore, these techniques are

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not suitable for screening of different biological samples on large scale. In such situations, immunological methods, being sensitive, specific and robust, offer several advantages over these tests and are the most common methods used for detection of microbial pathogens or their toxins.

The conventional detection methods and vaccine formulations rely mostly on crude native preparations and, thus, are error prone. Many a times, purification using traditional techniques such as molecular sieving, ion exchange chromatography and isoelectric focussing does not reach to the required homogeneity resulting into generation of less specific reagents with poor detection potential. In such conditions, production of recombinant SEs is likely to yield stable and purified SEB protein in large quantities that could be used effectively in the development of reliable and affordable diagnostic tools and vaccine formulations. Here, we report the applications of a recombinant form of SEB for the development of diagnostic test system.

Materials and Methods

Animals

Inbred BALB/c mice (15-20 g; 8-10 weeks old) and New Zealand White rabbits (1-1.5 kg; 12-13 months old) were obtained from the Experimental Animal Facility of the institute. Animals were housed under standard conditions of temperature and humidity with 12 hour light-dark cycle and were given standard rodent feed and water *ad libitum*. All animal care and experimental procedures were conducted in accordance with and approved by the Institutional Animal Ethics Committee, Defence Institute of Physiology & Allied Sciences, Delhi, India.

Toxins and Staphylococcal enterotoxin identification

Purified toxins (SEA, SEC, and TSST) obtained from Toxin Technologies (Sarasota, FL.) in lyophilized form were aliquoted and stored at -20°C. Production of SEs (SEA to SED) was tested by reversed passive latex agglutination test as per the manufacturer's instructions (SET-RPLA and TSST-RPLA; Denka Seiken, Japan).

RPLA Assay for SET production

Staphylococcal enterotoxins (A-D) production was assayed according to the instruction manual for the SET-RPLA (Denka-Seiken). In brief, each culture was plated on a Brain Heart Infusion (BHI) agar plate. After overnight culture on BHI agar plate, a colony from BHI plate was inoculated into BHI broth. Broth culture was centrifuged and serial 10-fold dilutions (up to 1024) of the supernatants were added to triplicate wells of a V-shaped-bottom microtiter plate. Twenty-five microliters of each of the anti-SEA, anti-SEB, anti-SEC, anti-SED and negative control latex reagents was added to the wells. After thorough mixing, the plates were incubated for 18 hrs at room temperature. SET production by each strain was judged according to the macroscopic observation of latex agglutination in the wells.

Bacteria

Escherichia coli M15 (*E. coli* M15) used for cloning and expression was purchased from Qiagen (Germany). This strain was maintained on Luria Bertani (LB) agar (Difco, USA) slants containing antibiotics as and when required. Glycerol stocks containing 20 percent glycerol of *E. coli* M15 were prepared and preserved at -70°C for long-term storage. SEB^+ *S. aureus* was isolated from mastitic milk sample of cattle, while *S.aureus* isolates from bacteremic patients were obtained from different outpatient departments in India. The *S.aureus* isolates were identified and characterized using standard tests and methods which included production of coagulase, DNAse, and hemolysin. All the *S.aureus* isolates were also assessed for coagulase typing and antimicrobial susceptibility pattern. Toxin profile of the *S.aureus* SEB^+ strain isolated from mastitic milk sample of cattle was determined using latex agglutination test kits (Denka-Seiken, Japan) for the production of Staphylococcal Enterotoxins A-D (SEA-SED) and PCR. The presence of the *seb* gene in clinical strains was determined by PCR with the primers indigenously designed by us. This isolate was subsequently used for cloning and other experiments.

In addition, clinical *S. aureus* strains from our own collection which included methicillin sensitive and resistant *S. aureus* strains were used as and when required. The stock cultures of staphylococci species were maintained on blood agar plates and grown in brain heart infusion (BHI) medium (Bacto; BD) at 37°C with shaking.

Chromosomal DNA preparation

Chromosomal DNA was isolated as described earlier with some modifications. Overnight grown SEB^+ *S. aureus* bacteria (Sa/EB/M/15) in 10 ml media were centrifuged and washed in PBS. The pellet was suspended in 1 ml of TE-glucose [25 mM Tris-HCl (pH 8.00, 10 mM EDTA (pH 8.0), 1.0% (wt/vol) D-glucose] and centrifuged at 7,500 × *g* for 5 min. The cells were resuspended in 100 µL of lysostaphin (1 mg/mL in TE-glucose; Sigma), 50 µL of lysozymes (50 mg/mL in TE-glucose; Sigma) and incubated at 37°C for 1 h. Eighty microliters of NaCl-cetyltrimethylammonium bromide solution (0.7 M NaCl, 10% [wt/vol] cetyltrimethylammonium bromide; Sigma) was added with mixing. The mixture was incubated at 65°C for 10 min. Sodium chloride (100 µL of a 5 M stock solution), sodium dodecyl sulfate (30 µL of 10% [wt/vol] sodium dodecyl sulfate; Sigma) and proteinase K (4 mg of proteinase K; Sigma) was added with mixing and incubated at 55°C for 30 min. Equal volumes of phenol-chloroform were mixed and the lysate was extracted. DNA was precipitated from the aqueous phase with 1 volume of isopropanol and resuspended in 100 µL of sterile distilled PCR-quality water (Sigma). The DNA concentration was determined by UV spectrophotometry at A_{260} and the extract was stored at 4°C. Approximately 50 to 100 ng of DNA was taken for PCR amplification. The gene sequence of *S.aureus* enterotoxin B gene (*seb*) was retrieved from GenBank (Accession No. M11118). A set of primers was designed indigenously with the help of Gene Runner software (Hastings Software Inc., USA). The designed primers were custom synthesized from M/s Operon, Germany.

