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Research paper

A novel tandem repeat cloning technique for creation of multiple short peptide repeats to differentiate closely related antigens

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ABSTRACT

Antibody cross-reactivity is a problem often associated with closely related antigens. This study was aimed to develop a method enabling differentiation of closely related toxins based on antigen designing strategy. The method involves identification of disparate amino acids (AA) confined to target antigen in comparison with two or more closely related antigens, their assembly into a DNA oligomer and further cloning as six tandem repeats (TR) using restriction and ligation strategy into a desired vector and finally generation of antigen specific antibodies. The practical utility of this method was demonstrated by generating and testing the specificity of polyclonal antibodies against staphylococcal enterotoxin C (SEC). Cross-reactivity is a problem often associated with SEC in immunoassays due to its amino acid sequence identity with staphylococcal enterotoxin B (SEB) (40–60%). To circumvent the same, the above-mentioned strategy was applied. Unique AA of SEC (36 AA) in comparison to SEB were selected, reassembled and with deduced corresponding nucleotides, an oligomer of 117 bases was designed. Using primers with restriction overhangs, three constructs were created each with two repeats using a common restriction site. The resulting three constructs were sequentially cloned into alternating restriction sites of pRSET A vector in directional orientation, expressed in *E. coli* for rTR/SEC protein which was used to generate specific polyclonal antibodies against SEC. Specificity was compared with antibody raised against whole SEC recombinant protein using Western blot and dot blot assays. High specificity was achieved through the developed strategy signifying its possible application to address cross-reactivity problem associated with closely related antigens.

1. Introduction

In diagnostic procedures, cross-reactivity or false positive result is a common occurrence manifested by closely related antigens due to conserved sequence identity and shared epitopes (Tedder et al., 1988; De Cock et al., 1991; George et al., 1992). Detection of such antigens based on immunoassays is thus undesirable as it leads to ambiguity in the end result. A classic example of closely resembling antigens are a class of staphylococcal enterotoxins (SEs) which are heat-stable proteins secreted by enterotoxigenic strains of *Staphylococcus aureus*. These toxins belong to a family of proteins called pyrogenic toxin superantigens (PTSAg) that are serologically defined, functionally related and share considerable AA sequence identity. The biological implications evinced by SEs include staphylococcal food poisoning (SFP), toxic shock syndrome (TSS), several allergic and autoimmune diseases (Balaban and Rasooly, 2000). > 24 types of SEs have been identified to date - SEA to SE*I*Y, which are named alphabetically in the sequence of their discovery (Argudín et al., 2010; Ono et al., 2015). Of these, SEA, SED, and SEE share 70–90% sequence identity, while SEB and SEC share 40–60% similarity (Balaban and Rasooly, 2000). Consequently, the antibodies raised against one toxin will show cross-reactivity or cross-protective immunity towards non-target antigen (Spero et al., 1978; Yoshioka et al., 2003). Even monoclonal antibodies are known to exhibit cross-antigen affinity due to shared epitopes (Thompson et al., 1984). As an alternative, molecular methods such as PCR and DNA probes could be employed to achieve specific detection and differentiation of related antigens (Neill et al., 1990; Nagaraj et al., 2014). However, they are restricted to providing information only about the presence/absence of gene and do not rely directly on presence of toxin.

Peptide-based enzyme immunoassays are profoundly used for

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Table 1
List of cloning primers used in the study.

Primer	Primer sequence (5′-3′)	Amplicon size (bp)
Mega-F	F - GATAATGTAGGTAAAGTAACA	117
Mega-R	R – GTTGATCAGGAAGTTACG	
TR XhoF	F – *CCG***CTCGAG**GATAATGTAGGTAAAGTAACA	150
TR XhoR	R – *CCG***CTCGAG**GTTGATCAGGAAGTTACG	150
TR BamF	F – CTGCCACACAACATA*CGG***GGATCC**GATAATGTAGGTAAAGTAACA	290
TR KpnR	R – GGCTTTACACTTTATGC*TTC***GGTACC**GTTGATCAGGAAGTTACG	
TR KpnF	F – CTGCCACACAACATA*CGG***GGTACC**GATAATGTAGGTAAAGTAACA	290
TR EcorIR	R – GGCTTTACACTTTATGC*TTC***GAATTC**GTTGATCAGGAAGTTACG	
TR EcorIF	F – CTGCCACACAACATA*CGG***GAATTC**GATAATGTAGGTAAAGTAACA	290
TR HindR	R – GGCTTTACACTTTATGC*TTC***AAGCTT**GTTGATCAGGAAGTTACG	
Overhang primers	F – CTGCCACACAACATACGG	–
	R – GGCTTTACACTTTATGCTTC	
sec clone	F – *CGC***GGATCC**ATGGCACTGATACTAGTTATTTC	776
	R – *CCC***AAGCTT**CCATTCTTTGTTGTAAGGTGGA	

The bold letters indicate bases coding for restriction enzyme recognition sequences, the italicized letters indicate restriction enzyme overhangs and underlined letters indicate overhang sequences for PCR amplification.

differential diagnosis of closely related antigens such as viral serotypes (Viscidi et al., 1991; Pau et al., 1993; Brattegaard et al., 1995). The discrimination is achieved through peptide-specific antibodies that evade cross-reactivity. Yet, peptide synthesis is an expensive means for small-scale production. Moreover, monomeric peptide antigens may fail to stimulate the necessary immune response themselves due to small size. Further, their usefulness is limited by reduced assay sensitivity due to loss of flexibility required for optimal antibody binding, low coating efficiency of peptides or a constrained orientation (Geerligs et al., 1988; Shin et al., 1996). An approach to increase the size of peptides may address the aforementioned problems.

