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Original article

Immunogenic properties of trivalent recombinant protein composed of B-subunits of LT, STX-2, and CT toxins

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Abstract

Infectious diarrhoea remains an emerging problem in the world health program. Among diarrheagenic agents, *Vibrio cholerae* and enterotoxigenic and enterohemorrhagic *Escherichia coli* are critical enteropathogens. AB5 toxin produced by these bacteria, heat-labile enterotoxin (LT), cholera enterotoxin (CT), and shiga-like cytotoxin (STX) can target the immune system and are subunit vaccine candidates. A chemically-synthesized chimeric construct composed of the binding subunits of these toxins (LTB, STXB, and CTXB) was developed based on bioinformatics studies. The whole chimeric protein (rLSC) and each of the segments (rLTB, rSTXB, and rCTXB) were expressed in a prokaryotic expression system (*E. coli*), purified, and analysed for their immunogenic properties. The results indicate that these recombinant proteins were effectively able to present appropriate epitopes to an animal model of the immune system which could result in and increase IgG in serum and immune responses that protect against the binding activity of these toxins. The immunological assays revealed that the sera of immunized mice prevented toxins from binding to their specific receptors and neutralized their toxic effects. The proposed construct should be considered as a potent immunogen to prevent toxicity and diarrhoea.

Keywords: Diarrhoea; ETEC; EHEC; *Vibrio cholerae*; Recombinant protein; Subunit vaccine

1. Introduction

Despite the progress and recent achievements in global health, infectious diarrhoea remains a leading cause of mortality in children under 5 years of age. It also has a long-term adverse effect on growth and cognitive development. Among the 7.6 million deaths annually of children under 5 years of age (reported in 2010), 0.75 million die as a consequence of diarrhoea [1]. *Vibrio cholerae* and enterotoxigenic and enterohemorrhagic *Escherichia coli* (ETEC, EHEC) are three critical enteropathogens that increase mortality in children and represent serious public health problems in developing countries [2,3]. It is believed that ETEC accounts for 20–25% of diarrhoea in children 1–4 years of age and up to 40% of traveller diarrhoea. It is also estimated that it causes approximately 280 million episodes of disease and about 400,000 deaths every year [1]. Several outbreaks in humans of *E. coli* O157:H7, the most common serotype of EHEC, have been reported globally in recent decades [4,5]. In addition to *E. coli* infections, the annual burden of cholera has been estimated 1.4–4.3 million cases with 28,000–142,000 deaths worldwide

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[6]. The pathogenic mechanisms of all three microorganisms (*V. cholerae*, ETEC, and EHEC) have much in common; they produce enterotoxins that cause diarrhoea with many similarities in structure and function [7,8].

The heat-labile enterotoxin (LT) secreted by ETEC and cholera enterotoxin (CT) produced by *V. cholerae* are hetero-oligomeric complexes composed of an enzymatic A subunit and 5 identical binding (B) subunits (AB5 toxins) [9]. The binding of LTB and CTB subunits facilitates the entrance of the toxic portion of the A subunit into the cytosol, which stimulates the epithelial cell adenylate cyclase-cyclic AMP signalling pathway. The cAMP level becomes significantly elevated, allowing massive secretion of electrolytes and water into the gut and provoking diarrhoea [10–12]. Shiga-like cytotoxin (STX), another AB5 toxin, is expressed by *E. coli* O157:H7 and can cause haemorrhagic colitis and the haemolytic uraemic syndrome [13].

Because current therapies are ineffective, prevention of ETEC and EHEC infections is a major public health issue [14]. Moreover, both LTB and CTB are potent immunogens, adjuvants, and ordinary candidates for mucosal vaccines [15,16]. Generation of effective combined vaccines for EHEC, ETEC and *V. cholerae* is highly desirable. In spite of the similarities in the sequential and conformational structures of CTB and LTB, LTB has epitopes which are not found on CTB [17,18]. It has been suggested that a synergistic effect may be attained by employing a mixture of CTB and LTB epitopes [19]. Development of a trivalent subunit vaccine could elicit specific neutralizing antibodies and confer effective protection against these bacterial toxins. Accordingly, several bacterial and yeast systems have been used for production of CTXB, LTB, and STXB along with a chimeric fusion construct against ETEC and EHEC [20–22].