PCR was performed using reagents from MBI Fermentas (Germany). The PCR conditions for the amplification were optimized to an initial denaturation step of 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min and a final extension step of 72°C for 10 min. PCR program was run in *i-Cycler* (BioRad, USA).

Development of recombinant SEB (r-SEB) protein

The *seb* cDNA (Accession No. M11118) encoding 717 bases long *S. aureus* enterotoxin B was amplified by PCR, using forward (5'-GACCTT**GGATCC**GAGAGTCAACCAGATCCTAA-3') and reverse (5'-AAGGTC**AAGCTT**CTTTTTCTTTGTCGTAAGATAAA-3') primers containing the restriction sites (bold and underlined) for *Bam*H1 and *Hin*dIII. Construction of *pQE30* vector was done by standard cloning procedure. Briefly, double-stranded cDNA was purified followed by restriction digestion with *Bam*H1 and *Hin*dIII. *pQE30* vector DNA was also digested with same enzymes, and ligation was performed using T4 DNA ligation kit (MBI Fermentas) at 4°C overnight. The ligation reac-

tion of 10 µl was set up according to manufacturer's instructions (Qiagen Inc., Germany). In brief, the 7.5 molar excess of PCR product over vector using the formula mentioned below was used, and the ligation was performed overnight at 16°C for 16 hr.

ng PCR product required = ng of vector × PCR product size (bp) × molar ratio

Vector size (bp)

The reaction consisted of the required amount (ng) of purified PCR product, 50 ng of linearized vector DNA and 1x ligation buffer. Subsequently, the *pQE30*-SEB construct was transformed into *E. coli* M15 competent cells. The colony with a recombinant plasmid was isolated, and confirmation of positive clones was done by colony PCR, using the same primers. The expression optimization of rSEB was induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 24 hours and confirmed by SDS-poly-acrylamide gel electrophoresis and western blotting using anti-SEB polysera. The expressed rSEB protein was purified by Ni-NTA metal-affinity chromatography. The purified fraction of rSEB protein was dialyzed against PBS. Endotoxin levels of the purified protein were determined using Limulus amoebocyte lysate analysis kit (Whittaker Bioproducts, Walkersville, MD).

Characterization of the recombinant SEB (rSEB)

To determine the antigen specificity and antibody responses of recombinant SEB, ELISA and Western blotting were performed. Antigen-specific antibodies were analysed by ELISA. Western blotting of the recombinant protein (rSEB) was performed as per the standard procedure [27]. The antigenicity of protein was determined by developing the reaction using commercial hyper-immune sera (Sigma Aldrich, USA) and also hyper-immune sera raised in mice and rabbits against cell supernatant of SEB^+ *S.aureus* bacteria.

Immunogenicity of recombinant SEB (rSEB)

Animals [Mice (n=5) and rabbits (n=2)] were administered with PBS containing r-SEB (20, 40 and 60 µg per mouse or 40 and 60 µg per rabbit) subcutaneously at 0, 7, 15 and 21 days either singly (SEB) or mixed with alum (aluminum hydroxide 2%) as an adjuvant (SEB+Alum). Pre-immune sera as well as sera collected after immunization were collected and stored frozen until testing. Recombinant SEB antigen specificity and antibody response were determined by ELISA and western blotting. Additionally, commercially available (Sigma Aldrich, USA) and in house hyper-immune sera raised in mice and rabbits against cell supernatant of SEB^+ *S. aureus* bacteria was used to determine the antigenicity of protein. While, western blotting was used to characterize polyclonal antibodies, the titer of Hyperimmune Sera (HIS) was checked using ELISA. Pre-immune sera samples from both mice and rabbits were determined to contain non-detectable levels of anti-SEB antibodies (data not shown).

Toxicity criteria

Since SEB is potent superantigen, all animals were examined daily for clinical signs of toxicity as described earlier [28]. In mice shivering, hunched posture, ruffled coat, inability to ambulate, loss of consciousness, inappetence, tachypnea, and death were considered as toxicity criteria; whereas, fever, hyperemia, lethargy, inappetence, conjunctival suffusion, and diarrhea were considered as toxicity criteria in rabbits. To evaluate the pyrogenic effects in rabbits, rectal temperature was measured, and the course of temperature changes was monitored at appropriate time points; whereas diarrhea in rabbits was defined as the occurrence of at least one liquid elimination episode with or without mucus.

Lymphocyte proliferation and determination of cytokines

Further, we went to evaluate the lymphocyte proliferation and cytokines expression in animals. BALB/c mice were sacrificed two weeks after the last immunization, and splenocytes were collected, cultured (5×10^4 cells/well) and stirred *in-vitro* with the recombinant protein (5 µg/ml) followed by incubation for 72 hrs. at 37°C in 5% CO_2. Quick Cell Proliferation Assay Kit (BioVision, USA) was used to determine lymphocyte proliferation, and the serum levels of IL-1, TNF-α, and IFN-γ in the culture supernatant were measured in parallel cultures using ELISA kits from BD Biosciences as per the manufacturer's instructions.

