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Original Article

Detection of Some Enteric Bacterial Toxin Via Modified ELISA Assay

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Abstract

Introduction: Heat-labile enterotoxin (LT) of Enterotoxigenic *Escherichia coli* (ETEC) and Shiga toxin (Stx) of *Shigella dysenteriae* serotype 1 are two important toxins of food-borne pathogens associated with diarrheal disease. These agents have been recognized as the first leading causes of neonatal diarrhea and the Hemolytic-Uremic Syndrome (HUS). These toxins have two subunits, A and B, that B subunit can be used as a diagnostic tool.

Materials and Methods: In this study, the *LTB* and *STXB* genes were amplified by using the Polymerase Chain Reaction (PCR) technique and cloned into the prokaryotic expression vector. Following the expression of recombinant LTB and STXB proteins, mouse polyclonal anti recombinant LT enterotoxin and Shiga toxin B subunits were produced for immunological detection. An Enzyme-Linked Immunosorbent Assay (ELISA) was developed for detecting toxins using clinical samples.

Results: Our results showed that the competitive ELISA has high specificity. In addition, the detection limits for LTB and STXB were 20 ng and 90 ng, respectively.

Conclusions: Our findings revealed that the B subunit of LT and STX can be of a great help in detecting these agents.

Keywords: Heat-labile Enterotoxin (LT), Shiga Toxin, B Subunits, Polyclonal Antibodies, ELISA
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Introduction

Annually, about 3 million people die due to gastroenteritis worldwide.[1,2] The ETEC is a major cause of diarrhea with 280-400 million infected cases and 400-800 thousands of deaths per year.[3,4] ETEC strains cause disease, especially in young children and travelers in developing countries.[5-7] The LT and a plasmid-encoded, high-molecular weight toxin are the main agents responsible for the disease caused by ETEC.[8,9] The crystal structure of LT shows that it is composed of two subunits: an A subunit (LTA) (27 kDa) and five non-covalently associated B-subunits (LTB) (11.6 kDa each) forming a pentameric ring.[10,11] Severe diarrhea is a consequence of ADP-ribosylation activity of LTA that activates Adenylyl Cyclase (AC), and increases the intracellular cAMP. LTB can bind to ganglioside GM1 which is, found ubiquitously on the cell membranes of mammalian cells.[12] The B subunit is highly immunogenic and the role of antitoxic immunity to these has been the subject of many studies.[12] On the other hand, shigellosis that usually is caused by *Shigella* species such as *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei* and *Shigella boydii* is an intestinal tract infection. The *Shigella* organisms cause dysentery and the ingestion of as few as 100-200 organisms will cause disease.[13] Shigellosis is a very common disease and over 165 million new cases appear in the world each year. Over 1 million people die mostly in developing countries which approximately 880,000 deaths occur in Asia.[14] The virulence of *S. dysenteriae* may be due to two properties of the organism i.e. invasiveness and toxin production. The real Shiga toxin (Stx) and Shiga-Like Toxin (SLT) are produced from *Shigella dysenteriae* serotype 1 and Shiga Toxin-producing *Escherichia coli* (STEC) (as also called *E. coli* O157:H7 or enterohemorrhagic *E. coli* (EHEC)), respectively.[15] Shiga toxin is associated with the clinical Hemolytic-Uremic Syndrome (HUS). The receptor of Stx is the neutral glycosphingolipid globotriaosylceramide (Gb3/CD77) which is expressed on many cells of the human body.[16,17] The 3D structure of Stx showed an A subunit (StxA) (32 kDa) and five non-covalently linked B-subunits (StxB) (7.7 kDa each) that formed a pentameric ring. The A fragment inhibits protein synthesis by eliminating an adenine from the 28S rRNA of the 60S ribosomal subunit.[16,18] The existing descriptive studies performed in different regions in Iran

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indicate the prevalence of ETEC, STEC and *Shigella*. Actually, 369 stool samples were ETEC positive (32.9%), 200 stool samples were ETEC (15.5%) and STEC (15%) positive, 55 stool samples were ETEC (6.8%), *Shigella* spp. positive (26.7%), 808 stool samples were positive for ETEC (38.8%) *Shigella* spp and STEC (46.6%), 1120 diarrheal specimens were positive for ETEC (28.7%) and *Shigella* species (27.8%).[4,19,20] Considering the importance of these toxin producing pathogens and due to the oral-fecal route of transmission of the bacteria, rapid diagnosic methods are required. Conventional methods and available techniques are sensitive and inexpensive but are very time-consuming.[21,22] Immunological-based methods (such as ELISA) are very practical to be used for the detection of the bacterial cells and toxins of food-borne pathogens.[23,24] Accordingly, in the present study, the rLTB and rSTXB proteins were produced and detected by ELISA.

