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% to 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 400000 deaths every year [1]. Several outbreaks in humans of *E. O157*, 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 to 4.3 million cases with 28000 to 142000 deaths worldwide [6]. The pathogenic mechanisms of all three microorganisms (*V. cholera*, 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 *Vibrio 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 uremic 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 *Vibrio 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 *Vibrio 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 DH5 α 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 crud 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, *EcoRI* and *HindIII*, 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 (OD₆₀₀ 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 NaH₂PO₄, 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 sulfate 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.

2.4. Western blotting analysis

Ten micrograms each of purified LTB, STXB, CTXB, and chimeric LSC were analyzed in 12% SDS-PAGE and an immunoblot assay was performed as previously described [4]. The chimeric protein (LSC) was incubated with anti-CTXB (1:10000; Abcam), anti-STXB (1:10000; Abcam), anti-LTB (1:10000; Sigma), and HRP conjugated anti-His-tag (1:10000; Sigma) separately. HRP-conjugated goat anti-mouse IgG (1:5000; Sigma) was used as the secondary antibody when using anti-LTB, anti-STXB, and anti-CTXB as the primary antibodies. Final detection of the membrane was carried out using a 3,3'-diaminobenzidine tablet (DAB; Sigma). To verify the

reactivity of the immune sera with each fragment, LTB, STXB, and CTXB were blotted and incubated with antisera from LSC immunized mice.

2.5. Mice group immunization

Forty-eight BALB/c mice (female, 8 wk of age; Pasteur Institute; Tehran, Iran) were divided randomly into four groups; half of the mice in each group were considered as controls. For immunization, 25 µg of LTB, STXB, CTXB, and chimeric LSC protein with complete Freund's adjuvant (CFA) was injected subcutaneously. The control groups were injected with PBS. Two booster injections of the same dose with incomplete Freund's adjuvant (IFA) were administered at 2-week intervals. Antisera from immunized mice were collected and stored in -20 until further experiments.

2.6. Determination of IgG responses to recombinant proteins

The wells of a Maxisorb 96-well plate (Nunc; Denmark) were coated with 100 µl coating buffer (0.015M Na₂CO₃, 0.035M NaHCO₃; pH 9.6; 4 °C; O/N) containing 500 ng of either LTB, STXB, CTXB, or LSC and enzyme-linked immunosorbent assay (ELISA) was performed as previously described [4].

2.7. GM1 inhibition assay

GM1-inhibition assay [27, 28] was performed to evaluate immune serum neutralizing activity against LT and CT toxin using ELISA. The wells of the plate were coated with 3 µg monosialoganglioside GM1 (Sigma) and blocked with 3% (w/v) skim milk/PBST. Thereafter, the plate was incubated with 250 ng/100 µl of CT or LT which had been treated previously with

the serum of immunized mice. The PBS and sera of injected mice with PBS were used as a negative control. Polyclonal anti-rCTXB was used as the primary antibody and HRP conjugated goat anti-mouse IgG was used as the secondary antibody. Each phase was completed with 1 h of incubation at 37 °C and was washed 3 times with PBST. The plate was developed using an OPD substrate and the reaction was stopped by sulphuric acid (2.5 M) and the OD₄₉₂ for each well was measured.

2.8. CHO cell elongation assay

CHO cell elongation assay was performed to evaluate the neutralizing activity of immunized mice serum against CT. The test was based on those of previous studies [29, 28]. In brief, the CHO cells (Pasteur Institute Cell Culture Bank; Tehran, Iran) were seeded in a cell culture plate and grown at 37 °C in a 5% CO₂ humidified incubator. The CHO cells were then incubated for 10 h with 250 ng CT pretreated with serum. The cells were then examined microscopically for morphological changes.

2.9. Hela cell cytotoxicity and neutralization assay

The Hela cell cytotoxicity test was performed as described [30, 31]. In short, 96-well microtiter plates were seeded with 10000 Hela cells (Pasteur Institute; Tehran, Iran) per well and incubated at 37 °C in the presence of 5% CO₂ for 20 h. An overnight culture of *E. coli* 0157:H7 (STX-2+) was centrifuged (1750×g; 30 min; 4 °C) and the supernatant was filter sterilized (0.22 µm) for use as a source of STX-2 toxin. The clarified supernatants were serially diluted in cell culture, added to each well, and incubated for a further 48 h. After washing, the adherent cells were fixed in 10% formalin and stained with crystal violet (0.13% w/v in 5% ethanol-2%

formalin-PBS). The absorbance at 600 nm was measured in each well using a microtiter plate reader (Bio-Rad). The 50% cytotoxic dose (CD₅₀) of toxin was defined as the dilution of *E. coli* O157 culture supernatant that killed 50% of the Hela cells as compared to the untreated control cells.