An advanced system to increase analytical sensitivity of short monomeric peptides is through multiple antigenic peptides (MAPs) (Kim and Pau, 2001), where number of peptides are covalently linked to a lysine core. However, the chemical synthesis of MAPs is once again an expensive approach. A convenient way to produce multiple peptides is to adopt cloning technique. It is possible to develop tandem repeat (TR) peptides in the form of polypeptides/proteins through cloning and expression of tandem DNA multimers. Aside from contributing enhanced stability in expression host, these TR proteins have demonstrated to evoke immune responses as well (Shen, 1984; Stevenson et al., 2004). Thomson et al. (1995) have followed PCR and overlap extension to develop DNA sequence coding for the poly-epitope protein. A method called ligation-PCR was proposed by Jiang et al. (1996) to construct tandem DNA repeats in a defined orientation. An attempt was made by Lee et al. (2000) to develop tandemly repeated DNA cassette using synthetic DNA with 3′ overhang cohesive ends which were self-ligated with T4 DNA ligase and were expressed in *E. coli*. Another group worked on TA cloning and ligation PCR strategy using 3-bp overhangs (Hongwei et al., 2012). In all the methods mentioned, a pre-defined, continuous stretch of peptide sequence will be considered for cloning purpose. However, in cases such as SEs where difference/variance is confined to certain amino acid sequences which are dispersed in discontinuous stretches, the above-mentioned methods are inappropriate.

In this study, we developed a novel tandem repeat cloning strategy for specific discrimination of SEC from SEB. The procedure includes selection of toxin-specific amino acid (AA) sequences, synthesis of deduced DNA oligomer, preparation of monomer by PCR with restriction site overhang primers, restriction-ligation mediated fusion of two monomers followed by sequential cloning into a vector resulting in recombinant plasmid (r-plasmid) containing six monomers in a directional orientation. Obtained r-plasmid was expressed in heterologous host to produce tandem repeat (TR) protein that was used to generate antibodies in mice. The specificity of resultant anti-TR/SEC antibodies was compared with anti-rSEC sera raised against whole SEC recombinant protein by employing Western blot and dot blot assays.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The NCIM 2127 (SEB^+) and ATCC 700699 (SEC^+) were used as reference strains in the study. In addition, *S. aureus* isolates possessing *seb* and *sec* reported in our earlier study were also used to check specificity (Nagaraj et al., 2014). For genomic DNA preparation, strains were grown in Brain Heart Infusion (BHI) broth (Himedia) for overnight, while for enterotoxin production, *S. aureus* strains were grown in Tryptic Soy Broth (TSB) (Himedia) for 24 h with aeration at 225 rpm at 37 °C. The *E. coli* strains DH5α and BL21 (DE3) pLysS (Novagen, Madison, WI) were used as cloning and expression host strains respectively for transformation and expression of recombinant proteins. BL21 (DE3) pLysS cells were maintained in LB broth with 35 μg/mL chloramphenicol, whereas DH5α cells were maintained in LB broth without any antibiotics. The T7 based expression vector pRSET A (Invitrogen) which was used for cloning was propagated in DH5α and maintained in Luria Bertani (LB) broth (Himedia) with 50 μg/mL ampicillin. LB agar plates supplemented with 50 μg/mL ampicillin were used for screening of recombinant clones.

2.2. Construction of recombinant clones

2.2.1. Construction of clone expressing rTR/SEC specific protein: a novel antigen preparation strategy

The protein sequences of SEC1 (**P01553**), SEC2 (**P34071**), SEC3 (**P0A0L5**) and SEB (**P01552**) were derived from UniProt protein database. Multiple sequence alignments were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to analyze conserved and variable sequences. The unique amino acid sequences present only in SEC (US1, US2, US3, US4) were selected and reassembled to form a continuous stretch of sequence comprised of 39 amino acids (36 AA unique to SEC and 3 conserved AA). The corresponding nucleotide sequences of SEC (GenBank accession No. **X51661.1**) were derived from GenBank database that produced a gene stretch of 117 bp. The codons of gene stretch were optimized according to *E. coli* codon usage frequency table to enhance translation rates/high expression in heterologous host. The single strand of gene comprised of 117 bases was synthesized as a megaprimer from Sigma (Table 1). Using two extreme primers, double-stranded DNA was obtained through PCR amplification. Restriction sites were incorporated in primers to facilitate restriction-ligation mediated fusion (Table 1). PCR was performed in 20 μL reaction volume containing 400 nM of each primer, 200 μM of each dNTPs, 1.0 U *Taq* polymerase (Sigma), 1.5 mM of $MgCl_2$ in 1× PCR buffer (Sigma) with 1 ng of megaprimer as template. PCR was