Materials and Methods

Bacterial Strains and Isolation of Genomic DNA

Standard strains of ETEC and *Shigella dysenteriae* were grown in LB broth at 37 °C and centrifuged at 15,000×g for 10 min. The CTAB-NaCl method was used to extract bacterial DNA. The extracted DNA was also detected by electrophoresis on 1% agarose gel.[25]

PCR Amplification and Gel Electrophoresis

The *ltb* and *stxb* genes (375,270 bp) were amplified by the PCR method with specific primers (*ltb* F: 5-tatagaattcatgaata aagtaaaatattatgtt-3 containing an *Eco*RI-engineered restriction site and *ltb* R: 5-tatataagcttctagttttccatactgattg-3 containing a *Hin*dIII-engineered restriction site) (*stxb* F: 5-tatagaattcatgaa aaaaacattattaatag-3 containing an *Eco*RI-engineered restriction site and *stxb*R: 5-tatataagctttcaacgaaaaataacttc-3 containing a *Hin*dIII-engineered restriction site). Complete sequence of *lt* and *stx* genes available on GenBank were used to design the primers (Sinaclon, Iran). The PCR was carried out based on the pervious study[26] in a 25 µl reaction mixture containing 10 pM of each gene-specific primers, 2 µl of 25 mM $MgCl_2$, 10 mM of each dNTPs, 2.5 µl of 10X enzyme buffer and 0.5 U of *Taq* DNA polymerase. The PCR amplification was performed with an initial denaturation of DNA at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s (*ltb* gene) and annealing at 53 °C for 45 s (*stxb* gene), extension at 72 °C for 1 min and then a final extension at 72 °C for 5 min. Moreover, the *ltb* and *stxb* genes were amplified using *pfu* DNA polymerase using 4 mM magnesium sulfate and 10 pM of each primer with an initial denaturation of DNA at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s (*ltb* gene) and annealing at 53 °C for 45 s (*stxb* gene), extension at 72 °C for 60 s and then a final extension at 72 °C for 5 min. Then the PCR products were run on a 1% agarose gel.[27]

Cloning and Expression of Recombinant Proteins in E. coli Cloning Host BL21

The PCR products were digested with *Eco*RI/*Hin*dIII (Sinaclon, Iran) and cloned into the prokaryotic expression vector pET-28a (+) in the same site of pET28a expression vector with kanamycin resistant selectable markers. Ligation of the *ltb* and *stxb* genes was performed by T4 DNA ligase (Fermentas, USA). The ligated products were transformed into competent *E. coli* strain DH5 α.[28] The colony PCR technique, restriction enzyme analysis and DNA sequencing methods were used to determine the accuracy of cloning. The recombinant plasmids were extracted by plasmid extraction kit (Intron, Korea) from *E. coli* DH5-α cells and were transformed into competent *E. coli* strain BL21DE3plysS. The rLTB and rSTXB expression were optimized for inducer in transformed BL21 (DE3) bacteria by adding 1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG) (OD600 = 0.6) at 37 °C, under constant shaking at 150 rpm. The collected samples were analyzed by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) after 24 h to detect expressed proteins.[27]

SDS-PAGE and Western Blot with Anti-his taq Antibody

Secreted protein samples were separated by 12% SDS-PAGE and then placed into polyvinylidene fluoride (PVDF) membranes (Roche, Germany). In addition, 5% w/v skimmed milk in PBS containing 0.05% Tween-20 (PBS/T), with pH 7.4 at 4 °C were used to block non-specific sites. Western blot was performed[29] to confirm the presence of his-tagged LTB and STXB proteins after purification. A 1:3000 dilution of mice anti-his tag specific antibody (Roche, Germany) in the PBS containing 0.05% Tween 20 (PBS/T) were used to incubate the membrane, with gentle shaking at room temperature for 1 h. Then, the PBS/T was used to wash membrane thrice, subsequently incubated with Horseradish Peroxidase (HRP) coupled with the goat anti-mouse IgG-HRP conjugate (Roche, Germany) diluted 1:1000 in PBS/T at room temperature for 1 h and once more washed with PBS/T thrice. The TMB substrate was used to visualize the membrane.[27]

Purification of the Recombinant LTB and STXB Proteins with His-Tag

To evaluate the solubility of the expressed proteins, recombinant *E. coli* cells were grown on LB agar containing kanamycin 20 µg/ml (Sigma, Germany) and then; the bacterial pellets were re-suspended in PBS and followed by a sonication step. The lysates were centrifuged at 12,000×g for 15 min and the supernatant were collected containing soluble fraction. The insoluble particles in pellet were re-suspended in 8 M urea. The extracts were examined by running

Table 1. Summary of Immunization Protocols

Group	n	Immunization 7d components		Immunization 14d components		Immunization 21d components	
Control	3	PBS rLTB	200 µl 10 µg	PBS rLTB	200 µl 10 µg	PBS rLTB	200 µl 10 µg
rLTB	7	complete Ferund's adjuvant	100 µl	incomplete Ferund's adjuvant	100 µl	incomplete Ferund's adjuvant	100 µl

Table 2. Summary of Immunization Protocols

Group	n	Immunization 7d components		Immunization 14d components		Immunization 21d components	
Control	3	PBS rSTXB	200 µl 10 µg	PBS rSTXB	200 µl 10 µg	PBS rSTXB	200 µl 10 µg
rSTXB	7	complete Ferund's adjuvant	100 µl	incomplete Ferund's adjuvant	100 µl	incomplete Ferund'sadjuvant	100 µl

running on 12% SDS-PAGE. Purification of the recombinant proteins containing poly-His tag were carried out using nickel divalent ions (Ni-NTA chromatographic) (Qiagen, USA). The protein concentrations were estimated by the Bradford method and the positive elutes were confirmed using SDS-PAGE.