Chimeric fusion of binding subunits of toxins such as CTXB, LTB, and STXB produces a cocktail vaccine that could be ideal for conferring protective immunity to multiple prominent virulence factors [17,23]. The current study designed a synthetic LSC gene (JX866680), composed of *ltB (L)*, *stxB* (S) and *ctxB* (C) from EHEC, ETEC and *V. cholerae,* respectively, that was successfully expressed in *E. coli* as the expression host. The immunogenicity of this synthetic fusion protein was evaluated under *in vitro* conditions and showed its potential as a vaccine candidate able to elicit effective and protective immune responses.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. coli O157:H7 (PTCC: 1763) producing only STX-2 and *V. cholerae* O1 (PTCC: 1611) were obtained from the Iranian Research Organization for Science and Technology. The ETEC isolate that produced only LT toxin was kindly provided by Prof. S.L. Mousavi (Shahed University, Tehran, Iran). The pET-28a plasmid was obtained from Novagen (USA), and the *E. coli* BL21 (DE3) and DH5α strains were acquired from Pasteur Institute of Iran. The bacteria were grown in Luria-Bertani (LB) broth or on LB agar at 37 °C. The media were supplemented with kanamycin (40 μg/ml; Sigma) for selection of recombinant plasmids. The crude preparation of LT, CT and STX2 was performed as previously described [24,25].

2.2. Genetic engineering and generating expression construct

A trivalent recombinant immunogen consisting of *ltB* (L), *stxB* (S), and *ctxB* (C) was designed and codon optimization was performed base on *E. coli* as expression host. Linear B-cell and discontinuous B-cell epitopes of chimeric construct were predicted using Bcepred server (http://www.imtech.res.in/raghava/bcepred/) and Ellipro server (http://tools.immuneepitope.org/tools/ElliPro). The different segments were fused together using the appropriate α-helix producing linkers [4,26]; the 6 histidine tag and ochre stop codon (TAA) sequences were added to the C-terminal of the chimeric gene (Fig. 1A). To facilitate subsequent cloning into pET28a, *Eco*RI and *Hind*III, restriction sites were embedded at the 5′ and the 3′ ends of the construct, respectively. The trimeric gene was chemically synthesized and cloned into pUC57 by Biomatik (Ontario, Canada) and approved by sequencing. The final sequence of the construct was submitted to GenBank (accession no. JX866680). To express each *ltB*, *stxB* and *ctxB* gene separately, the three segments were amplified by specific primers using the synthetic construct (*lsc*) as a DNA template and cloned into the pET28a vector. Table 1 lists the primers used in this study.

The chimeric (*lsc*) and *ltB*, *stxB* and *ctxB* genes were cloned separately into the pET28a expression vector, which provided the N-terminal 6 His-tag and approved by sequencing. The LSC recombinant protein had an additional 6 His-tag at its C-terminal. Each recombinant expression vector was transformed into a competent *E. coli* strain DH5α. The authentic recombinant constructs were transformed into a competent expression host (*E. coli* BL21 (DE3)) and the recombinant colonies were stored at −70 °C.

2.3. Heterologous gene expression and purification of recombinant proteins

Transformed bacteria harbouring pET28a-*ltB*, -*stxB*, -*ctxB*, and -*lsc* were grown in LB broth (50 μg/ml kanamycin, 37 °C). In the mid-exponential phase of growth (OD_{600} 0.5), the bacteria were induced by the addition of 1 mM isopropyl-β-D-galactopyranoside (IPTG; Sigma). The induced bacteria were harvested (5000 × *g*; 10 min; 25 °C) and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea; pH 8.0). After protein estimation using the Bradford method, the samples were analysed for recombinant protein bands by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant 6 His-tagged proteins were purified using denatured nickel-nitrilotriacetic acid (Ni-NTA; Qiagen) resin and the purification was verified on 12% SDS-PAGE. The stepwise dialysis against PBS (pH 7.5) was performed to remove urea from the purified recombinant proteins.