SEB antigen ELISA

Using recombinant SEB, an antigen (Sandwich) ELISA was developed and standardized for the detection of SEB in different biological samples. We coated flat-bottomed polystyrene plates with five µg/ml of rabbit polyclonal antibody to recombinant SEB for 18 hr at room temperature. Defatted skimmed milk powder was used for blocking. Purified rSEB/crude SEB (1 µg/mL) in 1% BSA–PBS was used as the standard. Further, polyclonal anti-mouse antibody to rSEB (100 µL; 1:5000) was used as secondary antibody. Next, after washing with PBST and incubation, goat anti-mouse IgG conjugated to horseradish peroxidase (HRP; 1:5000) was added followed by addition of 0.4% solution of the colorimetric substrate (100 µL) *o*-phenylenediamine (Sigma) dissolved in 0.1 M citric acid/0.2 M Na_2HPO_4 containing 0.006% H_2O_2. The O.D. was recorded at A_{492} using a Dynatech, microplate reader. The experiments were performed in duplicate sets of samples.

Determination of SEB in biological specimens

The ability to measure SEB in biological samples was determined by diluting a known amount (1 µg/mL) of SEB in solutions of BSA (1%), human serum (70%), rabbit urine (70%), milk (70%) and mouse lung lavage (25 mg/mL). The concentration of SEB in these samples and in a standard sample of SEB were measured by the ELISA described earlier. Prior to the experiments, these biological fluids were evaluated for the presence of pre-existing anti-SEB antibodies and were determined to have non-detectable levels. All the biological samples were analysed for the presence of pre-existing anti-SEB antibodies by ELISA and were determined to contain non-detectable levels (data not shown).

Specificity testing of antigen ELISA for SEB

We performed the ELISA assay of the purified SEB, SEC and TSST-1 (concentration two µg/ml) using the above-discussed protocol to determine the specificity of the ELISA for SEB and other staphylococcal enterotoxins.

Determination of staphylococcal protein A interference

To determine the possible staphylococcal protein A interference with the ELISA, purified SEB was diluted to 1 µg/mL into recombinant staphylococcal protein A (Sigma) at concentrations ranging from 20 to 1,000 ng/mL. The samples were assayed by the antigen ELISA with the anti-SEB antibodies described above, and the values were compared to SEB alone diluted in assay diluent to determine possible interference from protein A. Recombinant protein A was used to avoid possible enterotoxin contamination.

Application of capture ELISA: Animal experiments

New Zealand White rabbits (n=5 rabbits per group) were injected

i.p. with 50 µg of purified SEB dissolved in 100 µL of PBS. In another experimental setting, New Zealand White rabbits (n=5 rabbits per group) were injected intra-venously with 10^6 CFU of clinical SEB positive and negative *S. aureus* strains.

Statistical tests

Results were analysed using GraphPad PRISM 5.0 and SPSS 21 softwares. Student t-test was applied for the comparison of continuous data from two groups. One way and Two-way ANOVA with post hoc multiple comparison tests were applied. Data were presented in mean ± SEM. $p<0.05$ was considered significant.

Results

Cloning, expression, and purification of recombinant SEB

Development of field-based diagnostic test and therapy would significantly help in the containment and prevention of SEB intoxication in the present context of bioterror threat scenario. To develop diagnostic test systems or any therapeutic modality, protein in pure and stable form is an absolute necessity. Thus, to produce a stable and highly purified protein *en masse*, full-length *seb* gene was amplified from genomic DNA of SEB^+ *S. aureus* bacteria of bovine origin and cloned in frame into pQE30 expression vector downstream of the histidine hexapeptide (His_6) coding tag; thus, yielding the construct pQE30-SEB. The resultant construct was confirmed by restriction enzyme analyses, PCR and partial sequencing across the fusion borders. The engineered pQE30-SEB construct was used to transform *E. coli* M15 host strain harboring the pREP4 plasmid (Figure 1A). Thus, recombinant SEB (rSEB) was engineered as a hybrid protein containing the additional dodecapeptide fMet-Arg-Gly-Ser-(His_6)-Gly-Ser at its predicted mature N-terminus.

The protein was predominantly expressed in insoluble fraction and was purified by metal chelate affinity chromatography under denaturing conditions. SDS-PAGE analysis of the affinity-purified protein revealed a single band of approximately 30 kDa (Figure 1B). The protein was refolded *in vitro* and used for various studies. We performed a comparison of protein fractions of eluted and the crude lysate achieving purity of >99%. Solubilization under denaturing conditions was required for purification of the product by low-pressure affinity chromatography on Ni-NTA agarose with a recovery yield of 6-8 mg of purified protein. Endotoxin levels in the final protein preparations were found to be <100 U/mg, as determined using a Limulus amoebocyte lysate assay.

Antigenicity and antibody-capture properties of recombinant SEB

We determined the antigenicity and antibody-capture properties of recombinant SEB towards development of immunodiagnostic test for the diagnosis and detection of SEB intoxication. Validation of these properties for the affinity purified recombinant product was done by Western blot and ELISA. We used five different detection reagents like anti-His antibody as first reagent, used as a control for the recombinant fusion product. Second, rabbit polyclonal antibody elicited against recombinant protein. Third, sera from BALB/c mice immunized with His_6-tagged SEB. Fourth, sera from cows infected with SEB^+ *S. aureus* bacteria. Fifth Sera from human patients infected with SEB^+ *S. aureus* bacteria. Homogenously purified His_6-tagged SEB was shown to be antigenic in rabbits and mice and was efficiently used as capture antigen for detection of SEB-specific antibodies in cattle and human sera (Figure 2A). The authenticity of the recombinant protein as SEB antigen was also validated by demonstrating that the antibodies elicited in mice and rabbits recognized native SEB protein.

