Application of a Chimeric Protein Construct having Enterotoxin B and Toxic Shock Syndrome Toxin Domains of *S. aureus* in Immunodiagnosics

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Abstract Staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 are the super antigens responsible for diseases such as staphylococcal food poisoning and toxic shock syndrome. At low serum concentrations, SEB can trigger toxic shock, profound hypotension and multi organ failure and hence is recognized as biowarfare molecule. In this study, a multidomain fusion protein (r-TE) was generated with specificity for SEB and toxic shock syndrome toxin (Tsst-1). The fusion gene comprising the conserved regions of *seb* and the *tsst* genes was codon-optimized for expression in *Escherichia coli* and encoded a 26 kDa recombinant multidomain chimeric protein (r-TE). Hyperimmune antiserum raised against r-TE specifically reacted with SEB (~28 kDa) and Tsst-1 (~22 kDa) components during Western blot analysis and by plate ELISA in confirmed toxin producing strains of *S. aureus*. The antigenicity of the SEB component of the r-TE protein was also confirmed using TECRA kit. The described procedure of creating a single protein molecule carrying components of two different toxins whilst still retaining the original antigenic determinants of individual toxins proved highly advantageous in the development of rapid, reliable and cost effective immunoassays and may also have the potential to serve as candidate molecule for vaccine studies.

Keywords Seb · Tsst · Chimeric protein ·
S. aureus r-TE · Superantigens · Western blot

Introduction

Staphylococcus aureus, a Gram-positive cocci, with potential to cause clinical and food-borne infections by secreting a variety of extra cellular toxins is responsible for many nosocomial infections, besides being the main causative agent of food intoxication [1, 2]. Among the array of toxin components being possessed by this pathogen, enterotoxin B (SEB) and toxic shock syndrome toxin (Tsst-1) of *S. aureus* are the important virulence factors that play vital role in the pathogenicity. SEB is the primary cause of staphylococcal food poisoning and a potent mitogen, whereas Tsst may lead to toxic shock syndrome which is potentially fatal. Both Tsst-1, and SEB belong to a family of superantigens, at very low concentrations, these super antigens induces polyclonal immune response by direct binding to class II major histocompatibility complex proteins and T-cell receptors on the surfaces of B and T cells without being internalized and processed like a normal antigen [3–5]. At low serum concentrations, SEB can also trigger a toxic shock and profound hypotension resulting in multi organ failure [6, 7].

Both SEB and Tsst owing to their virulence potential, qualify as biowarfare molecules [8–10]. In the event of biological emergency, the disease is likely to be confused with naturally occurring epidemic, further confounded by difficulties for the timely diagnosis which could lead to delay in the initiation of treatment or control procedures. An early diagnosis of the disease will enable medical/rapid response teams to implement appropriate defensive measures for an effective action. Though the detection systems based on the PCR are specific and accurate, the main disadvantage happens to be their inability to correlate to the expression of toxin components by the organism. Certain immunoassays have been reported for the detection of

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these toxins individually, but so far there is no single system available to detect both of these toxin molecules simultaneously. Moreover, the commercially available immunoassays for the detection of individual toxin are costly. Simultaneous detection of both of these toxins in one assay can also make the product economical.

In the present study, we followed a strategy of combining together the conserved domains of *tsst-1* and *seb* to form a single fusion gene and to express the multidomain recombinant chimeric protein in *Escherichia coli* without exhibiting toxicity to the host cells. The polyclonal antibodies thus generated were successfully evaluated for the detection of Tsst-1 or SEB containing *S. aureus* from different sources by ELISA as well as by Western blot analysis. Detection of these two toxins simultaneously by a single immunoassay would have advantages in terms of rapidity, convenience and cost saving during biological emergencies.