To identify the serum capacity for toxin neutralization, a serial dilution of the sera of immunized mice was mixed with 10-fold CD₅₀ toxin, overlaid onto the Hela cells as described in the cytotoxicity assay, and incubated for a further 48 h at 37 °C. The cells were fixed, stained, and their OD₆₀₀ was read with a microtiter plate reader. The neutralization titre was defined as the reciprocal of the highest dilution of the sera of immunized mice with a greater OD₆₀₀ than those with 10CD₅₀ of STX-2 alone, or toxin incubated with sera of control mice.

2.10. Statistical analysis

The data were representative of three separate experiments and described the mean $\pm$ standard deviation (SD). SPSS 12.0 was used for all statistical analyses. Student t-test was performed to analyse the antibody response data of the immunized and non-immunized animal groups. A value of $p < 0.05$ was considered statistically significant. Mice survival differences were studied by one-sided Fisher's exact test.

3. Result

3.1. Construction and subcloning of synthetic gene

The LSC chimeric gene (Fig. 1A) was designed according to bioinformatics analysis (data not shown) and chemically synthesized. The synthetic gene was ligated into the pET28a expression vector and the recombinant clone was fully sequenced. Each of the three major fragments (*ltB*, *stxB*, and *ctxB*) were amplified and separately inserted into pET28a. The recombinant plasmids

were confirmed by restriction enzyme digestion and DNA sequencing (data not shown). All four recombinant expression plasmids were then transformed into *E. coli* BL21 (DE3) for protein expression.

3.2. Expression, purification and characterization of recombinant proteins

The recombinant proteins, rLSC (38.9 KDa), rLTB (15.7 KDa), rSTXB (11.3 KDa), and rCTXB (15.4 KDa), harbouring 6 polyhistidines were expressed in *E. coli* BL21 (DE3) and their expected bands were analysed on SDS-PAGE (Fig. 1B). The denatured protein was purified by nickel affinity chromatography (Ni-NTA agarose gel) (Fig. 1C) and the authenticity of the purified protein was confirmed by Western blotting with anti-polyHis-tag antibody.

Fractions of purified proteins were analysed on SDS-PAGE (Fig. 1D). The immune reactivity of LTB, STXB, and CTXB in the structure of chimeric LSC protein was verified by Western blotting with specific commercial anti-LTB, anti-STXB, and anti-CTXB antibodies (Fig. 1E). The anti-LSC antibody produced by the immunized mice recognized all recombinant purified LTB, STXB, and CTXB (Fig. 1F).

3.3. Immunogenicity of rLSC, rLTB, rSTXB and rCTXB

Enzyme-linked immunosorbent assay showed hyper-immunization in rLSC mice compared to the control group. Fig. 2A shows the high titres of IgG class antibodies achieved against LSC after the third injection. The data from Western blot results showed that the serum of LSC-immunized mice identified rLTB, rSTXB, and rCTXB proteins. The ELISA results validated these data and demonstrated that the higher dilutions of anti-rLSC antibody reacted with the lowest amount (100 ng) of purified rLTB, rSTXB, rCTXB, and rLSC. Although some

discrepancies were seen in the titres (end-point titre), statistical analysis showed they were not significant ($p < 0.05$; Fig. 2B). Both sera from mice immunized with rCTXB or rLTB recognized pure rLSC, rCTXB, and rLTB, but failed to react with pure rSTXB (Fig. 3A).

3.4. GM1 inhibition assay

GM1-ELISA assay was performed to determine the neutralizing activity of the sera of immunized mice against CT and LT. The antisera from immunized mice against rLTB, rSTXB, rCTXB, and rLSC were used as test samples and control mice were tested using GM1-ELISA to determine neutralizing activity. The results showed that serum samples isolated from mice immunized with rLTB, rCTXB, and rLSC blocked binding of CT and LT to the coated GM1 ganglioside, while the sera of the control mice and the mice immunized with rSTXB could not inhibit toxin-binding (Fig. 3B).

3.5. CHO elongation assay

In the CHO elongation assay, no morphological changes were detected in the cells cocultured with CT or LT which had been pretreated with the sera of mice immunized with rLSC, rCTXB, and rLTB. On the contrary, the rSTXB or PBS anti-sera not only showed no effect on elongation, but also like native CT and LT, a massive elongation in CHO cells (Fig. 4).