Immunogenicity and toxicity of recombinant SEB

Immunogenicity and toxicity of purified recombinant SEB were

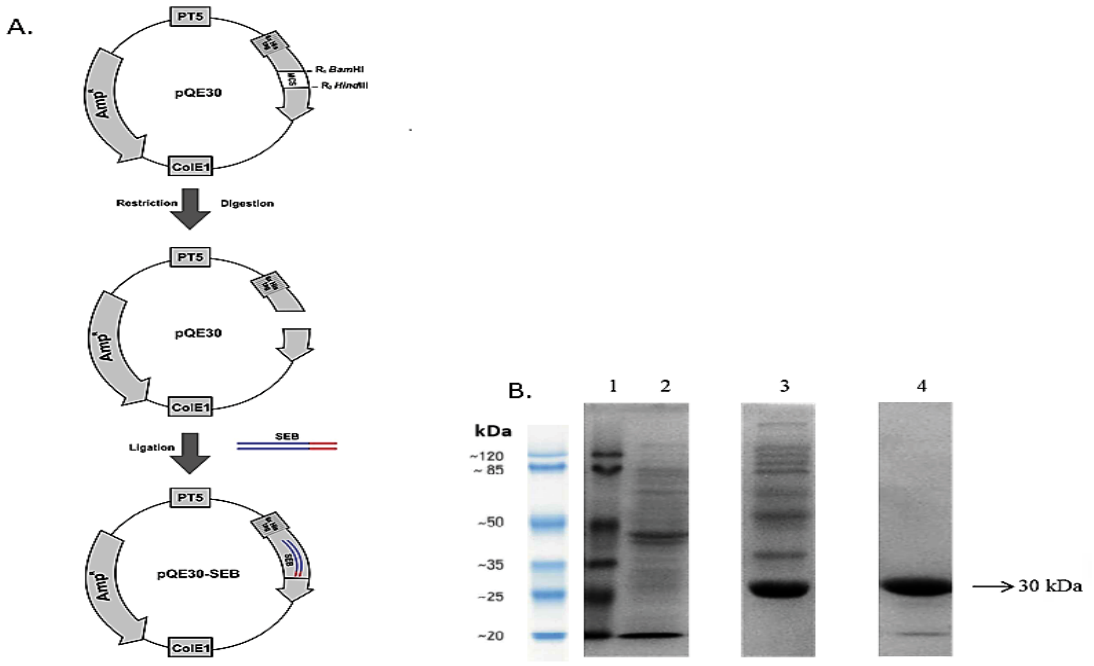

Figure 1: Cloning, expression and purification of recombinant SEB (A) Cloning of recombinant SEB gene into pQE30 expression vector (B) SDS-PAGE showing expression of rSEB toxin; 1-Protein MW ladder, 2- *E.coli* M 15 host cells, 3-Induced recombinant protein and 4-Purified recombinant protein.

evaluated in mice and rabbits that were immunized subcutaneously with rSEB alone or in combination with adjuvant in different experimental settings. We determined the antibody titers by ELISA with rSEB as a bound target. Administration of His_6-tagged SEB induced an antigen-specific humoral immune response with average endpoint titers varying from 1:1360 to 1:3040 and 1:2720 to 1:4160, respectively in rSEB and rSEB+Alum groups in mice. The intra-group differences were not statistically significant. Shivering and shuffling were observed at 12-72 hrs after each administration in some (2/5) animals. However, no mortality was observed in any group.

In rabbits, administration of His_6-tagged SEB elicited good antibody responses that ranged from 1:60000 to 1:100000 and 1:100000 to 1:120000, respectively in rSEB and rSEB+Alum groups (Figure 2B). However, the differences in antibody response between non-adjuvanted and adjuvanted groups were found non-significant at all doses of immunization (Figure 2B). Varying degrees of symptoms of intoxication viz., fever, lethargy, in appetence, sleepiness and diarrhea were observed in all the rabbits in both the dose groups. Moreover, symptoms were comparatively more prominent in the 60 µg dose group. The episodes of diarrhea were more severe and lasted for two to three days with recovery period of 3-4 days in the rabbits which were administered with 60 µg dose. Nevertheless, none of the animals died or necessitated sacrifice.

Lymphocyte proliferation and cytokine profile of rSEB

To study the antigen-specific lymphocyte response induced by recombinant protein, an *in vitro* lymphocyte proliferation assay was performed. Spleenocytes from both adjuvanted (rSEB+Alum) and non-adjuvanted mice elicited a significant lymphocyte proliferation response as compared to the control ($p<0.01$). Spleen cells from control mice did not proliferate against this antigen (Figure 3A). To further characterize the immune-modulation properties of this antigen, we analyzed by ELISA the concentration of cytokines in supernatants of spleen cells stimulated with rSEB. Mice immunized with rSEB+Alum showed a significant increase in the amount of IL-1, IFN-γ and TNF-α (Figure 3B) as compared to control group ($p<0.05$).

Configuration, sensitivity and specificity of capture ELISA

Using rSEB, we developed an in-house antigen ELISA for the detection of SEB in different biological samples with BSA as a standard. This ELISA was standardized employing rabbit polyclonal antibodies as the capture antibody and mouse polyclonal antibodies as revealing antibodies. A standard curve was constructed for in-house ELISA by plotting the absorbance as dependent variable against the concentration by using the results for recombinant toxin diluted to 10 µg/mL. Dilutions were made either in BSA (1%), human serum (70%), and rabbit urine (70%). Linear regression trend lines were generated for the lower part of the standard curve to calculate the concentrations in unknown SEB containing samples. Besides human sera and rabbit urine; milk (70%) and mouse lung lavage (25 mg/mL) were also included in this study to determine the sensitivity and accuracy of the sandwich ELISA developed. The samples were analyzed in triplicates, and the calculated concentrations from different diluents were compared to determine the sensitivity and accuracy of the assay. The lower limit was defined as the lowest dilution from which the original concentration of the toxin could be calculated from the equation for the linear fit.