carried out through 30 cycles in Eppendorf master gradient (Hamberg, Germany) thermal cycler at 94 °C for 20 s denaturation, 57 °C for 20 s annealing and 72 °C for 20 s extension. The DNA was denatured for 2 min in the beginning and finally extended for 5 min at 72 °C. The 117 bp amplicon was further amplified with same PCR conditions using primers containing restriction sites to produce six monomers. The primers used to construct TR clone are enlisted in the Table 1. *Xho*I site was kept in between two monomers for restriction-ligation mediated fusion. Two monomers with common XhoI site were digested with the enzyme and ligated to get a fused product of two repeats. The ligation mixture was run in 1.5% agarose gel and the fused band (~250 bp) was excised. The band was purified using Genelute kit (Sigma) and the same was used as a template for bulk amplification using overhang primers which were designed from non-target DNA sequence. Similarly three constructs were obtained with common XhoI site in between. The constructs were digested with respective restriction enzymes and purified. The first construct with BamHI and *Kpn*I site was inserted into BamHI/KpnI site of pRSET A using T4 DNA ligase. The resulting recombinant plasmid (r-plasmid) was transformed into chemically competent *E. coli* DH5α cells and clones were screened. The recombinant plasmid containing first construct (~250 bp insert) was extracted from positive clone using Plasmid miniprep kit (Sigma), digested with KpnI and *Eco*RI enzymes and was inserted with second construct with respective sites. Similarly, the plasmid with two inserts (~500 bp insert) was extracted and the last construct was inserted into EcoRI and *Hin*dIII site to get tandem repeat of six monomers. The recombinant plasmid with all six inserts (~750 bp insert) was then extracted and transformed into chemically competent BL21 (DE3) pLysS expression host for expression of rTR/SEC protein. The recombinant plasmid containing first and second constructs were also transformed into BL21 (DE3) pLysS cells to accomplish expression studies. Each time, the transformants produced/developed on LB-ampicillin agar plates were screened by direct colony PCR using the T7 forward and reverse universal primers.

2.2.2. Construction of recombinant clone expressing whole SEC antigen (rSEC)

The open reading frame (ORF) of *sec*3 without signal peptide was used to generate whole recombinant-SEC (rSEC) protein. The primer sequence used for amplification of *sec*3 is given in the Table 1. The gene was amplified by genomic DNA of *S. aureus* ATCC 700699 strain, double-digested with BamHI and *Hin*dIII enzymes and inserted into BamHI/HindIII sites of pRSETA expression vector. The recombinant plasmid was transformed into BL21 (DE3) pLysS expression host.

2.3. Validation of fusion gene sequence orientation by sequencing

The orientation of fusion gene and the sequence information was confirmed by dideoxy terminal sequencing analysis of plasmid containing all six inserts using T7 forward and reverse sequencing. Recombinant plasmid sequencing was performed at Eurofins Genomics sequencing facility located in Bangalore, India.

2.4. Expression and purification of fusion proteins

The transformed BL21 (DE3) pLysS host cells containing recombinant plasmids were grown in LB broth (containing 50 μg/mL of ampicillin and 35 μg/mL of chloramphenicol) at 37 °C until OD_{600} reached 0.6 following which IPTG (Fermentas) was added to a final concentration of 1 mM. After 5 h of induction, cells were harvested from one mL of culture by centrifugation at 10,000 rpm for 10 min at 4 °C. Cell harvests were resuspended and lysed in 1 × Laemmli sample buffer followed by centrifugation to remove cell debris and were fractionated by 12% SDS-PAGE. Recombinant protein expression was identified by Coomassie brilliant blue staining and Western blot analysis using mouse monoclonal anti-histidine antibodies (Sigma).

2.5. Purification of recombinant fusion proteins

As recombinant proteins were mainly localized as the inclusion bodies, the *E. coli* cells were lysed by sonication. The cell lysate was denatured in 8 M urea (8 M urea, 10 mM Tris-HCl and 100 mM NaH_2PO_4) and the cell debris was removed by centrifugation. The supernatant containing recombinant proteins was subjected to affinity chromatography using a Ni-NTA agarose (Qiagen, Chatsworth, CA) by gravity flow method.

Fractions of 1 mL elutions were collected and analyzed by SDS-PAGE. Peak fractions were pooled together and stored at −80 °C until use. The protein content was estimated by the method of Lowry et al. (1951).

2.6. Production of hyperimmune polysera

Six-to-eight week old female BALB/c mice were immunized intraperitoneally with purified rTR/SEC and rSEC proteins separately. Primary immunization was done with 50 μg of purified recombinant proteins in Freund's complete adjuvant (FCA) (Sigma, India). Two boosts of 10 μg each of recombinant proteins in Freund's incomplete adjuvant (FIA) (Sigma, India) was followed on days 14 and 28. Sera were collected 7–10 days after last booster dose to analyze antibody titers. Murine antibodies from sera were purified using protein A IgG affinity column purification kit (Genei, Bangalore) following manufacturer's instructions.