Materials and Methods

Bacterial Strains and Materials

The bacterial strains used in this study were obtained from ATCC and National Collection of Industrial Microorganisms (NCIM), clinical isolates from SDM Medical college, Dharwad and twelve *S. aureus* strains isolated from food samples collected from different sources of Mysore. Characterization of this important foodborne micro-organism was carried out by standard conventional biochemical identification procedures. All of the standard strains and isolates were checked for the presence of the genes under study (*seb* and *tsst*) using PCR primers developed in house (Table 1). All these standard strains and isolates were used

for evaluation of immunogenicity of r-chimeric protein. The enzymes and chemicals used for DNA manipulation were purchased from Fermentas (India). The oligonucleotides were obtained from MWG Biotech Pvt Ltd. (Bangalore). The plasmid pRSET A and *E. coli* host BL21 (DE3) pLysS were purchased from Invitrogen (India).

PCR Amplification of *tsst* and *seb* Genes and Plasmid Construction

Construction of fusion gene and plasmid were carried out as per the procedure described by [11], the conserved portions of *tsst-1* and *seb* were amplified by PCR, using *S. aureus* TSST-Japan and ATCC 51740 whole genomic DNA respectively as template. Primers of *tsst-1* gene fragment (323 bps) were designed to insert *Bam*HI and *Sac*I sites to the 5' and 3' ends, similarly, primers of *seb* gene fragment (332 bps) were designed to insert *Sac*I and *Hin*-dIII sites to the 5' and 3' ends via PCR (Table 2). PCR was performed in 25 µl reaction volume containing 400 nM of each primer, 200 µM of each dNTP, 1.0 U Pfu polymerase (MBI Fermentas), 1.5 mM of MgSO₄ in 1× PCR buffer (MBI Fermentas) with 50 ng of template DNA. PCR was carried out through 30 cycles in Eppendorf master cycler gradient (Hamburg, Germany) thermal cycler at 94 °C for 1 min denaturation, 54 °C for 1 min annealing and 72 °C for 2 min extension. The DNA was denatured for 5 min in the beginning and finally extended for 15 min at 72 °C. The amplified PCR products were restricted with appropriate enzymes, separated on 1 % agarose gel and purified by Genelute gel extraction kit (Sigma).

Fusion gene, codon-optimized for *E. coli* expression, was first generated by ligation of PCR amplified products encoding conserved domains of *tsst-1* and *seb* using T4 DNA ligase and subsequent PCR amplification with

Table 1 Primers for detection of *seb* and *tsst* genes of *S. aureus*

Genes targeted	Primer sequence (5'–3')	Accession number	Amplicon size (bps)
<i>seb</i>	For—CCAGATCCTAAACCAGATGAGTT	AY856382.1	325
	Rev—GTTTTTCGTTTGTCAGTTTGATG		
<i>tsst</i>	For—CCCTTTGTTGCTTGCGAC	J02615.1	569
	Rev—TGGATCCGTCATTCATTGTTA		

Table 2 Primers for cloning of *seb* and *tsst* genes of *S. aureus*

Genes targeted	Primer sequence (5'–3')	Accession number	Amplicon size
<i>Tsst</i>	For—ATGGGATCCTTGCTTGCGACAATCG	J02615.1	323
	Rev—CACGAGCTCATATAAGTTTCCTTCGCTAGTATG		
<i>Seb</i>	For—ATAGAGCTCACCAGATCCTAAACCAGATG	AY856382.1	332
	Rev—TAGAAGCTTGTTTGTCAGTTTGATGCG		

forward primer of *tsst-I* and reverse primer of *seb* to get fusion gene (655 bp). The resultant 655 bp gene (r-TE) was ligated in frame into the *Bam*HI and *Hind*III sites of pRSET A to generate the plasmid pR-r-TE, which expresses a chimeric protein of 26 kDa. The ligated product was transformed into chemically competent *E. coli* BL21 (DE3) pLysS cells and selected on ampicillin plates [12]. Transformants were subjected to direct colony PCR screening, using *tsst-I* forward primer and *seb* reverse primer to identify recombinants harboring the synthetic r-TE gene.