Immunization Assay

A total of 20 female BALB/c mice weighted 18-22 g were randomly divided into two groups, one group for LTB Ag (n = 10) and another group for STXB Ag (n = 10). The mice were kept under recommended conditions according to the Institutional Animal Care and Use Committee (IACUC) guidelines. The mice in each group were divided into a test group (n = 7) and a control group (n = 3). For this purpose, 10 µg recombinant proteins (LTB or STXB) in Complete Freund's Adjuvant (CFA) were administered to each mouse in the test group. Also, 10 µg of each rLTB and rSTXB protein in CFA was administered as the booster after 14 and 28 days. The sterile PBS was administered to the control group using the same protocol. One week after the second and third booster doses, the blood was obtained from the tail vein of the mice.[27]

Antibody Production and Titration

The concentration of immunoglobulin G (IgG) was measured by indirect ELISA. About 3 µg of rLTB diluted in 100 µl coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) was used to coat the plates. After overnight incubation at 4 °C, the PBS/T (PBS containing 0.05% w/v Tween 20) was used to wash plates thrice. In addition, 200 µl of 3% w/v skimmed milk in PBS/T (1 h, 37 °C) were used to block nonspecific binding sites. The collected serum samples were serially diluted 1:500 using PBS containing 0.03% w/v Tween 20. Then, the treated samples were used for the ELISA analysis. After washing the samples thrice, 100 mL of the goat anti-mouse IgG peroxidase conjugate (Sigma, Germany) were added which was diluted 1:5000 in PBS/T. The plates were incubated at 37 °C for 1 h. Next, the PBS-T was used to wash samples thrice. Also, 100 µl of TMB substrate (Sigma, Germany) was added to each well and incubated at room temperature for 15 min. To stop the reaction, 100 µl of 2 M H_2SO_4 was added and the result was read by ELISA reader spectrophotometer (Bio-Rad, USA) at 450 nm. The aforementioned ELISA method was used for rSTXB.

Protein G Sepharose 4B Column for Purification of Anti-LTB and Anti-STXB IgG

For the purification of IgG antibodies, the column purchased from Sigma, Germany was used. Then, the columns were washed with Tris (10 mM, pH 8) until they reached equilibrium. Serum samples were eluted from the columns with 100 mM and 10 mM Tris until additional proteins were washed up. Antibodies were eluted from the columns using 100 mM glycine buffer (100 mM, pH 3). Finally, the purity of IgG was analyzed by 12% SDS-PAGE (12%).

Table 3. Indirect-Competitive ELISA for Clinical Samples with LTB: Samples with OD<0.968 Were Considered Positive

rLTB (Ag) (µg/µl)	Ab (µg/µl)	OD	Clinical samples	Ag (µg/µl)	Ab (µg/µl)	OD
8	0.5	0.419	1	3	0.5	1.219
7	0.5	0.429	2	3	0.5	1.261
6	0.5	0.463	3	3	0.5	1.237
5	0.5	0.509	4	3	0.5	1.413
4	0.5	0.518	5	3	0.5	**0.519**
3	0.5	0.528	6	3	0.5	1.318
1.5	0.5	0.635	7	3	0.5	1.229
0.75	0.5	0.662	8	3	0.5	1.234
0.375	0.5	0.675	9	3	0.5	1.423
0.187	0.5	0.727	10	3	0.5	**0.619**
0.093	0.5	0.816	11	3	0.5	1.219
0.046	0.5	0.933	12	3	0.5	1.208
0.023	0.5	0.968	13	3	0.5	**0.804**

Table 4. Indirect-Competitive ELISA for Clinical Samples with STXB: Samples with OD<1.1 Were Considered Positive

rLTB (Ag) (µg/µl)	Ab (µg/µl)	OD	Clinical samples	Ag (µg/µl)	Ab (µg/µl)	OD
8	0.5	0.559	1	3	0.5	1.19
7	0.5	0.632	2	3	0.5	1.284
6	0.5	0.691	3	3	0.5	1.337
5	0.5	0.728	4	3	0.5	**0.413**
4	0.5	0.77	5	3	0.5	1.519
3	0.5	0.778	6	3	0.5	1.348
1.5	0.5	0.826	7	3	0.5	**0.429**
0.75	0.5	0.933	8	3	0.5	1.424
0.375	0.5	1.029	9	3	0.5	1.543
0.187	0.5	1.046	10	3	0.5	0.654
0.093	0.5	1.17	11	3	0.5	**1.659**
0.046	0.5	1.213	12	3	0.5	**0.919**
0.023	0.5	1.262	13	3	0.5	1.724

Table 5. Indirect ELISA for Clinical Samples with LTB

Samples	Ag (µg/µl)	Ab (µg/µl)	OD
1 (rLTB)	3	0.5	**1.97**
2 (sLTB)	3	0.5	**1.376**
3	3	0.5	0.162
4	3	0.5	0.203
5	3	0.5	0.252
6	3	0.5	0.23
7	3	0.5	0.284
8	3	0.5	**0.112**
9	3	0.5	0.221
10	3	0.5	0.185
11	3	0.5	**1.215**
12	3	0.5	0.206
13	3	0.5	0.197
14	3	0.5	0.223
15	3	0.5	0.213
16	3	0.5	**1.256**
17	3	0.5	0.169
18	3	0.5	0.201
19	3	0.5	0.231
20	3	0.5	1.302
Without Ag	-	0.5	0.117
Without Ab	3	-	0.138