This ELISA yielded good standard curve that allowed the fitting of trend lines to quantify the SEB concentration in BSA, human serum and rabbit urine (Figure 4). The ELISA developed was more sensitive with SEB toxin detected at a level of 20 ng/mL. The sensitivity of the assay was comparable in serum, urine and BSA; while the accuracy remains comparable in all the samples except moderate variation in mouse lung lavage at 40 and 20 ng/mL concentrations (Table 1). Thus, this ELISA showed that the quantification of SEB was near to accuracy

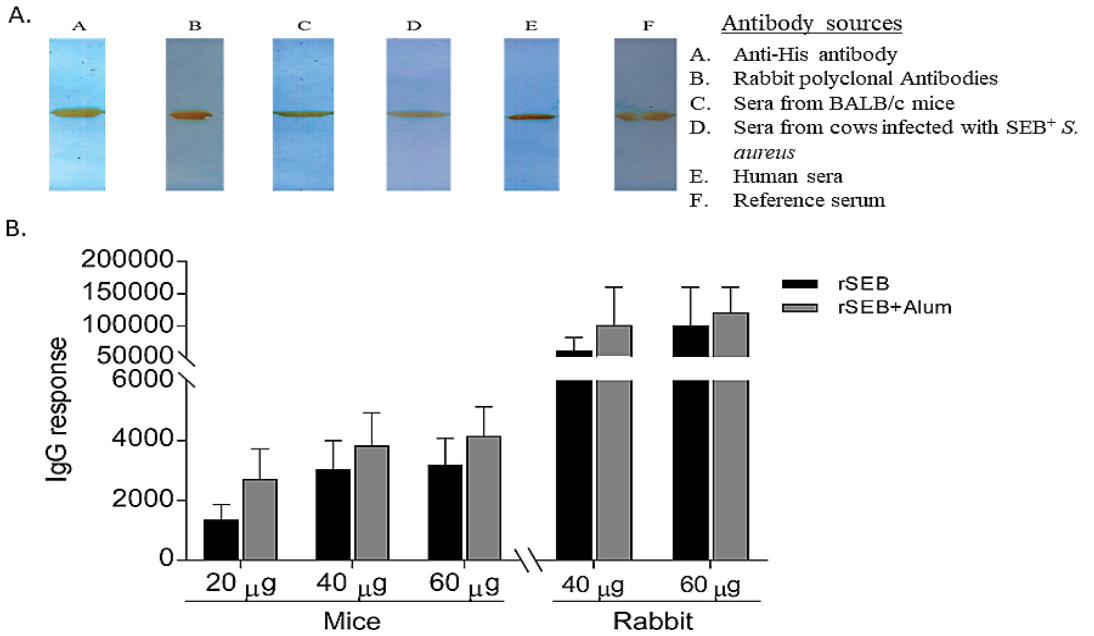

Figure 2: Antigen specificity and IgG antibody response of rSEB. (A) Western blot assay using purified rSEB bovine (Strips A-E) or native SEB (strip F). (B) Determination of specific humoral immune response in mice (n=5 per group) and rabbits (n=2 per group) immunized subcutaneously with rSEB with or without alum. Antibody titres were determined by ELISA with rSEB as the bound target. Data is expressed as Mean ± SEM.

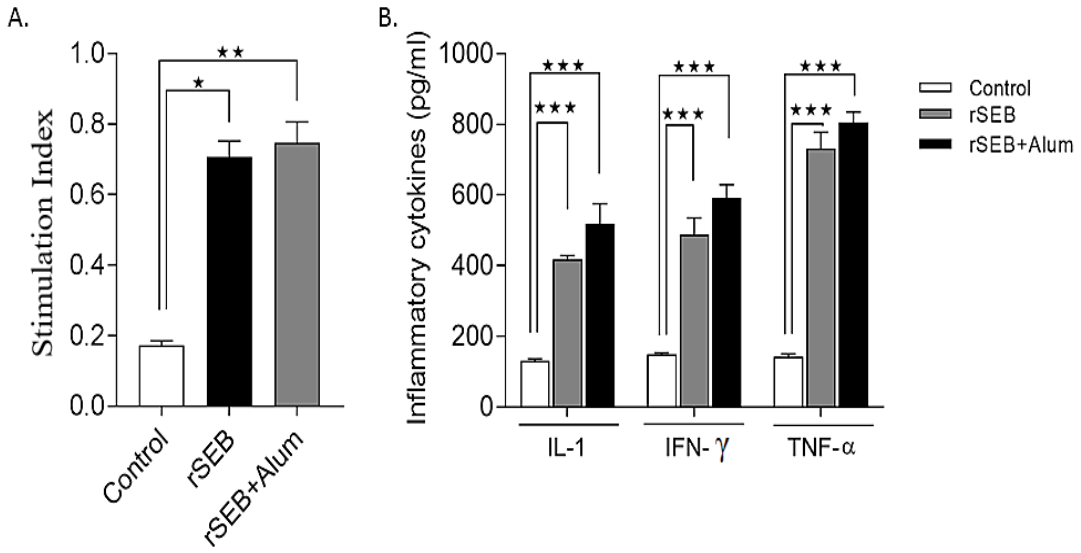

Figure 3: Lymphocyte proliferation (A) and cytokine profile (B) of rSEB in the spleen cells from mice vaccinated with rSEB alone and combined with alum compared with control (PBS+alum). Each bar represents the group mean (n=5) ± SEM. The results presented are representatives of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001(Immunized *vs*. control).