3.6. Hela cells neutralization assay

The result of Hela cell cytotoxicity assay with the culture supernatant of *E. coli* O157:H7 as a source of STX-2 indicated that the CD 50 of toxin was equivalent to a 10^{-5} dilution of prepared toxin containing 1.5 pg of STX-2. The Hela cells neutralization assay was performed with 10 CD

50 of STX-2 toxin. The sera of mice immunized with rSTXB and rLSC neutralized the activity of STX-2, but the sera of mice immunized with PBS, rLTB, or rCTXB had no neutralizing effect on toxicity (Fig. 5).

4. Discussion

Intestinal pathogenic bacteria such as enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and *Vibrio cholerae* are the major causes of bacterial diarrhoea. An effective prevention program could be a beneficial and applicable approach to reducing the morbidity and mortality rate of these diseases. In recent decades, the toxins of these bacteria have been targeted by researchers to design subunit vaccines. The B subunits of heat labile (LT), Shiga-like (STX), and cholera (CT) toxins are potent immunogens with the ability to induce neutralizing and protective immune responses; they can be considered as dominant vaccine candidates [32, 33, 19, 34, 35]. Multi-component antigens are powerful candidate vaccines because they are cost-effective, induce specific immune responses, and present fewer bio-safety hazards [32, 33, 19, 34, 35].

STX, LT and CT play crucial roles in bacterial pathogenesis and have potent immunogenic properties [36-38]. A trimeric vaccine candidate construct composed of the binding subunits of these three important toxins (LTB, STXB, CTXB) was designed and chemically synthesized based on bioinformatics. To maintain their conformational structures, appropriate linker peptides were applied to the design construct.

Despite the limitations of STX-2B expression in previous studies [39, 40], application of the strategies of elimination of the signal peptide along and optimization of the codons according to the host codon preference, were applied and resulted in an appropriate level (76 mg/L) of recombinant gene expression.

Immunoblotting analysis with specific antibodies demonstrated the existence of specific bands of appropriate size, as expected, which showed full-length protein expression. This suggests that the protein synthesis machinery translated the recombinant proteins from the mature mRNA and no deletion or degradation occurred in expression, as had been expected from the results of previous bioinformatics analyses.

The immunological response in animal models revealed that these recombinant proteins could trigger humoral immune responses and produce high levels of IgG immunoglobulin. These results were consistent with the findings of previous research using similar chimeric or one-part proteins for immunogenicity in animal models [41, 4, 27].

Western blotting and ELISA results revealed that the anti-rLSC antibody could identify pure rLTB, rSTXB and rCTXB proteins. The data reveal the ability of the chimeric protein to provoke immune system responses to all nearby epitopes in each part of the LSC protein. The ELISA assay revealed that anti-rLSC reacted with rLTB, rSTXB, and rCTXB proteins. Although it appears that the antibody responses to these recombinant proteins were not equal, a comparison of the endpoint titres showed no statistical significant differences among them.

A topographical view of the results indicates that, although the STXB is located between LTB and CTXB in the chimeric protein structure, their epitopes were exposed for the immune system. In other words, LTB and CTXB could not induce serious conformational changes in the STXB protein. This finding also suggests the efficiency of the linkers for separation of these three segments in the final structure of the chimeric protein and prohibition of inaccessibility of STXB epitopes. As described [42, 43, 27, 28], LTB and CTXB have remarkable structural similarities (~80%) which could be a reasonable explanation for the cross-reactivity of anti-rLTB and anti-rCTXB in this study. [According to available reports each selected monomer \(STXB,](#)

LTB) can create immunity because of its effective epitope; however pentameric structure could provide better results [44, 45].

The GM1 binding inhibition experiment determined that antibodies produced in mice groups immunized with rLSC, rLTB, and rCTXB could inhibit both LT and CT toxin-binding and activity. This indicates that these antibodies could neutralize the epitopes of the native toxins which play a key role in binding the toxin to the GM1 receptor. Result shows that the anti-LTB and anti-CTXB inhibition indices were better than that for anti-LSC. This finding implies that the rLTB and rCTXB epitopes could have experienced minor changes in the chimeric form, as predicted by the bioinformatics software [14]. These results were in agreement with a previous study using CTXB and LTB antigens to stimulate the mucosal immune system [28]. Similarly, the CHO elongation results indicated that LT and CT toxins were effectively inactivated by anti-rLSC, anti-rLTB, and anti-rCTXB antibodies and prevented any change in CHO cell shape.