Table 6. Indirect ELISA for Clinical Samples with STXB

Samples	Ag (µg/µl)	Ab (µg/µl)	OD
1 (rSTXB)	3	0.5	**2.34**
2 (sSTXB)	3	0.5	**1.614**
3	3	0.5	0.362
4	3	0.5	0.293
5	3	0.5	0.252
6	3	0.5	0.335
7	3	0.5	0.384
8	3	0.5	**1.252**
9	3	0.5	0.241
10	3	0.5	0.285
11	3	0.5	**1.315**
12	3	0.5	0.216
13	3	0.5	0.294
14	3	0.5	0.237
15	3	0.5	0.213
16	3	0.5	**1.376**
17	3	0.5	0.269
18	3	0.5	0.202
19	3	0.5	0.232
20	3	0.5	**1.302**
Without Ag	-	0.5	0.123
Without Ab	3	-	0.142

Optimization and Construction of Standard Curve

The standard curve was illustrated by the competitive ELISA. In this regard, various concentration of the antigens were used. In this assay, the concentrations of coating antigen (LTB, STXB) and the antibody were 3 µg and 0.5 µg, respectively. To attain antibody-antigen complexes, unlabeled primary antibody incubated with obtained antigen and the obtained complexes were added to well plates pre-coated with the same antigen. The plates were washed to remove unbound antibodies, and then secondary antibody was added that was specific to the primary antibody and was conjugated with an enzyme. Finally, a substrate was added and the remaining enzymes showed a chromogenic or fluorescent signal.

The Competitive ELISA with Standard and Clinical Samples

To identify the specificity of antigen and antibody, a competitive ELISA at an antigen concentration of 3 µg and at an antibody concentration of 0.5 µg was performed. In this assay, standard and clinical samples (LTB, STXB) were used.

The Indirect ELISA with Standard and Clinical Samples

An indirect ELISA at an antigen concentration of 3 µg and at an antibody concentration of 0.5 µg was performed. In this assay, standard and clinical samples (LTB, STXB) were used.

Statistical Analysis

The data obtained from three independent experiments were represented as the mean ± Standard Deviation (±SD). The results were analyzed using SPSS and Excel to achieve mean and SD and the student's t-test was used for independent samples. A P value less than 0.05 has been considered statistically significant.

Results

PCR Amplification and Cloning of (ltb and stxb) Genes

The ETEC and *Shigella dysenteriae* genomes were extracted (Figure 1a). Then, *ltb* and *stxb* genes were amplified by PCR using specific primers. The PCR products (375 and 270 bp) are shown in Figure 1b and 1c. The pET-28a (+) vector was used to clone fragments and then transformed into competent *E. coli* DH5-α strain. After extracting pET28a plasmids from *E. coli* it was digested by *Eco*RI/*Hin*dIII and analyzed using agarose gel electrophoresis (Figure 1d and 1e). The recombinant pET28a-*ltb* and pET28a-*stxb* plasmids were extracted from