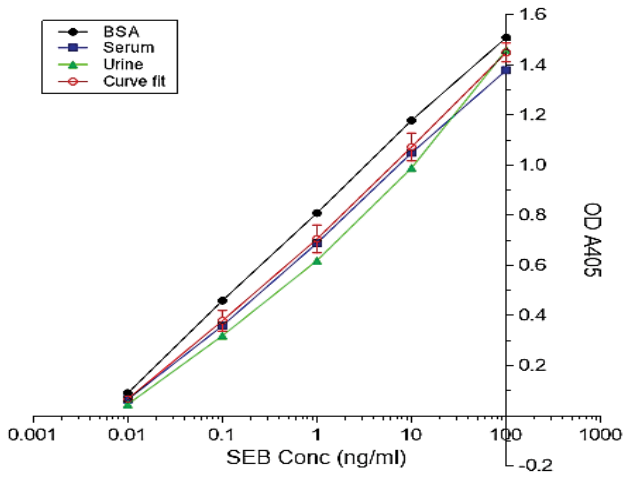

Figure 4: SEB detection by sandwich ELISA. A comparison of SEB detection in BSA, human serum and rabbit urine is shown. The test was performed in triplicate, and error bars of the curve fit represent standard error of mean.

and yielded calculated values that were very close to the actual amount of toxin added (Table 1).

Specificity testing of antigen ELISA for SEB

Specificity of antigen ELISA for SEB was determined with other staphylococcal enterotoxins viz., SEC, and TSST-1. Our data demonstrated that ELISA detected all the other toxins viz., TSST-1 and SEC at higher concentrations. However, the sensitivity of these two proteins was less than that of SEB (Figure 5A).

Staphylococcal protein A interference

It is recognized that staphylococcal protein A (spA), a staphylococcal cell wall protein produced by *S. aureus* bacteria, exhibits the interference to ELISA because of binding of N terminus of protein to IgG. To address

SEB used in ELISA (ng/mL)	BSA (1%)	Human serum (70.00%)	Rabbit urine (70.00%)	Milk (70.00%)	Mouse Lung Lavage (25 mg/mL)
100	88.12 ± 0.00033	85.84 ± 0.0007	91.11 ± 0.0041	86.41 ± 0.0061	76.51 ± 0.0081
60	56.76 ± 0.0026	53.88 ± 0.0008	62.18 ± 0.0006	68.23 ± 0.0051	54.61 ± 0.0032
40	43.81 ± 0.0007	46.51 ± 0.0061	37.66 ± 0.0008	44.42 ± 0.0006	66.78 ± 0.0003
20	17.16 ± 0.00041	15.11± 0.0001	16.35 ± 0.0044	31.67 ± 0.0051	40.81 ± 0.0047

Table 1: Quantification of SEB in different biological samples.

the possible interference of protein A, the sensitivity of the ELISA was examined in the presence of increasing concentrations of protein A. To compare the results, protein A interference was also tested with ELISA developed using native SEB (nSEB).

Results indicated statistically significant differences between two ELISAs (rSEB *vs*. nSEB) at every protein A concentration. In the presence of high concentrations of protein A (1000 ng/ml), the levels of SEB measured were increased non-significantly when tested with rSEB ELISA. On the contrary, the levels of protein A were found increased significantly ($p< 0.005$) by ELISA where native SEB was used and the absorbance was comparable to the background level (Figure 5B). The findings demonstrated that staphylococcal protein A did not interfere with SEB measurements by ELISA developed using recombinant SEB. Non-interference of protein A on the detection property of SEB by ELISA developed using recombinant SEB suggest complete removal of protein A during purification of recombinant SEB; while such proteins were remained intact in native SEB.

Application of sandwich ELISA

We performed animal experiments with New Zealand White rabbits that were injected i.p. with 50 µg of purified SEB dissolved in 100 µL of PBS. This concentration was suggested by other investigators to be the sub-lethal dose for rabbits. Next, we injected New Zealand

White rabbits intravenously with 10^6 CFU of clinical SEB$^+$ and SEB$^-$ *S. aureus* bacteria. SEB levels were measured using sandwich ELISA in the serum, urine and faeces of rabbits at 2, 8, and 24 h post-exposure. Sera were collected by bleeding ear vein and stored frozen; while urine release was induced and collected by rubbing the rabbit's lower abdomen. All the rabbits developed mild symptoms of intoxication (decreased mobility) within 24 h, but survived.

Results indicated detection of SEB in all the body fluids (serum, urine and faeces) of rabbits. The SEB concentration was 0.18 µg, 0.14 µg, and 0.09 µg, respectively in rabbit serum, urine, and faeces after 2 hr exposure. Increased concentration of SEB was observed after 8 hr exposure to 1.04 µg, 0.76 µg, and 0.33 µg, respectively in the samples investigated. However, we observed decrease in serum SEB concentration after 24 hr of exposure; whereas, slight increased concentration of SEB was seen in urine and faeces after 24 hr indicating excretion of SEB in urine and faeces (Figure 6A). Upon infection with SEB$^+$ *S. aureus* bacteria, similar pattern was observed. Serum SEB levels were heightened to 1.1 µg, 1.3 µg and 0.38 µg respectively at 8 hr interval in serum, urine, and faeces samples (Figure 6B).