Expression and Purification of r-Chimeric Protein

The *E. coli* cells carrying the recombinant plasmid were grown at 37 °C in Luria–Bertani broth with 50 µg/ml of ampicillin at 200 rpm. When A_{600} reached 0.6, IPTG was added to a final concentration of 1 mM. After 5 h of induction, cells were harvested by centrifugation at $4,000 \times g$ for 20 min. The recombinant protein with amino terminal hexa histidine residues was subjected to purification by IMAC method using Ni–NTA (Qiagen) as a chelating resin under denaturing conditions using guanidine hydrochloride as denaturing agent. All the samples were examined on 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein content was estimated by the method of Lowry et al. [13]. For immunization purpose, the six-histidine tag was removed from 200 µg of purified chimeric protein by cleavage with 0.1 U of biotinylated thrombin (Novagen, India) at 16 °C for 16 h. The cleaved recombinant protein was then purified by passage through streptavidin-agarose (Novagen) to remove the biotinylated thrombin and then a nickel charged Hi-Trap column to remove cleaved six-histidine tags and any remaining uncleaved His-tagged chimeric protein moieties.

Preparation of Hyperimmune Polyclonal Antiserum

Hyperimmune anti-r-chimeric protein polyclonal antiserum was generated by subdermal immunization of male BALB/c mice (6 weeks old) with affinity-purified r-chimeric protein. Primary immunization of each animal was done with 50 µg of purified r-chimeric protein in Freund's complete adjuvant. Two boosts of 10 µg each of r-chimeric protein in Freund's incomplete adjuvant followed on days 14 and 28.

Antibody Titer by Indirect Plate ELISA

The antibody titer of immunized mice sera was measured by indirect plate-ELISA using r-TE protein as antigen [14]. 50 µl of 10 µg/ml r-TE protein in 50 mM carbonate buffer (pH 9.6) was coated on to microtiter plates, incubated at 4 °C overnight, followed by blocking with 5 % non fat milk

solution at 37 °C for 2 h. Plates were washed twice with PBST (tween 20–0.05 %) solution and incubated with 50 µl of hyperimmune sera raised against r-TE protein from 1:1,000 to 1:64,000 dilutions in PBS. Later plate was washed four times with PBST buffer and incubated in HRP conjugated anti-mouse antibodies raised in goat for 30 min. Plates were washed vigorously with PBST for six times and developed with o-phenylenediamine dihydrochloride solution (0.1 mg/ml) and 1 µl of 30 % H_2O_2 in 1 ml citrate phosphate buffer. The reactivity of the same hyperimmune serum was tested by Western immunoblot analysis as described below.

Western Blot Analysis

The affinity-purified r-chimeric protein and crude toxin preparation of Tsst-1 and SEB of *S. aureus* standards as well as isolates were used for Western immunoblot analysis. Briefly, crude SEB and Tsst-1 toxin and r-chimeric protein separated by 15 % PAGE were transferred to a nitrocellulose sheet and then the sheet was immersed in TBS (150 mM NaCl, 50 mM Tris–HCl pH 7.4) containing 0.05 % Tween 20 and 5 % milk at 4 °C overnight to saturate protein-binding site. The antigen electroblotted onto the nitrocellulose membranes was incubated with the hyperimmune mouse sera at a 1:1000 dilution and then with peroxidase-conjugated anti-mouse IgG (Sigma India) at a 1:1,000 dilution and developed with 0.3 % diaminobenzidine tetrahydrochloride (Sigma, India) and 0.03 % H_2O_2 at room temperature for 5 min. Washing the sheet in 0.1 M H_2SO_4 terminated the enzyme reaction.

SEB Analysis

The r-TE protein was evaluated for the presence of native antigenic determinants of SEB using a TECRA VIA Staphylococcus enterotoxin kit (TECRA International).