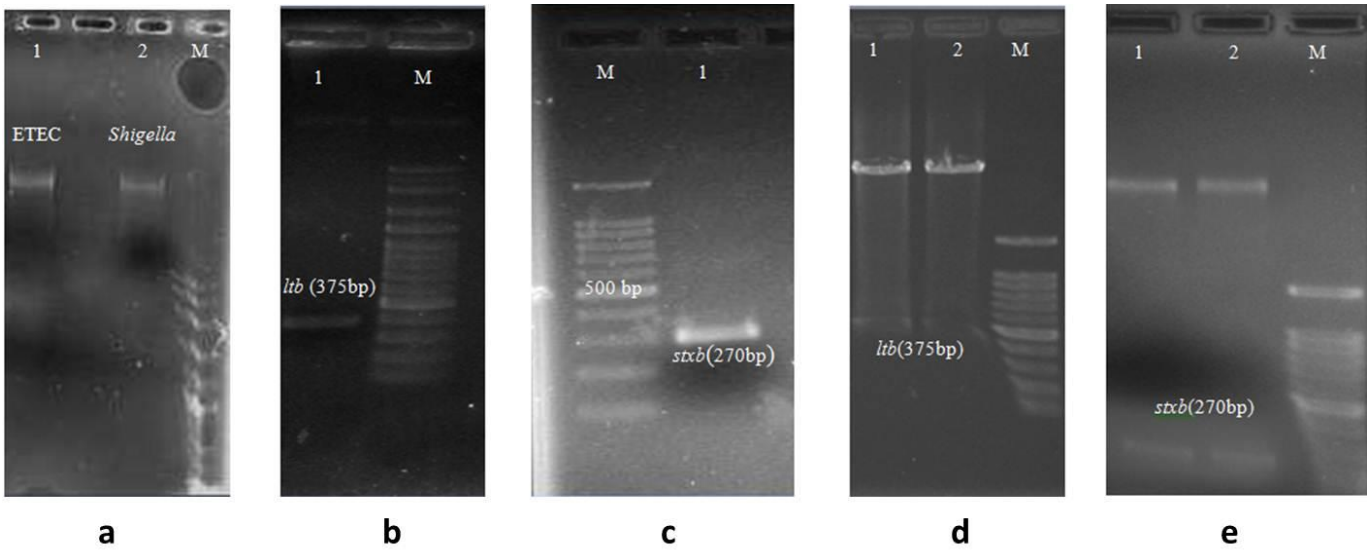

Figure 1. Genome Extraction, PCR Products and Digestion Analysis on Agarose Gel. **a)** Lane M: DNA size marker; Lane 1: Enterotoxigenic *Escherichia coli* (ETEC) *genome*. Lane 2: *Shigella dysenteriae* genome; **b)** Lane M: DNA size marker; Lane 1: *ltb gene*; **c)** Lane M: DNA size marker; Lane 1: *stxb gen*; **d)** Lane M: DNA size marker; Lanes 1 & 2: digested constructs of by *Eco*RI and *Hind*III restriction enzymes; **e)** Lane M: DNA size marker; Lanes 1 & 2: digested constructs of by EcoRI and HindIII restriction enzymes.

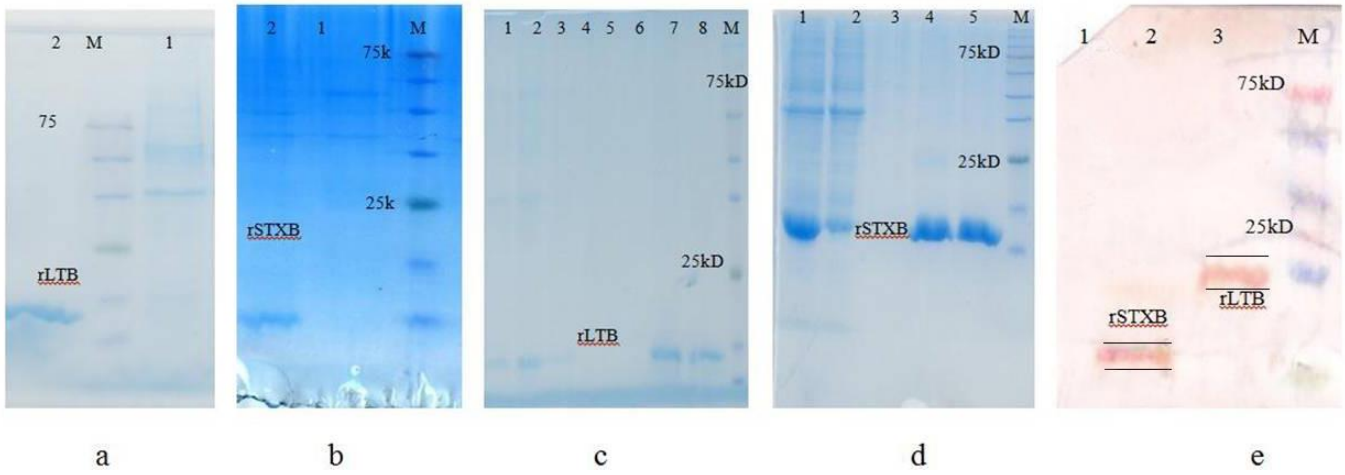

Figure 2. Expression, Purification and Identification of rLTB and rSTXB. **a)** Expression of rLTB. M: protein size marker; Lane 1: non-induced transformed (pET28a without insert) BL21DE3 as control; Lane 2: induced transformed (pET28a with insert). **b)** Expression of rSTXB. M: protein size marker; Lane 1: non-induced transformed (pET28a without insert) BL21DE3 as control; Lane 2: induced transformed (pET28a with insert). **c)** Purification steps of rLTB. M: protein size marker; Lane 1: cleared lysate before passing through column; Lane 2 & 3: Flow through; Lane 4 & 5: Wash; Lane 6, 7 & 8: elution with 250 mM imidazole. **d)** Purification steps of rSTXB. M: protein size marker; Lane 1: cleared lysate before passing through column; Lane 2: Flow through; Lane 3: Wash; Lane 4 & 5: elution with 250 mM imidazole. **e)** Western blotting of rLTB and rSTXB. M: protein size marker; Lane 1: non-induced transformed (pET28a without insert) BL21DE3 as control; Lane 2 & 3: purified recombinant proteins.

E. coli DH5-α cells and transformed into competent *E. coli* strain BL21DE3plysS.

Expression, Purification and Confirmation of Recombinant Proteins (LTB and STXB)

LTB and STXB recombinant proteins plus N-terminal His-tag that were expressed in *E. coli* BL21DE3 (Figure 2a and 2b) purified using Ni-NTA affinity chromatography. The purified product was analyzed by SDS-PAGE which is demonstrated in Figure 2c and 2d. Protein concentrations were estimated by the Bradford protein assay. The presence of proteins plus anti-his tag antibodies were confirmed by Western blot (Figure 2e).

Immunization with Recombinant LTB and STXB Proteins

The concentration of IgG was measured with indirect ELISA. The results showed that antibody titer increased after each immunization (Figure 3a and 3b). The control samples did not reveal any significant level of the anti-LTXB and anti-STXB antibody.