2.7. Antibody titer and isotyping

Antibody titers from polyclonal serum of mice immunized with rTR/SEC and rSEC was estimated by indirect ELISA. Briefly, 96 well microtitre plate (Nunc) was coated with 100 μL (1 μg) of 10 μg/mL of rTR/SEC and rSEC protein in carbonate-bicarbonate buffer (pH 9.6) separately and incubated at 4 °C overnight. Following antigen coating, unbound sites in the wells were blocked with 5% skim milk solution prepared in PBS and incubated overnight at 4 °C. Milk solution was discarded and wells were washed with PBST (0.05% tween 20) solution four times to remove excess milk protein. Two-fold dilutions of sera starting with 1:1000 dilutions were added to wells in triplicates and incubated at 37 °C for 1 h. Horse Radish Peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, India) was used as secondary antibody. Reaction was developed with 100 μL substrate *o*-phenylenediamine dihydrochloride (OPD- 0.5 mg/ml) and 30% H_2O_2 in citrate-phosphate buffer (pH -5.0). Reaction was terminated by adding 20 μL of 2 M H_2SO_4 followed by taking absorbance at 490 nm in Tecan Infinite M200 pro multimode reader (Austria). Titers were determined by following end point dilution method as the highest dilution of serum having a mean OD two times greater than naïve serum samples.

For determining antibody isotypes, the antibodies were analyzed by isotyping kit (Sigma). Naïve serum (antibody from immunization with only PBS) was used as control during antibody titer and isotyping testing.

2.8. Determination of antibody specificity

The *S. aureus* strains NCIM 2127 (SEB^+) and ATCC 700699 (SEC^+) as well as isolates were grown in TSB broth for 24 h for enterotoxin production and the native toxins were extracted by methanol-chloroform protein precipitation method according to Wessel and Flügge (1984). Concentrated native SEs were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The unbound sites were saturated by immersing membrane in 5% milk solution. One set of the antigen electroblotted onto the nitrocellulose membranes was incubated with the appropriate dilution of anti-rSEC antibody and the other with anti- rTR/SEC antibody followed by incubation for 1 h at 37 °C. The blots were washed with PBST followed by incubation with

peroxidase-conjugated secondary antibodies. After thorough washing, the blots were immersed in developing solution containing diaminobenzidine tetrahydrochloride (DAB) solution (0.5 mg/mL) with 30% H_2O_2 in PBS at room temperature for 5 min. The enzyme reaction was terminated using distilled water and the peroxidase-positive bands were visualized.

The specificity of antibodies was also analyzed using in-house recombinant proteins through dot-blot assay. Briefly, rSEs (rSEA, rSEB, rSEC and rSED) were spotted onto nitrocellulose membrane and further procedures (blocking, primary and secondary antibody steps) were performed similar to Western blot assay as mentioned above. The result was interpreted by visual inspection of nitrocellulose membrane.

3. Results

3.1. Multiple sequence alignment studies

Among the SEs, staphylococcal enterotoxin C (SEC) variants are a group of highly conserved proteins with significant immunological cross-reactivity. These variants share > 97% of amino acid identity among them. Therefore, comparison was emphasized mainly among SEB, SEC1, SEC2 and SEC3. We observed that about 40–60% sequence was conserved between SEB and SEC and the variation occurred as different amino acids randomly. Though numerous polymorphic amino acids were encountered, considering antigen-antibody interactions in subsequent immunoassays which generally require a segment comprising several amino acids, a discontinuous stretch comprising of 39 amino acids that are unique to SEC were selected (36 AA unique to SEC and 3 conserved AA) (Fig. 1). Four such portions (US1-US4) each containing 8–12 amino acids thus obtained were re-assembled to form a continuous sequence (Fig. 2). In order to enhance expression of recombinant protein, the codons were modified using codon usage table. Finally, restriction-ligation mediated sequential cloning resulted in r-plasmid containing six monomers in tandem repeats composed of ~750 bp insert oriented as an open reading frame as analyzed by DNA sequencing (Fig. 3). The obtained DNA sequence post sequencing was in agreement with the expected sequence. To confirm the expression of each construct, pilot-scale induction was undertaken after each sequential cloning, where each construct yielded expected (calculated using Gene Runner software) protein band as analyzed by SDS-PAGE and Western blot (Fig. 4A and B). An expressed band with ~14 kDa was observed with clone having first construct. Similarly, clones bearing second and third constructs showed band at ~22 kDa and ~30 kDa respectively (Fig. 4A). rSEC showed a protein band corresponding to ~35 kDa. Further, the recombinant proteins were purified to homogeneity with no extra bands detected by Coomassie brilliant blue staining (Fig. 4A).