Inactivation of the STX toxin in the Hela cells neutralization assay by the sera of mice immunized with recombinant proteins is considered as an *in vitro* marker for protective immune response. The effects of neutralization of anti-rLSC and anti-rSTXB were observed in the Hela cell cytotoxicity assay, as expected. There was no significant difference between anti-rSTXB and anti-rLSC neutralization titres, which suggests that antibodies to STXB produced in the rLSC chimeric construct and STXB as a separate fragment (rSTXB) were equally able to neutralize the STX toxin. This result suggests that the neutralizing epitopes of rSTXB were conserved in the chimeric protein.

The results indicate that LSC chimeric protein could provide an effective neutralisation against toxins produced by ETEC, EHEC, and *Vibrio cholerae* bacteria and could be applicable as a vaccine component for diarrhoea.

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458

Figure Legends

Fig 1. (A) Schematic of chimeric LSC; the connections of LTB (L)-STXB (S) and STXB-CTXB (C) were created in two repetitions of YAPQDP and three repetitions of EAAAK linkers, respectively. The numbers denote the start and end of each fragment. **(B)** SDS-PAGE analysis of total bacterial proteins containing pET28a-*lsc* (lane 3), *-ltB* (lane 5), *-stxB* (lane 6), and *-ctxB* (lane 7); lane 2 and lane 4 are total proteins before induction. **(C)** SDS-PAGE analysis of purified rLTB (lane 2), rSTXB (lane 3), rCTXB (lane 4), and rLSC (lane 5). **(D)** Western blotting analysis of purified recombinant rCTXB (lane 2), rSTXB (lane 3), rLTB (lane 4), and rLSC (lane 5) with anti-His tag. **(E)** Western blotting analysis for characterisation of rLSC with specific commercial anti-LTB (lane 2), anti-STXB (lane 3), and anti-CTXB antibodies (lane 4). **(F)** Western blotting analysis with anti-LSC antibodies from mice immunized with rLSC for detection of rCTXB (lane 2), rSTXB (lane 3), rLTB (lane 4), and rLSC (lane 5); in all sections, lane 1 is the protein molecular weight marker.

Fig 2. (A) IgG response to rLSC in sera of immunized mice. **(B)** Titration of sera from rLSC mice immunized with rLTB, rSTXB, rCTXB and rLSC.

Fig 3. (A) Characterisation and cross-reactivity analyses of antibodies from immunized mice versus all purified recombinant proteins. **(B)** GM1 binding index of toxins incubated with sera of mice immunized by recombinant proteins. The GM1 index is calculated as $\left(\frac{AB_{492} \text{ sample}}{AB_{492} \text{ PBS}} \right) \times 100$; the GM1 binding index of toxins incubated with PBS alone (PBS) and sera of control mice (control) are shown.

Fig 4. Effect of sera of immunized mice on the elongation of CHO cells. Serum antibodies of mice immunized with LTB, CTXB and LSC blocked the effect of CT and LT toxins, whereas anti-STXB and the sera of control mice showed no inhibitory effect on elongation of CHO cells.

Fig 5. *In vitro* neutralization of culture supernatant of *E. coli* O157:H7 (STX-2⁺) with anti-sera from mice immunized with rLTB, rSTXB, rCTXB, and rLSC. The bars represent the geometric mean of the log of the neutralization titre to STX-2 and the error bars are ± 1 SD. Comparison of samples was conducted using the Duncan test at the 1% level.

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Table 1

Table 1. Primer sequences used in this experiment.

Primer	Sequence ^a	Restriction site	PCR product (length; bp)
Lf	5'-GGATCC GAATTC <u>ATG</u> CCCCGCAGAG-3'	<i>Eco</i> RI	<i>ltB</i> (312)
Lr	5'-GGCATA CTCGAG <u>TTA</u> ATTTCCATCGAGATGGCC-3'	<i>Xho</i> I	<i>ltB</i> (312)
Sf	5'-GATCCG GGATCC <u>ATG</u> CCGACTGTG-3'	<i>Bam</i> HI	<i>stxB</i> (210)
Sr	5'-TGCTTCA AGCTT <u>TTA</u> ATCATTGTTGAACTGAACTTC-3'	<i>Hind</i> III	<i>stxB</i> (210)
Cf	5'-GCGAA GAATTC <u>ATG</u> ACCCCGCAGAACATC-3'	<i>Eco</i> RI	<i>ctxB</i> (312)
Cr	5'-GTGATGA AGCTT <u>TTA</u> ATTTGCCATGCTGATCGC-3'	<i>Hind</i> III	<i>ctxB</i> (312)

^a The start (ATG) and the reverse complement of stop (TTA) codons are underlined and the restriction sites are shown in bold.