ELISA

To determine whether the anti-r-TE antibody also bound native toxins, an antibody ELISA was performed according to the method of Harlow and Lane [15] using plastic 96-well microtiter plates (PolySorb; Nunc) as the solid phase. The plates were coated with 100 µl diluted antigen and incubated at room temperature for 1 h. Each well was washed twice in PBS, and the remaining wall area was blocked with 3 % BSA in PBS for 2 h at room temperature. A dilution of the anti-r-TE antibody in 1 % BSA in PBS/0.05 % Tween was added and incubated for 2 h. Each well was washed three times with PBS/0.05 % Tween. Anti-mice IgG conjugated to horseradish peroxidase was added

and incubated for 1 h, washed three times and developed using o-phenylenediamine dihydrochloride (Sigma) and H_2O_2 in 0.15 M phosphate citrate buffer (pH 5.0), and the A_{450} was measured. Initial assays were performed to determine the antigen concentration that best discriminated between ELISA reactions of culture supernatant from confirmed toxin-producing organisms and non-toxin producing organisms. Chequer-board titrations were performed with 50-, 100- and 200-fold serum dilutions and antigen concentrations per well of 5, 25, 50 and 100 ng. In subsequent assays to determine sensitivity and specificity, plates were coated with the predetermined antigen concentrations (5, 25, 50 and 100 ng). Incubations were performed with 50- and 20,000-fold dilutions of primary sera and secondary antibody conjugate, respectively. Individual culture supernatant samples were tested in duplicate and the means of the two measurements were calculated for analysis. Paired measurements that differed by greater than 10 % were retested. One negative control serum sample from unimmunized mice was included, in duplicate, on each plate as a quality control measure.

Evaluation of the Detection Systems

Evaluation of the hyper immune sera based Western blot detection system was carried out using 216 food, clinical and environmental samples. Simultaneously the samples were tested for the presence of *seb* and *tsst* genes by PCR and expression of SEB toxin using commercially available TECRA kit.

Results

Design of a Novel Tsst–SEB Toxin Chimeric Protein

In order to create a recombinant chimeric protein that could be useful in simultaneous detection of Tsst and SEB toxins, we focused on antigenic domains of the Tsst and SEB toxin by selecting truncated portions. Care has been taken during designing of primers for amplification of *tsst*–*seb* genes to accommodate open reading frame of one gene in view of open reading frame of preceding gene. The fusion gene designated r-TE contains conserved domains of *tsst* and *seb* was designed to be in frame with the initiator codon and N terminal six histidine tag encoding sequence provided by the vector. The fusion gene (r-TE) generated a 26-kDa recombinant multidomain chimeric protein (Fig. 1). Restriction enzyme digestion of recombinant plasmid revealed a 2.9 kb linearised vector and 655 bps insert as predicted. DNA sequence analysis of plasmid showed right open reading frame of insert. *E. coli* BL 21 DE3 (plysS) cells were transformed with the recombinant plasmid.

Expression and Purification of Recombinant Tsst–SEB Chimeric Protein

The presence of Tsst–SEB chimeric protein was determined by SDS-PAGE analyses of cytosol and inclusion bodies. The r-chimeric protein appeared to be aggregating to form inclusion bodies (Figs. 2 and 3). The recombinant Tsst–SEB chimeric protein was purified from inclusion bodies under denaturing conditions. The protein after affinity chromatography was about 90 % pure with small amount of degraded and partially synthesized protein (Fig. 4). The pooled fractions containing affinity purified Tsst–SEB chimeric protein were dialyzed against T10E5 buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.5).