3.2. Antibody isotypes

Since rTR/SEC is an unusual protein, the generated antisera was subjected to isotyping and compared with that of anti-rSEC antibody to ascertain type of immune response. The isotyping assay performed for anti-rTR/SEC antibodies showed adequate immune response similar to anti-rSEC antibodies. The histogram analysis suggested elevated production of IgG subclass (particularly significant amount of IgG1, IgG2a and IgG2b isotypes) in both types of antisera tested. However, the level of isotypes varied. IgG2a level was high in case of rSEC antibody, whereas rTR/SEC antibodies exhibited primarily IgG2b isotype (Fig. 5). Further, the level of IgG3 and IgM isotypes were also slightly increased. Yet, there was no production of IgA in both anti-sera.

3.3. Specificity of polyclonal antibodies

The specificity of anti-rTR/SEC antibody generated was assessed by Western blot analysis. For comparison, anti-rSEC antibody was also tested against native toxins. Antibody raised against whole rSEC showed reactivity with cell lysates of all SEB and SEC producing strains. However, specificity was confined to SEC producing strains when murine anti-TR/SEC was used (Fig. 6). The bands seen at higher molecular weight regions correspond to immunoglobulin (Ig) binding proteins of *S. aureus* namely staphylococcal protein (SpA) and staphylococcal binder of immunoglobulin (Sbi). These proteins due to their innate Ig binding nature interfere with primary and secondary

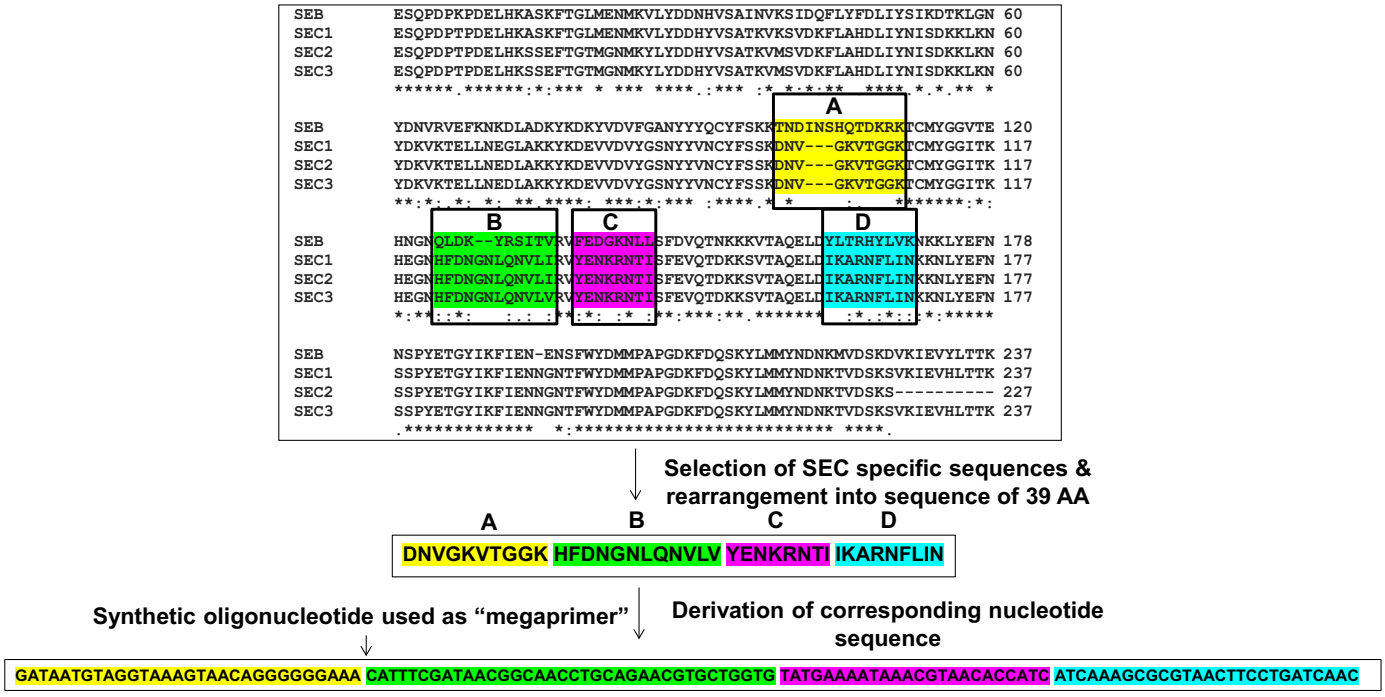

Fig. 1. Clustal Omega analysis of SEB and SEC (3 variants) protein sequences and designing of synthetic oligonucleotide (megaprimer) using unique sequences specific to SEC.

