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Recombinant expression of *in silico* identified B-cell epitope of epsilon toxin of *Clostridium perfringens* in translational fusion with a carrier protein

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Abstract:
Epsilon toxin secreted by *Clostridium perfringens* types B and D has been directly implicated as the causative agent of fatal enterotoxemia in domestic animals. The aim of the present study is to use *in silico* approach for identification of B-cell epitope(s) of epsilon toxin, and its expression in fusion with a carrier protein to analyze its potential as vaccine candidate(s). Using different computational analyses and bioinformatics tools, a number of antigenic determinant regions of epsilon toxin were identified. One of the B cell epitopes of epsilon toxin comprising the region (amino acids 40-62) was identified as a promising antigenic determinant. This Etx epitope (Etx_{40-62}) was cloned and expressed as a translational fusion with B-subunit of heat labile enterotoxin (LTB) of *E. coli* in a secretory expression system. Similar to the native LTB, the recombinant fusion protein retained the ability to pentamerize and bind to GM_1 ganglioside receptor of LTB. The $rLTB.Etx_{40-62}$ could be detected both with anti-Etx and anti-LTB antisera. The $rLTB.Etx_{40-62}$ fusion protein thus can be evaluated as a potential vaccine candidate against *C. perfringens*.

Keywords: Epitope, Bioinformatics, Epitope prediction algorithms, *In silico*, Epsilon toxin, *Clostridium perfringens*, LTB, fusion protein, vaccine.

Abbreviations: aa, amino acid(s), Etx, epsilon toxin of *Clostridium perfringens*, LTB, B-subunit of heat labile enterotoxin of *E. coli*

Background:

Spore-forming, gram-positive *Clostridium perfringens* belong to a heterogeneous group of anaerobic bacteria which are major human and animal pathogens **[1, 2]**. Different species of *Clostridium perfringens* produce a variety of lethal toxins, based on which they are divided into five sub-types, A to E. Of these, the *Clostridium perfringens* type D is a major causative organism of fatal enterotoxaemia or pulpy kidney disease in domestic animals. The host pathology is attributed mainly to the epsilon toxin (Etx), a potent angiotoxin that damages endothelial cells leading to cell death/necrosis.

Overfeeding of domestic animals leads to an imbalance in gut microflora and an anaerobic environment that is highly

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conducive to the overgrowth of Clostridia in the gut of infected animal. As a result, large amounts of epsilon toxin produced by the pathogen gets absorbed by the gut mucosa and results in severe vascular damage **[3, 4]** and lesions in various major organs viz. brain, heart, lung and kidney **[5, 6]**.

As the disease progression is quite rapid, often leading to fatal consequences, vaccination is the best defence against the disease. Conventional vaccines are based on heat inactivated culture supernatants leading to non-specific and uncontrolled immune response. Several recombinant vaccine approaches are also being researched and offer a convenient alternative to the conventional vaccines. A protective immune response against the pathogen is largely based on B-cell mediated immune pathways. Therefore, the major task during recombinant vaccine development is to identify the surface antigens containing antibody binding epitopes (B-cell epitopes). Epitope-based vaccines provide a better approach compared to conventional vaccine strategies since they can be easily produced and do not elicit non-specific responses commonly observed in conventional vaccines.

Experimental identification of epitopes is relatively time consuming, and laborious. On the other hand *in silico* epitope analysis helps in rapid identification of promising epitopes with higher confidence and accuracy. B cell epitope prediction softwares use different algorithms based on various physico-chemical properties of amino acids such as hydrophilicity, flexibility, beta-turns, and surface accessibility. Since an epitope is a small entity and not highly immunogenic by iteslf, a suitable carrier protein is required as an adjuvant for triggering an immune response of high magnitude.

Vaccines development based on B cell epitope identification has been great success in many cases of infectious diseases **[7].** The present study was therefore undertaken to identify the B cell epitopes on epsilon toxin, which may aid in generating potential vaccine against the epsilon toxin infection. As an epitope is likely to only generate limited immunogenicity, it is proposed to couple the identified epitope with a carrier protein such as LTB to generate chimeric antigen, which is likely to have augmented immunogenecity due to the presence of fusion partner. LTB is selected as a fusion partner as it has been reported to be highly immunogenic and leads to a better immune response against the specific epitope fused to LTB **[7].**

Methodology:

In silico analysis of surface exposed B-cell epitopes of epsilon toxin

Bioinformatics approach employing Kyte- Doolittle **[8]**, Parker Hydrophilicity plots **[9],** BepiPred **[10]** and Chou- Fasman epitope prediction algorithms **[11]** was used to predict different hydrophilic regions, potential B cell epitopes, in the Etx (accession no. AJ250956.1). The amino acid sequence common among hydrophilic areas of different sizes (epitopes) identified by various algorithms was chosen to decrease the probability of inaccuracy from these predictions.