Antibody Titer by Indirect Plate ELISA

Hyperimmune from immunized mice was collected and antibody titer was tested against the recombinant r-TE as antigen by indirect ELISA on a microtiter plate. The absorbance was read at 492 nm in microplate reader. An

Fig. 1 Construction of the plasmid used for expression of the TE fusion protein. *Left panel* plasmid pRSET A, an expression vector containing the T7 promoter (P), a synthetic ribosome-binding site (R), a His6 affinity tag sequence (H), an express epitope (EX), a multiple cloning site (MCS), a transcriptional terminator (T), an origin of replication (O) and an ampicillin-resistance gene (amp^r). BXSBBPKNEBH in the MCS represents the sites for the restriction endonucleases BamHI, XhoI, SacI, BglII, PstI, PvuII, KpnI, NcoI, EcoRI, BstBI and HindIII, respectively. *Right panel* Tsst (encoding the toxic shock syndrome toxin of *S. aureus*) and entB (encoding staphylococcal enterotoxin B) were amplified by PCR with new restriction sites added at the ends of the primers and cloned into the BamHI and HindIII sites of pRSET A to generate the fusion gene construct r-TE

absorbance at O.D₄₉₂—0.3 was read at even 32,000 dilution of the hyperimmune sera.

Western Immunoblot Analysis

Western immunoblot analysis showed that mouse anti-r-chimeric protein serum strongly reacted with both Tsst-SEB of the different *S. aureus* strains (Fig. 5). All of these strains were previously having been checked for the presence of enterotoxin by commercially available TECRA kit as well as by the PCR method for the detection of *seb* producing gene and by PCR for the presence of *tsst* producing gene (Table 3). Hyperimmune anti sera of r-chimeric protein did not exhibit any reaction with other non-*S. aureus* group strains tested.

Fig. 2 Electrophoretic analysis of r-TE protein expression in *E. coli*. All samples were separated by 12 % SDS-PAGE. The gel was stained with Coomassie blue to visualize the protein components. Lanes 1 to 4 cells expressing TE fusion protein, L5 uninduced *E. coli* host cells BL 21 (DE3) pLysS cells, L6 M molecular weight standards, L7 cells expressing TE fusion protein, L8 cytosolic proteins of cells expressing TE fusion protein, L9 cells expressing fusion protein, L10 *E. coli* host cells negative for expression

Fig. 3 Electrophoretic analysis of cytoplasmic, periplasmic and inclusion bodies of cells expressing of r-TE chimeric protein

L1:uninduced clone; L2: induced clone; L3:cytoplasmic fraction; L4: periplasmic fraction; L5:unstained protein marker; L6: culture supernatant; L7 & 8: inclusion body fractions.

SEB Analysis

The r-TE protein sample tested positive for SEB moieties using a TECRA VIA Staphylococcus enterotoxin kit (TECRA International).

Development of an ELISA

An ELISA-based system was also developed that could be used in both clinical and food research laboratories. This would also have the advantage of quantifying the toxin. Maximum absorbance values were observed at antigen concentrations of 50 ng ml⁻¹. The ratio of mean absorbance values between standard toxin-positive and individual test samples was not significantly different at 50-, 100- and

Fig. 4 Purification of *E. coli* expressed r-TE chimeric protein using Ni-NTA chromatography. LM molecular weight standards, L2 first elution 1, L3 second elution 2, L4 third elution, L5 fourth elution

200-fold dilutions of the primary antiserum, and therefore a 50-fold dilution was chosen for subsequent assays.

Evaluation of the Detection Systems

When evaluated on 216 food, clinical and environmental samples the hyper immune sera detected 12 SEB positive *S. aureus* strains which were unequivocally detected positive for presence of *seb* gene by the PCR and the toxin expression by TECRA kit (Table 3). Three *tsst* PCR positive isolates recovered from the different samples and were reactive with hyperimmune sera.