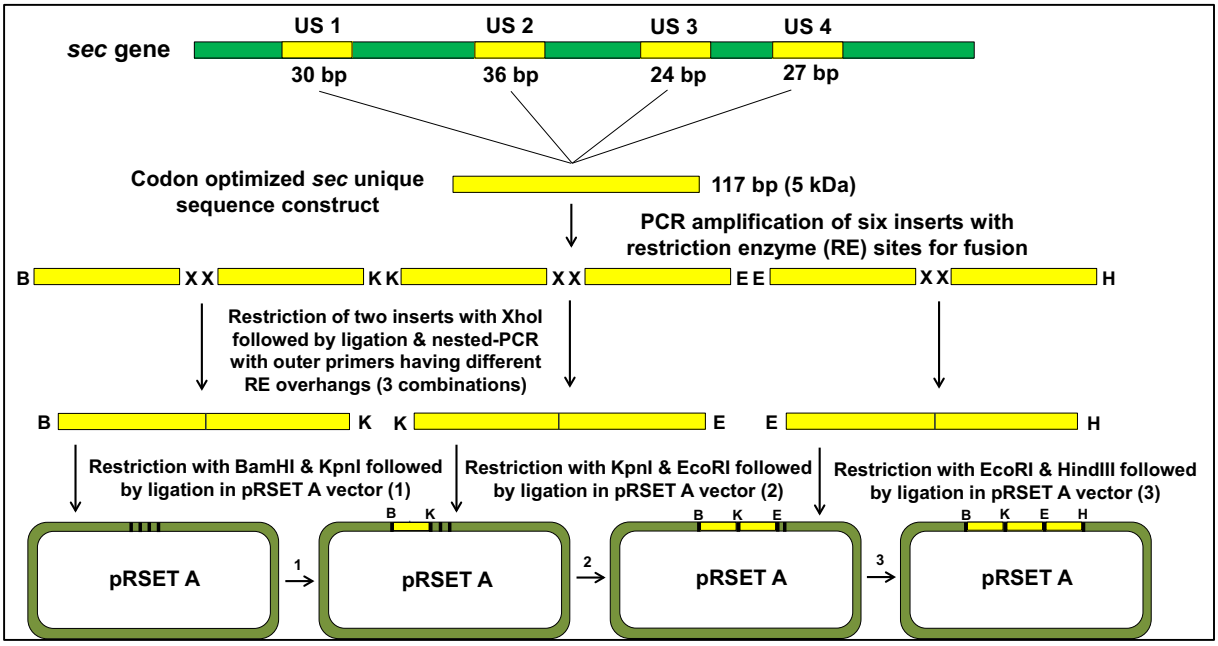

Fig. 2. Schematic representation of TR-cloning strategy for expression of SEC unique sequence (in comparison to SEB) in six tandem repeats.

antibodies in immunoassays (Reddy et al., 2013; Upadhyay et al., 2008).

Further, dot blot assay performed using recombinant SEs generated in our earlier studies (Reddy et al., 2013) also yielded specific reaction with rSEC protein when rTR/SEC antibody was used. Similar to Western blot, anti-rSEC antibody showed reactivity with both rSEB and rSEC, whereas rTR/SEC antibody showed reactivity with rSEC only (Fig. 7). Since the test antigens used in this experiment are homogenous recombinant protein, no cross-reactivity was observed unlike the samples from *S. aureus* as seen in Fig. 6.

4. Discussion

Antibodies are the backbone for developing immunological tools which aid in detection of low concentrations of pathogens, analytes and other contaminants. Immunological assays are hampered by cross-reactivity problems or false positives when trying to detect closely related antigens. It is possible to develop assays free from cross-reactivity using monoclonal antibodies specific for the epitope of interest. However, monoclonal antibody production and purification in large scale is costly process. On the other hand, high titer polyclonal antibodies in large quantities can be produced easily and inexpensively using experimental animals such as rabbit, chicken, goat and horse. But polyclonal antibodies bind multiple epitopes of an antigen and if the epitopes are shared between closely related antigens it leads to false positives. In this work we developed a method for generating specific polyclonal antibodies devoid of cross-reactivity with closely related proteins/antigens by antigen engineering/designing approach. As evidence for the proof of concept, we demonstrated the strategy by the closely related enterotoxins of *S. aureus*. Among the 20 or more of enterotoxins secreted by *S. aureus*, we chose SEB and SEC due to their similarity at amino acid sequence and structure.

Four stretches comprising of 39 different amino acids were found at dispersed regions (36 amino acids unique to SEC and 3 conserved AA) as analyzed by clustal omega multiple alignment tool. Since they were in discontinuous stretches, cloning and expression was strenuous. To facilitate cloning, the amino acid sequences were assembled and corresponding nucleotide sequences were used to get a gene construct. However, the expression of the constructed gene would have produced a peptide of < 10 kDa which is not sufficient to elicit immune response and subsequent antibody generation. Therefore, to express an immunogenic protein in heterologous host, the gene sequence was inserted into vector in tandem repeats of six units by restriction-ligation fusion followed by restriction mediated sequential insertion. The resultant r-plasmid could express a polypeptide/protein of 30 kDa (rTR/SEC) comprised of tandem repeats of SEC sequence which would be an ideal immunogen. In fact, tandem repeats are proved to be immunogenic in other studies also (Francis, 1990). A fair immune response (IgG1 and IgG2 production indicating an influence on both Th1 and Th2 responses) was elicited by this protein analogous to normal rSEC antigen which was evidenced by isotyping assay.