Generation of ltb-epitope fusion construct

To make recombinant fusion of the predicted epitope, the amino acid sequences of the epitope was translated into nucleotide sequences. Complementary oligonucelotides corresponding to the coding and non-coding strand of the identified epitope were designed with a spacer of nucleotide sequence coding for 5-glycine residues at the 5′ end and termination codon at the 3′ end. *Pst*I and *Hind*III sites were included at the spacer and termination ends, respectively. A unique *Xho*I restriction site was also added just before the *Hind*III site for restriction analysis of the putative recombinants. Strategy for cloning the epitope at the C-terminus of *ltb* in pQE32 is given in **(Figure 1)**. The two oligonucleotides were annealed and ligated to the *Sac*I-*Pst*I digested pQELTB plasmid, harbouring the LTB gene in plasmid pQE32. The ligated product was transformed into *E. coli* DH5α cells and the putative recombinants were selected on LB agar plate containing ampicillin (100 µg/ml). The recombinants were analyzed by *Xho*I digestion and confirmed by automated DNA sequencing (DNA Sequencing Facility, University of Delhi South Campus, New Delhi). The insert carrying epsilon toxin epitope in fusion with LTB was subsequently cloned into pMMB secretory expression vector (derived from pMMB66EH vector, GenBank Accession no. X15234) digested with *Sac*I and *Hind*III.. The resultant plasmid is designated as pMMB*ltb.etx*$_{40-62}$.

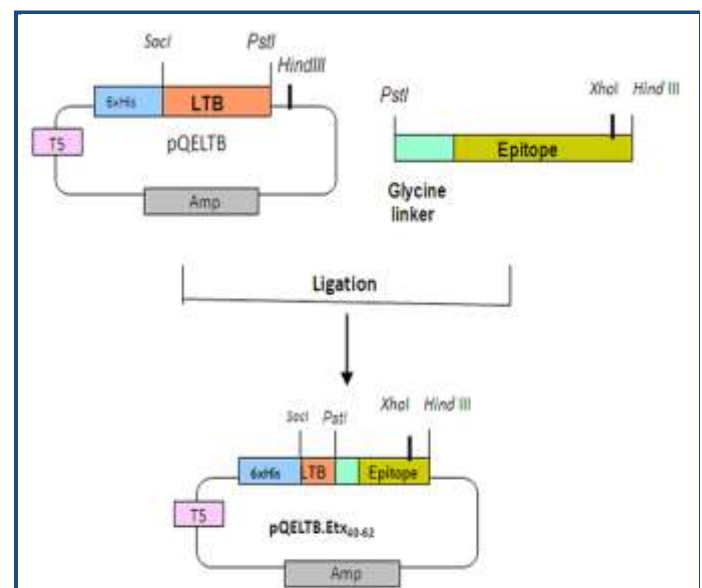

Figure 1: Cloning strategy for epitoes in fusion with LTB. Pairs of oligonucleotides corresponding to the Etx epitope spanning 40-62 residues of the Etx were annealed and ligated to *Pst*I-*Hind*III digested vector pQELTB clone. The final construct carried the *E. coli* LTB gene under the control of T5 promoter in C-terminal translation fusion with the Etx epitope with 5-Glycine residues in between. The fusion gene is tagged with 6-Histidine residues on the 5' end.

Conjugal transfer of the pMMB positive construct to Vibrio cholerae cells for secretory expression

E. coli DH5α cells harbouring the secretory expression plasmid pMMBltb*Etx*$_{40-62}$, JBK70 and *E. coli* pRK2013 cells were used as donor, recipient and helper strains, respectively. The pMMB*ltb.Etx*$_{40-62}$ from *E. coli* DH5α cells was transferred to the *Vibrio cholerae* JBK70 cells essentially as described earlier **[12].** The putative transformed JBK70 cells were selected in the

presence of both ampicillin (50 µg/ml) and polymyxin B (50 units/ml). Expression analysis of the fusion construct was carried out by growing the transformed cell culture till the absorbance (A) reached to 0.6-0.8, followed by addition of 1mM IPTG ((isopropyl-.beta.-D-thio-galactopyranoside, Sigma Aldrich Chemical Co., USA) to induce the expression of the fusion protein. The cells were grown further for 6 hr at 37^{0}C and secretory expression of the recombinant fusion protein was analyzed by SDS-PAGE (15%) of the culture supernatant.