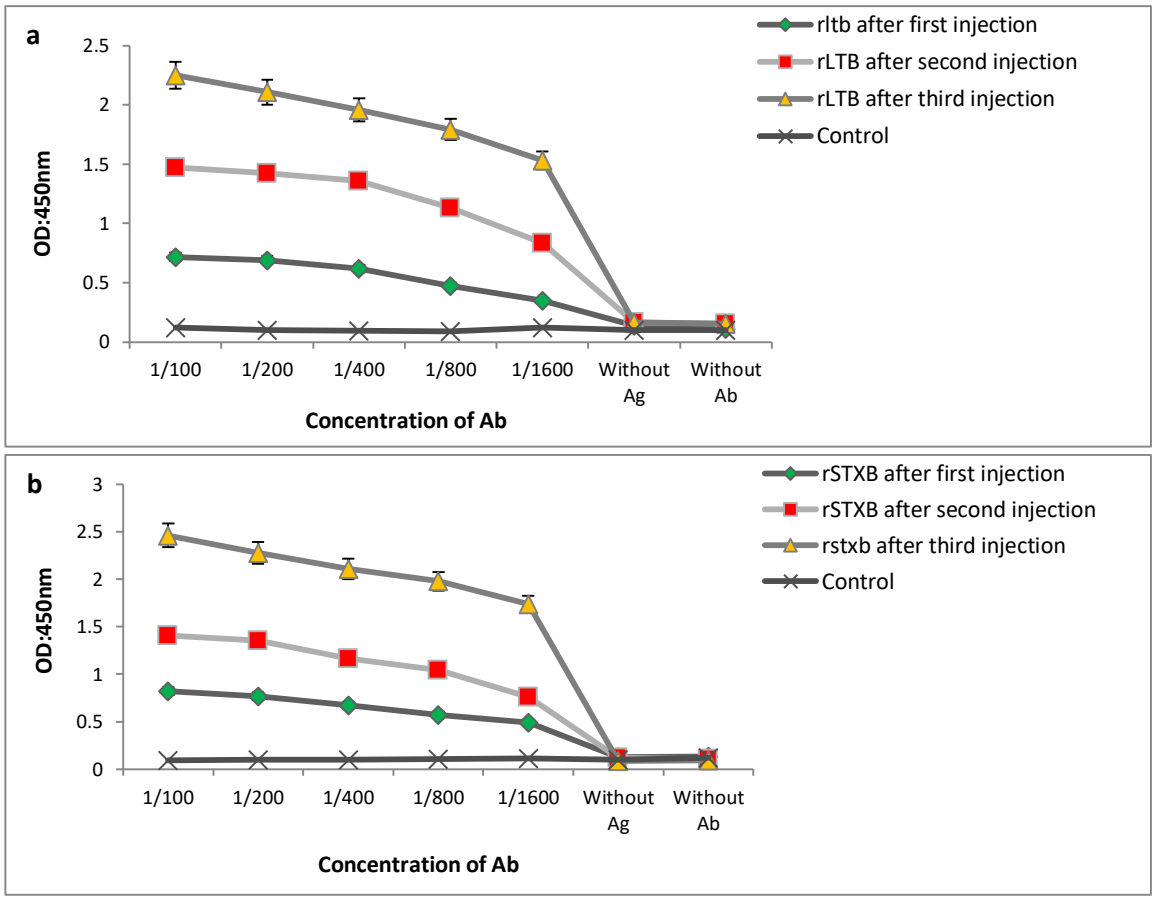

Figure 3. a) Serum Antibody Response of BALB/c Mice Immunized with Recombinant LTB Proteins; **b)** Serum Antibody Response of BALB/c Mice Immunized with Recombinant STXB Proteins.

Protein G Sepharose 4B Column for Purification of Anti-LTB and Anti-STXB

After purification of Anti-LTB and Anti-STXB IgG with protein G Sepharose 4B column, the concentrations of IgG were measured by the Bradford method (2.30 and 2.969 mg/mL). Finally, the purity of IgG was analyzed by 12% SDS-PAGE (Figure 4).

Evaluation of Optimum Antibody and Concentration of LTB and STXB for Specific ELISA

An indirect competitive ELISA was designed to determine the optimal concentration of antibody and the final optimum concentration of IgG was 0.5 µg/ml (Figure 5a and 5b). For determination of antigen, 3 µg was considered for antigen coating. For competitive ELISA, the higher the concentration of the sample antigens was, the weaker the final signal recorded. To reach the primary antibody binding sites, the labeled antigens conquer with unlabeled sample antigens. As the number of antigens in the sample increases, the amount of labeled antigens reserved in the well decreases and the signal become weaker. The detection limits for LTB and STXB were 20 ng and 90 ng, respectively. The cut-off value in ELISA for these two proteins was determined as Optical Density (OD) value of 0.968 and 1.1.