Although it is possible to obtain tandem repeats using ligation PCR,

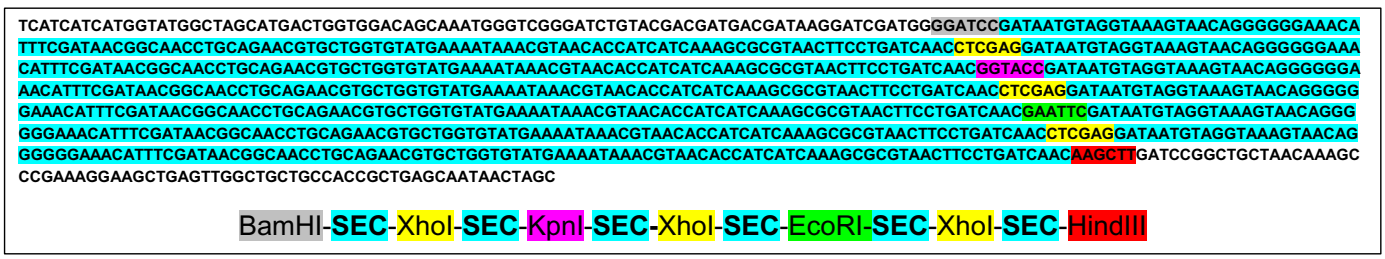

Fig. 3. Alignment of r-TR/SEC plasmid sequence after sequencing analysis clearly showing six constructs inserted within specific restriction sites in a directional orientation. The sequence alignment was color-coded for easy identification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

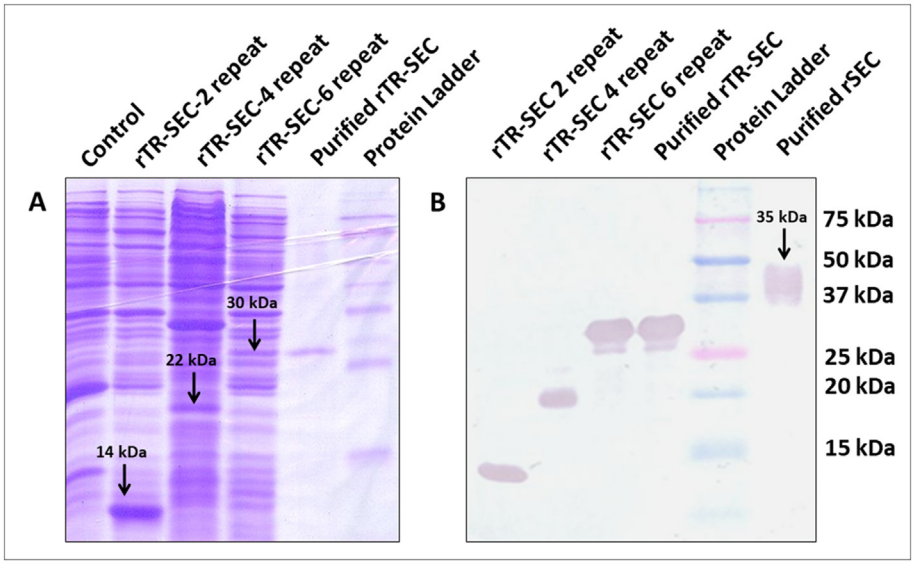

Fig. 4. Expression, purification and Western blot analysis of rTR/SEC and rSEC proteins. A. Coomassie brilliant blue stained SDS-PAGE gel showing expression patterns of r-TR/SEC in different repeats. **B.** Western blot analysis with anti-histidine antibodies showing expression of r-TR/SEC (in different repeats) and whole r-SEC proteins. Whole-cell lysates were used for both experiments.

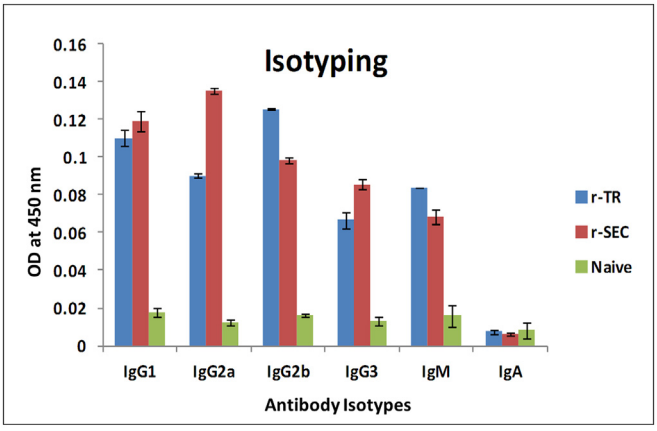

Fig. 5. Graph showing isotypes of generated antibodies. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