GM_1 receptor binding assay

In vitro GM_1 receptor binding activity of the fusion protein was determined using sandwich ELISA as described earlier **[13].** For this, GM1 ganglioside receptor (100 µl, 20 µg/ml) was coated onto ELISA plates and incubated at 4°C for 12-16 hr. Non-specific sites were saturated with 1% BSA in 50 mM PBS. Immunoplates coated with GM_1 ganglioside were incubated with the secretory fusion protein. This was followed by incubation with goat anti-LTB polyclonal antiserum at half-log dilutions ranging from 1:30 to 1:100,000. The final incubation was carried out with rabbit anti-goat horse radish peroxidase (HRP- conjugated secondary antibody (1:2000). Colour was developed by the addition of the substrate (o-phenylenediamine dihydrochloride) and the absorbance was read at 490 nm.

Western blot analysis

Expression of the rltb.Etx40-62 in *V. cholerae* was checked by immunoblotting of the induced culture supernatants with anti-LTB **[14]** available in the lab. The samples were analysed on SDS-PAGE (12%) and electro transferred on to nitrocellulose membrane at 90 mA for 2 h in transfer buffer. Nonspecific sites were blocked by 5% non-fat milk in 1×PBST (0.15 M PBS, pH 7.3, and 0.2% Tween 20) for 1 hr on a rocker. The membrane was then incubated with the primary antibody (1:500) for 1h at room temperature followed by incubation with HRP-conjugated secondary antibody (1:3000) for 1h. The blot was then washed three times thoroughly with 1×PBST, and developed with 3, 3'diaminobenzidine solution (0.05% DAB) after addition of hydrogen peroxide (30%, 1µl H_2O_2/ml of DAB solution). The reaction was terminated by washing the membrane several times with water.

Results & Discussion:

Antigenic epitopes are made of amino acid residues involved in antigen-antibody interactions. Therefore, epitope analysis plays an important role in the development of effective vaccine and diagnostic tools for different infections. To predict the linear B cell epitopes of epsilon toxin, the causative agent of enterotoxemia in domestic animals, a combination of algorithms such as hydrophobicity plot **[8]**, hydrophilicity **[9]**, BepiPred based on hidden Markov model **[10]** and Chou-Fasman secondary structure predicton **[11]** were used. Using these programmes, we were able to map several short hydrophilic regions that can be potential epitopes of the Etx **(Figure 2).** Use of number of algorithms in predicting the putative epitopes is critical as a single programme may not reliably identify the epitopes. Thus, when an epitope is predicted by multiple programmes, it is likely to be a correctly identified epitope, resulting in an increased confidence in the predicted epitope.

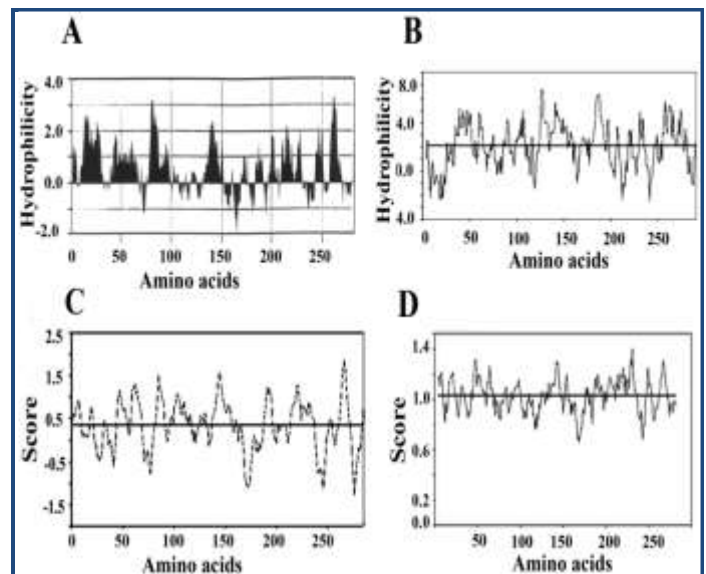

Figure 2: *In silico* analysis of surface exposed B-cell epitopes of Epsilon toxin: Computaional prediction of surface exposed region of epsilon toxin was done by using different algorithms **(A)** Kyte Dolittle plot **(B)** Parker Hydrophilicity prediction, **(C)** BepiPred linear epitope prediction, **(D)** Chou Fasman β turn prediction.

Therefore, in the present study, a single linear stretch of hydrophilic residues spanning amino acid residues 40-62 (Etx_{40-62}) was selected for further studies as it was common in all the programmes used for identification, and had the highest predicted antigenic and physicochemical scores among all the identified epitopes.