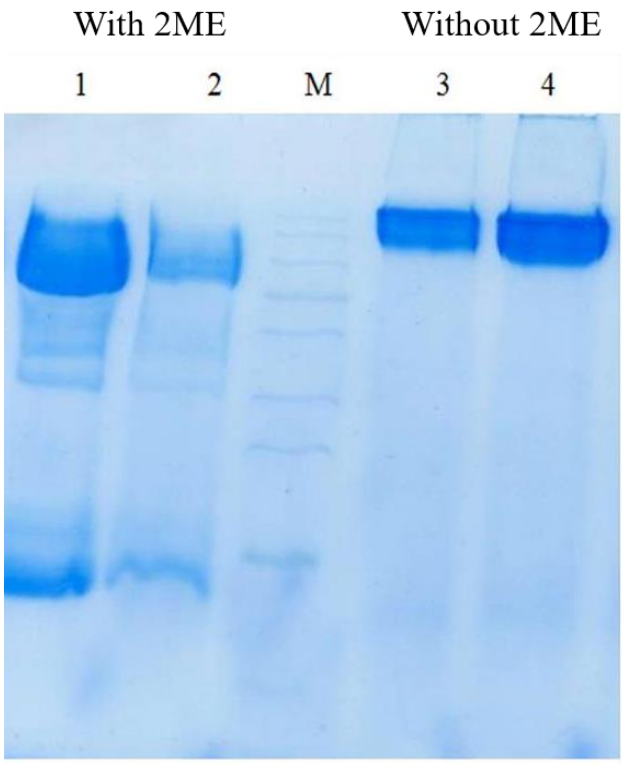

Figure. 4 Lane 1 & 2: SDS-PAGE of Purified IgG with 2ME, M: Protein Size Marker; Lane 3 & 4: SDS-PAGE of Purified IgG Without 2sME.

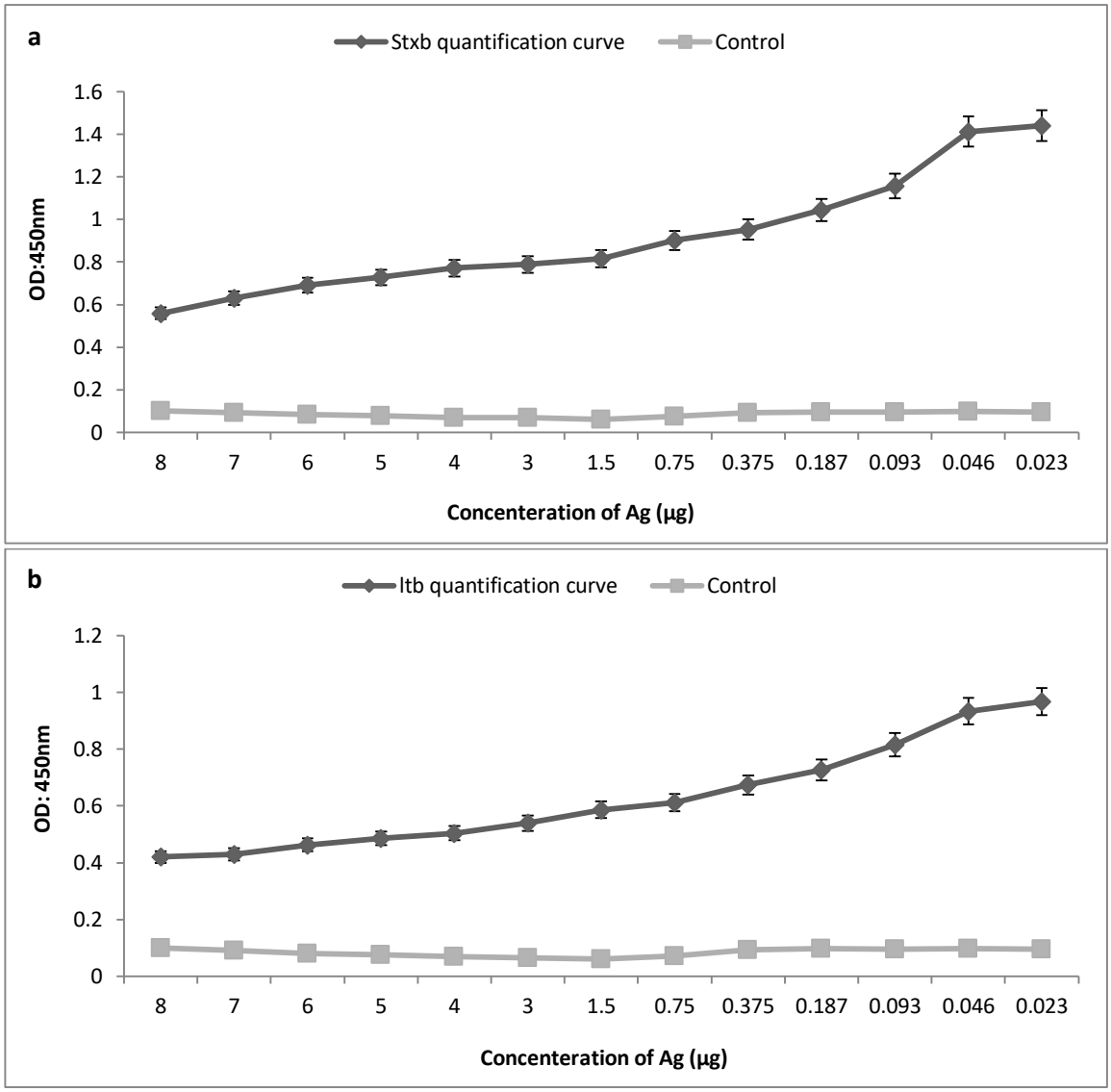

Figure 5. a) Standard Curve for LTB by Indirect-Competitive ELISA. Assays were conducted intriplicate. The data were represented as mean ± SD; **b)** Standard Curve for STXB by Indirect-Competitive ELISA. Assays were conducted in triplicate. The data were represented as mean ± SD.