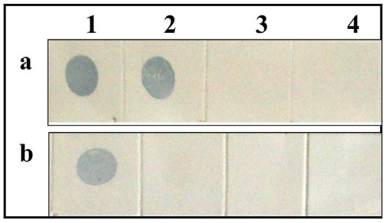

Fig. 7. Dot blot analysis of recombinant staphylococcal enterotoxin proteins with anti-SEC antibodies. **a** and **b** represents blots treated with rSEC and rTR/SEC antibodies respectively as primary antibodies. Lane 1: rSEC; Lane 2: rSEB; Lane 3: rSEA; Lane 4: rSED. The dilution of antisera used is 1:1000 in 1× PBS.

the activity of ligase depends on phosphorylated bases in which case phosphorylated primers must be employed. Moreover, this method is disadvantageous due to lack of control on number of repeats. Therefore, we preferred restriction-ligation mediated cloning for generating tandem repeats. Furthermore to avoid multiple steps, we have fused two inserts using common *Xho*I site in between two monomers followed by ligation and PCR. However, PCR amplification would have yielded

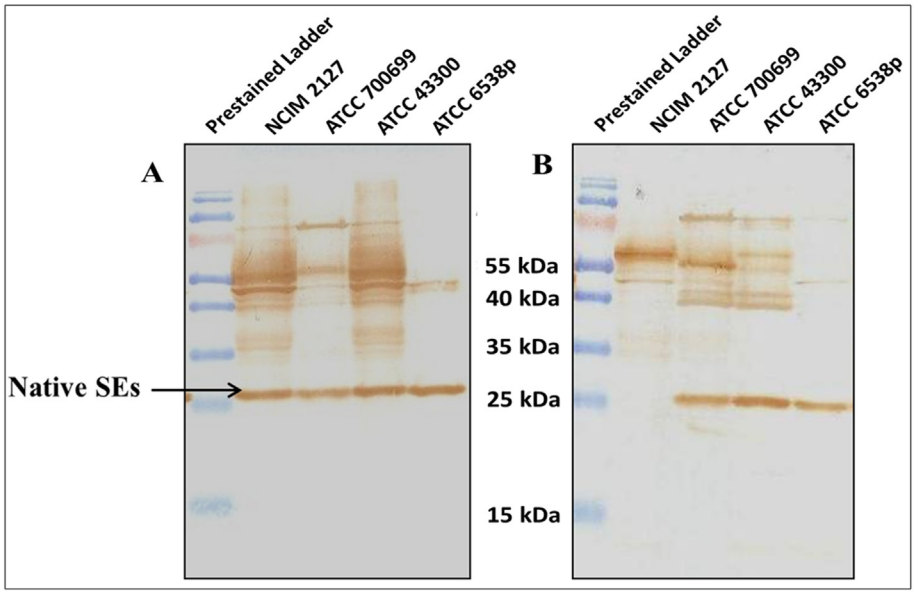

Fig. 6. Western blot results showing specificity reactions. **A.** Blot reacted with anti-rSEC antibody. **B.** Blot reacted with anti-rTR/SEC antibody. The dilution of antisera used is 1:1000 in 1× PBS. Antibodies raised against rSEC reacted both with SEC and SEB (*S. aureus* NCIM 2127) producing strains, whereas anti-rTR/SEC antibodies showed specificity towards SEC producing strain only.

multiple non-specific bands since repeated units exist. To prevent this, we have used primers containing non target DNA (overhang primers) that were placed extreme to restriction sites so that they will be cleaved during digestion and will not alter reading frame.

The method followed could be feasible to get controlled numbers of tandem repeats in proper orientation. To confirm the orientation, sequence of all inserts was analyzed by di-deoxy terminal sequencing and the result revealed that the arrangement, frame as well as orientation of the sequence were proper as expected. Yet, to verify proper insertion of each tandem unit, expression studies were carried out after every sequential cloning. After insertion of each insert, expressed protein was allowed to react with anti-rSEC murine antibodies. The results showed specific expressed band in all cases thus fulfilling the requirement. Finally, the reactivity of anti-rTR/SEC antibodies were compared with that of anti-rSEC antibodies using native as well as recombinant SEs, where selectivity/specificity towards SEC was achieved only through anti-rTR/SEC antibodies.

Though polyclonal antibodies were used, the specificity was not interfered by mixture of multiple epitope specific antibodies since only SEC specific sequences were in tandem repeats. Also, the broader range of epitopes enhances antigen binding avidity thus increasing the sensitivity of polyclonal antibodies than mAbs (Fischer et al., 2007). Therefore, the developed method could be used as an alternative to monoclonal antibody approach which is being used to achieve specificity in immunoassays. In fact, often it is uncertain to attain specificity even with the application of mAbs as in case of SEs (Thompson et al., 1984; Lapeyre et al., 1987). We speculate that, mAbs may fail to show specificity if the epitope against which it is raised is shared by the other antigens.

The presented method is simple and inexpensive to generate antibodies where specificity of detection of closely related antigens is of utmost importance. The same method could be followed to avoid cross-reactivity problem associated with similar antigens as in case of enterotoxins. As mentioned, the method could replace mAb approach saving cost and labor and could be extendable to other closely related antigens.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics statement

All animal experiments in this study have been approved and performed in accordance with guidelines of Institutional Animal Ethical Committee, D.F.R.L., Mysore.

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