The epitope being a very small entity may not be able to elicit immunogenic response, it has to be linked with a carrier or adjuvant to increase its immunogenicity. Since LTB has been reported to be a potent immunogen, and possesses adjuvant properties **[15, 16]**, the identified immunodominant region Etx_{40-62} was cloned at the C-terminus in translation fusion with LTB, with a glycine spacer between the epitope and LTB in a secretory expression vector. Strategic placement of glycine spacer allows proper and independent folding of the fusion partners. Release of an insert of ~380 bp of the recombinant clone with *Sac*I (present at the 5'- end of the *ltb*) and *Hind*III (at the 3' end of the synthetic oligonucleotide encoding the epitope) confirmed successful cloning of the epitope in fusion with *ltb* **(Figure 3A).**

Nucleotide sequencing of the recombinant plasmid further confirmed the in frame cloning of the epitope (*etx_{40-62}*) with *ltb.* Subsequently the fused *ltb.etx_{40-62}* DNA fragment released by digestion of pQE*ltb.etx_{40-62}* was cloned into pMMB68 plasmid digested with the same enzymes. Release of a fragment of the expected size by digestion of the putative recombinant with *Xho*I confirmed the cloning of fusion gene (*ltb.etx_{40-62}*) in pMMB68 **(Figure 3B, lane 1)**. *E. coli* is incapable of transporting proteins to extracellular milieu, lacks main terminal secretory branch of the general secretory pathway, therefore a closely related strain, *Vibrio cholerae,* having the ability to secrete some of its natural toxins to the medium is of great interest **[17].**

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Therefore, transfer of the fusion gene construct from *E. coli* cells to *Vibrio cholerae* cells was carried out for secretory expression of the recombinant fusion protein (rLTB.Etx_{40-62}).

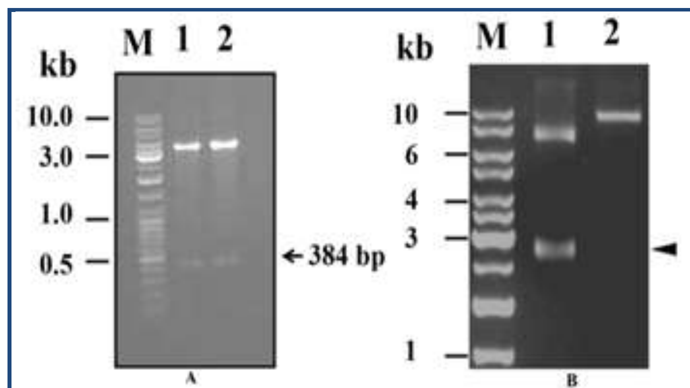

Figure 3: (A) Restriction analysis of pQE*ltb.etx*$_{40-62}$ putative recombinants harboring the DNA fragment encoding the Etx_{40-62} epitope as C-terminus fusion of LTB. Lanes 1 and 2 show the two putative clones digested with *Sac*I- *Pst*I. Arrow points to the released *ltb.etx*$_{40-62}$ fusion insert. M indicates DNA molecular weight marker; **(B)** Restriction analysis of the putative pMMB*ltb.etx*$_{40-62}$ clone. The insert released by digestion with *Xho*I (present at the c-terminus of the epitope and the vector) is indicated by arrowhead in lane 1. Lane 2 shows the linearized pMMB68, digested with *Xho*I. M indicates DNA molecular weight marker.

V. cholerae cells harbouring the recombinant plasmid were induced with IPTG. The culture supernatant of the induced culture was analysed for presence of the fusion protein by SDS-PAGE. Presence of a band of the expected size under non-reducing conditions in the induced culture supernatant **(Figure 4A, lane 1)** confirmed successful expression and secretion of the fusion protein to the extracellular milieu. Large-scale production **[18]** and overexpression of many heterologous proteins including LTB have been achieved using *Vibrio cholerae* **[8, 19].** In the present study also, the presence of the fusion protein in the induced culture supernatant of *V. cholerae* cells, showed that the protein is efficiently expressed, processed and secreted into the extracellular milieu.

LTB, used as a fusion partner in the present study, is a 55 KD, homo-pentamer of 11.6 KD polypeptide **[9].** LTB in its pentameric form binds to GM_1 ganglioside receptors present on the surfaces of mammalian cells **[20].** GM_1 receptor binding is regarded as an essential property for the stimulation of immune response and adjuvant activity of LTB is also associated to its receptor binding activity. Presence of a band at the expected pentameric position in **(Figure 4A),** lane 1 (under non-reducing and non-denaturing condition) suggests that the LTB present in the fusion protein retained the ability to pentamerize. Also, efficient binding of rLTB.Etx_{40-62} fusion protein to the receptor, comparable to that of the native LTB, was observed **(Figure 4B),** in GM1 receptor binding assay, No binding was detected with negative control BSA. The ability of the rLTB.Etx_{40-62} to pentamerize and bind to GM_1 ganglioside receptor indicates that the carrier protein LTB has retained its functional activity even after the C-terminal fusion with epsilon toxin epitopes.