Discussion

Staphylococcus aureus bacteria produce a wide range of virulence factors including enterotoxins that are known to cause extensive diseases in humans and animals [28,29]. Among all enterotoxins, staphylococcal enterotoxin B is the most lethal biological agent that can be conveniently used as bioterror agent [16]. However, at present, no simple to use diagnostic test systems or any vaccine formulations are available to screen the possible bioterror attack and/or to contain the SEB intoxication in the resource limited settings in developing countries. In this study, we report the suitability of recombinant form of staphylococcal enterotoxin B (rSEB) produced in *E. coli* for diagnosis and therapeutic vaccine potentials against SEB intoxication.

The cloning strategy adopted in the present investigation employing pQE30 expression vector produced SEB as 6X histidine fusion protein with appreciably high yields of recombinant protein. With this strategy, it was possible to produce a stable form of SEB protein in large quantities, free from other contaminating proteins, thereby providing a reliable source of protein for further investigation of its role in diagnosis

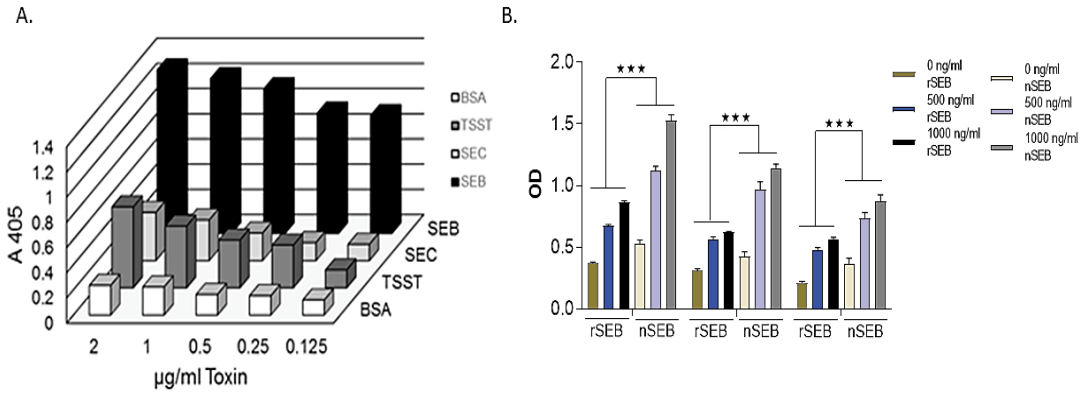

Figure 5: Specificity of antigen ELISA for SEB and Staphylococcal protein A interference. (A) Equal quantity of SEB, SEC, and TSST was measured using ELISA. (B) Measurement of SEB in the presence of Staphylococcal protein A with increasing concentration. The measurements were affected by high levels of protein A for both recombinant SEB (rSEB) and native SEB (nSEB), however, rSEB presented greater interference than nSEB. The results presented are representatives of three independent experiments and error bars represents standard deviation. * p<0.05; ** p<0.01; *** p<0.001.

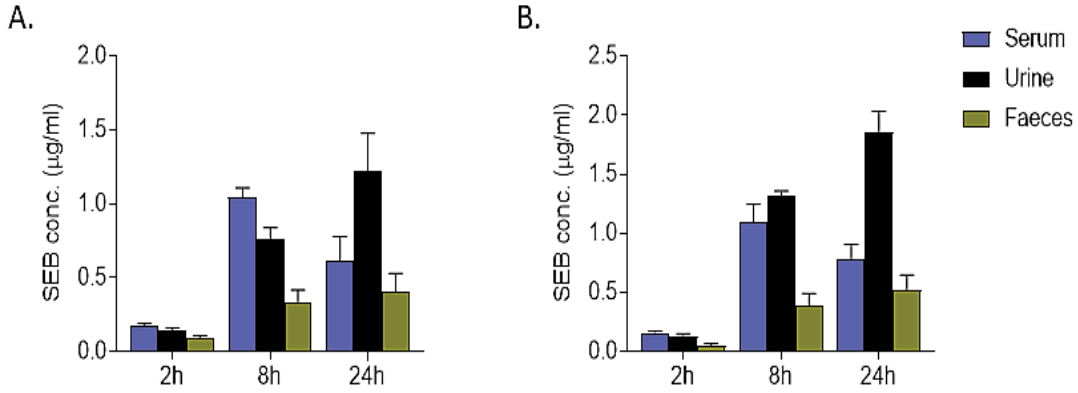

Figure 6: Determination of SEB using rSEB ELISA in different body fluids (serum, urine, and faeces) of experimentally infected rabbits. A group of rabbits was exposed to SEB toxin (A) while another group was infected with SEB producing *S. aureus* bacteria (B). SEB was determined by the antigen ELISA. The average SEB levels that were measured by antigen ELISA in different body fluids at given time points (2, 8 and 24 hrs) are shown. The test was performed in triplicate, and error bars of the curve fit represent standard deviation of mean. Prior to intoxication, SEB concentration was determined in these body fluids. No SEB was detected in any body fluids tested (data not shown).

and vaccine development. High-level expression of His_6-tagged proteins in *E. coli* M15 cells using pQE vectors are based on the T5 promoter transcription-translation system. The vector also contains a ribosome binding site, His_6-encoding sequences, followed by a multiple cloning sites, translational stop codons and two strong transcriptional terminators, t0 and T1 [30].

In order to develop immunodiagnostic tests for detection of SEB intoxication, antigenicity and antibody capture properties of recombinant SEB was tested using detection reagents from various sources. It was observed that, affinity purified His_6-tagged SEB reacted with antibodies from cattle and hyper-immunized rabbits and mice, thus confirming the antigenic authenticity of rSEB to SEB. These data provide evidence for the potential use of His_6-tagged SEB in immunological assays, which are more sensitive and specific than biological assays. The detection of serum IgG of human and cattle origin that are reactive to rSEB, further authenticate the antigenicity of recombinant SEB to native SEB from different species and can be used for *in vivo* detection of virulence factors during SEB^+ *S. aureus* infection and/or exposure to the SEB toxin.