Discussion

The ETEC and *Shigella dysenteriae* are the major causes of bacterial food-borne diseases and the most common source of infectious diarrhea in the world.[30] Several clinical studies have been performed on these two agents in different countries. In a survey conducted by Keskimaki et al., it has been revealed that Shiga toxin-producing *E. coli* was associated with bloody diarrhea in patients.[31] Andargie et al., 2008, in Gondar Town, Northwest Ethiopia, isolated *Shigella* and enterotoxigenic *E. coli* from the subjects.[32] Qu et al., in China obtained *Shigella* from clinical samples.[33] The results of a few other studies revealed that *Shigella* and enterotoxigenic *E. coli* were the most important agents isolated from clinical samples.[3,20,34,35] *S. dysenteriae* and the shigatoxigenic group of *E. coli* (STEC) are the most common sources for Shiga toxin.[36] The conventional bacterial culture methods for identifying bacteria are extremely time-consuming. Therefore, the rapid and proper detection of these bacteria and their toxins are of great importance. Recently, several screening methods are used for the rapid detection of the Enterotoxigenic *E. coli* (ETEC) and *Shigella dysenteriae* of which nucleic acid-based assays and immunology-based assays are the most commonly used ones. Cloning, expression, purification and production of recombinant LTB and STXB in large scales are pretty simple in the laboratory. During the last decade, several studies have been performed by using these proteins for detecting enterotoxigenic *E. coli* and *Shigella dysenteriae*.

In the current research, the standard strain of enterotoxigenic *E. coli* and *Shigella dysenteriae* were used to produce the recombinant LTB and STXB as a high immunogenic antigen. Moreover, the gene encoding for LTB and STXB were isolated from standard strains. The PCR technique was used to amplify a DNA fragment with the expected size (LTB: 375 bp and STXB: 270 bp) and for the expression, chemically

inducible T7 promoter (pET28a) was recruited. For the optimal expression of recombinant proteins, 1 mM IPTG was used at 37 °C and well-defined induction intervals. Proteins expression enhanced when the induction period lasted up to 18 h. The SDS-PAGE was employed to separate the recombinant proteins and the immunoblotting using mice anti-his tag specific antibody was recruited to confirm their biochemical composition. The recombinant proteins were purified using 6XHis-tag and Ni-NTA column. For immunizing the mice, the purified rLTB and rSTXB were used. All the immunized mice after the third injection produced high titer of anti-LTB and STXB antibody which was confirmed with the ELISA test. Then, an indirect-competitive ELISA and indirect ELISA were designed for the detection of LTB and STXB. Generally, these accurate and quick tests are essential to control enteric bacteria. In the developing world, rapid tests such as lateral flow (LFRTs) have been offered. This technique is very fast, easy to be performed and the obtained results are comparable to ELISAs. On the other hand, this technique has some disadvantages. For example, the required equipment is costly and the user has to prepare the sample. Actually, the diagnostic methods such as PCR, microarrays and loop-mediated isothermal amplification (LAMP) are usually used for the detection of infections in clinical settings. All of these techniques are fast, have high specificity and sensitivity, but, often DNA extraction is needed which is time-consuming. These analyses based on molecular methods are proper for the acute phase of the disease but are not quantitative. ELISA is a quantitative test, without the need to pre-assay handling and the overall running cost is lower in comparison with other techniques. In addition, a simple standard curve can be used to read the results of ELISA test. ELISA can detect the presence of an infectious agent in the body. Therefore, the newly developed molecular methods can also be used instead of ELISA. In recent decades, new methods have been rapidly developed for quick diagnosis of infectious diseases. Yet, PCR is the most usual and precise molecular method that can even detect a small number of pathogenic bacteria. Therefore, for screening various samples and elements, the ELISA test can be used. As the B subunit of heat-labile enterotoxin has high immunogenicity, many studies are performed based on the ELISA method using antibodies produced against bacterial toxin.

As the B subunit of heat-labile enterotoxin has high immunogenicity, many investigations are based on the detection of antibodies produce against the bacterial toxin as ELISA assay. For example, the studies conducted by Salimian et al., and Norton et al., showed that LTB is highly immunogenic and as the natural receptor for LT is GM1 ganglioside, its further purification resulted in an advancement of ganglioside-capture enzyme-linked immunosorbent assay.[6,37] Khalesi et al., produced rLTB and anti-LTB antibody and have developed the GM1 gangelioside receptor-ELISA based method.[38] For detecting enterotoxigenic *E. coli*, Menezes et al., developed antibodies against heat-labile enterotoxin.[39] For the detection of Shiga toxin, Oloomi et al., used ELISA.[40] In the study conducted by Madanchi et al., the CtxB-StxB fusion protein was expressed in *E. coli* in order to enhance immune response against StxB.[41]

Conclusion

The competitive ELISA has high specificity and is suitable for complex samples. The detection limits for LTB and STXB were 20 ng and 90 ng, respectively. The cut-off value in ELISA for these two proteins were determined as OD value of 0.96 and 1.1. These results can help us to design indirect ELESA for the detection of LTB and STXB in clinical samples.

Authors' Contributions

SAM, HS, JA, and AAIF made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. SAM, HS, and EB participated in drafting the article and critically revising it.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Ethical Approval

All animal experimentations were performed according to the guidelines of the Baqiyatallah University of Medical Sciences Animal Care with prior approval of the Institutional Animal Care.

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