For the rLTB.Etx_{40-62} fusion protein to be effective as a vaccine, it is important to retain its ability to pentamerize and bind to GM_1 ganglioside **[21].** Studies have shown that the fusion of HBV surface antigen epitopes and glycotransferase B epitope **[22, 23]** with CTB retained not only the biological function of CTB but also the antigenicity of both the fusion partners. Genetic fusion of small peptides/epitopes to protein carriers has long been an established practice both to improve intrinsic immunogenicity and for conformational expression of the epitopes.

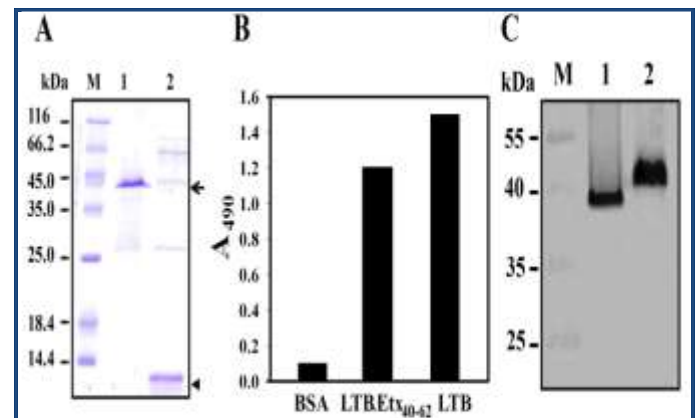

Figure 4: **(A)** Expression analysis of rLTB.Etx40-$_{62}$ in *V. cholerae*: *V. cholerae* cells harboring the plasmid pMMB*ltb.etx*$_{40-62}$ were induced with 1 mM IPTG for 6 h. Culture supernatant (after ammonium sulphate precipitation) was analyzed on 12% SDS-PAGE. Lanes 1 and 2 indicate the samples prepared under non-denaturing and denaturing conditions, respectively. The arrow points to fusion protein pentamer and the arrowhead points to fusion protein monomer. M indicates protein molecular weight marker; **(B)** GM_1 ganglioside receptor binding of rLTB.Etx_{40-62} fusion protein. Binding of the rLTB.Etx_{40-62} fusion protein to GM_1 ganglioside was checked by sandwich ELISA using anti-LTB antibodies. Immunoplates coated with GM_1 ganglioside receptor (10 ng/µl) were incubated with 5 ng/µl of purified rLTB.Etx_{40-62} fusion protein. 1% BSA was used as a non-specific protein and pure LTB protein was used as a positive control. Log dilutions of goat anti-LTB antibodies and 1:5000 dilution of anti-goat IgG-HRP antibodies were then used to detect the bound protein; **(C)** Western blot analysis of the rLTB.Etx_{40-62} fusion protein using anti-LTB antibody. The rLTB.Etx_{40-62} fusion protein and rLTB was electrophoresed on 12% SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted with anti-rLTB antibody. Lanes 1 and 2 show the rLTB and the rLTB.Etx_{40-62} fusion protein bands detected by the anti-LTB antibodies. M indicates protein molecular weight marker.

Immunoblot analysis of the expressed protein was carried out in order to assess the ability of the fusion partners to be recognized by the antisera raised against the native LTB. As evident, the fusion protein was detected by anti-LTB **(Figure 4C)** antisera, thus confirming the integrity and antigenicity of the fusion partner in the recombinant protein. Slower migration of the rLTB.Etx_{40-62} **(Figure 4C, lane 2)** in comparison to the native LTB (lane 1) is due to the addition of the Etx epitope at the C-terminus of LTB. Also, the purified fusion protein could efficiently form a pentamer (required for GM_1-receptor binding) indicating that the epsilon toxin epitope fused to the C-terminal

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of LTB did not alter the conformation/protein folding of the LTB, while maintaining its own integrity.

Conclusion:
In conclusion, the present study demonstrates the utility of this novel 2 step approach of identifying epitopes using bioinformatics/computational analysis and generating fusion proteins comprising the epitope and LTB a potent adjuvant. These approaches were successful in maintaining the antigenicity of both the epitope and the carrier protein and significantly minimize the time and efforts in vaccine design.

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