With the exception of SEC serotype [31]; there are no reports on molecular heterogeneity for any other staphylococcal enterotoxin [14]. Thus, given the reported conservation (>95% amino acid sequence identity) among SEB variants from different species [32], it is expected that His_6-tagged SEB will be recognized by antibodies from different sources and that polyclonal antibodies elicited to the recombinant protein can be used for detection of SEB variants, as well as other SE serotypes across the species. Here, we showed that rabbit polyclonal IgG to recombinant SEB recognized native SEB human variants.

After confirming the antigenicity and antibody capture properties of rSEB, we developed a capture enzyme-linked immunosorbent assay for the detection of SEB in biological samples. The generation of SEB-specific murine and rabbit antibodies allowed us to identify the combinations that could be successfully used to generate a simple to use sandwich ELISA. This ELISA was utilized to detect SEB levels both *in vitro* and *in vivo*. Neither serum nor urine or milk products interfered with the performance of this ELISA. With this assay, the peak levels of SEB in serum, urine and faeces can be measured in exposed individuals.

The ELISA described here provides excellent sensitivity for detection of enterotoxin B upto 20 ng/mL level and thus, comprehensively satisfies the general expectation of detection of low levels of SEB toxin in the biological samples. The ELISA was also evaluated to detect SEB in body fluids of rabbits those were exposed to SEB and SEB producing *S. aureus* bacteria. This ELISA can be a useful tool for the detection and diagnosis of SEB intoxication as well as infection with SEB producing *S. aureus* bacteria in humans and other species. Our data further indicated that SEB injected rabbits developed significant levels of SEB in their serum and excreted it through urine and faeces in a given time frame. Thus, this ELISA could be utilized for testing of vaccine efficacies or therapeutic reagents and further can be used for investigation of different aspects of *in vivo* regulation and clearance of SEB. A highly sensitive ELISA of sensitivity upto 20 pg/mL has been demonstrated for detection of SEB in serum and culture supernatant [17]. The high sensitivity described [17] can be due to use of monoclonal antibodies as capture and revealing antibodies. More advanced detection methods have been documented for detection of SEB in different environmental matrices [21-25]. However, these systems are not suited to resource poor settings because of their sophisticated instrumentation, requirement of expert personnel and high cost. In such situations, the ELISA described in this study with reagents generated using recombinant SEB would be highly useful for preliminary screening and early detection of SEB intoxication in case of any bioterror act.

Approximately 30% of the human population is colonized with *S. aureus* and SEB is produced by 5 to 10% of clinical *S. aureus* strains [33]. However, contribution of SEB to the outcome of staphylococcal infections in clinical settings is still unclear, mainly because tools for the measurement of SEB levels in large quantities and field conditions are lacking [33,34]. Furthermore, *S. aureus* is the second most common pathogen recovered in nosocomial bloodstream infections in other parts of the world [35]. However, not much information is available on prevalence of SEB or SEB producing *S. aureus* bacteria in the Indian scenario. This ELISA could be useful in answering both these aspects, too.

The immunogenicity observed with and without adjuvant yielded significant antibody production and protection index in both mice and rabbits. However, the immunogenicity and protection index observed with recombinant SEB alone without any adjuvant is an added characteristic that differentiates it from previous reports wherein adjuvants were used [36,37].

Regarding our recombinant SEB preparation, following things are worth mentioning:

Recombinant SEB was extracted and affinity purified using 8 M urea, which is known to cause unfolding of most proteins resulting into impairment of monomer/monomer interactions. Although enterotoxin folding can be restored to some extent after removal of urea, the resulting product is very labile [38]. This study indicated that affinity purified recombinant protein exhibited strong mitogenic activity *in vitro* for murine lymphocytes, comparable to native SEB.

Staphylococcal protein A (SpA) is a cell wall associated protein present in nearly all strains of *S. aureus*. SpA binds to Fc region of all the major classes of mammalian immunoglobulins and produce false positives during immunoassays [39]. While native preparations require additional protein extraction step to avoid possible interference by protein A and/or DEPC treatment to inhibit binding of SpA to capture antibody, the production of recombinant SEB here yielded SEB that was devoid of protein A which facilitated us to generate SEB specific reagents and develop ELISA with high specificity to SEB without any interference of SpA [40-49].

Conformational integrity is an important consideration for vaccine studies, as the vast majority of epitopes are conformational dependent [31]. Partial unfolding processes play a major role in toxin membrane insertion and translocation. Biological properties and immunogenicity showed by rSEB indicated maintenance of conformational integrity by recombinant molecule.

Conclusion

In conclusion, the cloning strategy adapted in the present investigation yielded safe, pure and stable recombinant protein. The data are encouraging and underscore the feasibility of rSEB molecule for development of reliable and affordable diagnostic tools and vaccine formulations. Availability of the recombinant *SEB* clone will enable us to develop isogenic mutants to further examine its role in the early diagnosis and management of SEB associated toxic shock syndrome in humans and animals.

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Contributions

RT: Conceived and Designed the experiments, Performed the experiments, Analysed the data, wrote the manuscript; AP: Performed the experiments, Analysed the data, helped in writing the manuscript; SK: Edited the manuscript and interpretation of data.

Declaration

Authors declare no conflict of interest.

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