Discussion

The strategy described in the present work was successful in generating a recombinant multidomain protein, r-TE,

comprising truncated regions of Tsst and SEB, for utilization in toxin detection or disease diagnosis. The fusion gene in the current study, which was codon-optimized for *E. coli* expression, was first generated by ligation of PCR-amplified products encoding Tsst-1 and SEB of *S. aureus*. The resultant 655 bps fusion gene was sequenced and the sequence was submitted to NCBI Genbank (Accession No. FJ527613). The pRSET A vector containing fusion gene yielded high expression which resulted in a recombinant two domain chimeric protein, r-TE, in the *E. coli* host (Fig. 2). The TECRA kit was able to identify the SEB antigenic determinant in the r-TE protein. The immunodominant nature of the chimeric protein could be reflected by virtue of obtaining a high titered hyperimmune antiserum raised in mice. Furthermore, the antigenicity of the both toxin components was demonstrated in Western blot, wherein, antisera specifically reacted with *S. aureus* strains producing native SEB and Tsst toxin molecules providing two specific reaction bands at ~28 kDa and at ~22 kDa regions respectively (Fig. 5).

From among the various kinds of food, clinical and environmental samples (216) processed for *S. aureus* isolation, 12 isolates were found to have *seb* gene by PCR were also detected positive by the hyperimmune sera raised against r-TE in ELISA as well as in Western blot. From among the three *tsst* positive isolates recovered from the different clinical samples, all were found to be reacting with hyperimmune sera. Though PCR has proven itself as a robust technique in pathogen detection for vast number of toxin associated genes but mere presence of certain genes

Fig. 5 Western blot analysis of culture-free supernatants of SEB and Tsst positive *S. aureus*

Table 3 ELISA, Western blot and PCR analysis of culture-free supernatants of *S. aureus* standards and isolates

Organism	ELISA	PCR	SEB	Tsst	Western blot	TECRA
<i>S. aureus</i> ATCC 51740	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2654	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2120	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2492	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2672	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2794	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2490	+	+	+	—	+	+
<i>S. aureus</i> NCIM 2901	+	+	+	—	—	+
<i>S. aureus</i> 3 food isolate	+	+	+	—	+	+
<i>S. aureus</i> 6 food isolate	+	+	+	—	+	+
<i>S. aureus</i> 12 food isolate	+	+	+	—	+	+
<i>S. aureus</i> 7 clinical isolate	+	+	+	—	+	+
<i>S. aureus</i> E 2147 clinical isolate	+	+	+	+	+	+
<i>S. aureus</i> clinical isolate SDM103	+	+	—	+	+	—
<i>S. aureus</i> (Tsst positive) Japan	+	+	—	+	+	—
<i>A. hydrophila</i> ATCC 49140	—	—	—	—	—	—
<i>S. typhimurium</i> NCIM 2501	—	—	—	—	—	—
<i>E. coli</i> MTCC 730	—	—	—	—	—	—

like *tsst*, *pvl* and others might not always associated with clinical syndromes [16, 17]. On the other side, targeting the expressed toxin employing appropriate immunoassays rather than the gene could provide more useful clinical relevance of the disease.

The presently described ELISA and/or Western blot analysis find its utility in situation related to bioterrorism/biowarfare involving *S. aureus* toxins. Its application in the development of a comprehensive toxin detection immunoassay beyond Western blot and ELISA can also be explored. The strong seroreactivity of r-TE that we observed with hyperimmune mice sera and also the fact that it recognizes the immuno dominant epitopes found in the full-length native toxin proteins of the organism suggests that the recombinant chimeric molecule might have potential as a candidate vaccine molecule.

In conclusion, this paper has described the successful construction and expression of a multidomain r-TE fusion gene with specificity tailored towards Tsst-1 and SEB of *S. aureus* coupled to *E. coli*-based expression system. This recombinant protein was found useful in developing a simple, rapid and reliable immunoassay for the surveillance studies and or for disease diagnostics. The recombinant chimeric protein-based immuno detection approach eventually provides advantages over PCR formats as the main disadvantage of PCR happens to be their inability to correlate to the expression of toxin components by the organism. PCR technologies would have limited impact in detecting these biowarfare agents unless the toxin producing bacteria were used as the bioweapons rather than the purified toxins. Additionally, this non toxic r-TE molecule can be further explored for its possible application in generating neutralizing antibodies against the native toxins using appropriate animal model.

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