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Research Article

Cloning and Expression of a Fusion Protein Containing Highly Epitopic Regions of *Clostridium perfringens* Epsilon Toxin and *Clostridium novyi* Alpha Toxin

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Abstract

Clostridium perfringens and *novyi* species are two important toxin-producing pathogens which pose a risk to the livestock health. Epsilon and alpha toxins are major toxins of these two pathogens, respectively. Advances in current vaccine industrialization lead to the utilization of toxin epitopes instead of the whole pathogen/toxoids to produce novel vaccines. In the present study, bioinformatics approaches were applied to design a fused protein containing both toxin fragments of interest with the highest antigenicity score for B-cells. To do so different specialized algorithms including I-TASSER, IEDB, ElliPro, PyDock and CLC Main Workbench were applied. The chimeric protein was successfully cloned, expressed, and purified using an immobilized-metal affinity chromatography for His-tagged proteins. During *in vivo* experiments on rabbits, the levels of immunization provided by the recombinant protein or native alpha and epsilon toxins were compared based on serological studies. Results indicated that the designed protein was able to stimulate effective immune responses against both alpha and epsilon toxins. This can be used as a proper strategy to design novel peptide-based subunit vaccines.

***Keywords*:** Clostridium, Alpha toxin, Epsilon-toxin, Fusion protein, ELISA, Immunization

Introduction

Clostridium novyi (*C. novyi*) type B is the causative agent of infectious necrotic hepatitis (black disease, German Bradsot), especially in sheep. The major pathological determinant of the disease is the edema-inducing and lethal exotoxin alpha (2178 aa, MW: 250 kDa) which is produced by *C. novyi* type B (Busch et al., 2000). *Clostridium perfringens* produces four main lethal toxins (alpha, beta, epsilon, and iota) which are divided into 5 toxin types (A–E). Spores and toxins of *Clostridium perfringens* (*C. perfringens*) have reportedly been considered as biological warfare agents. Epsilon-toxin (ETX), a 34-kDa pore-forming protein, is considered as the major virulence factor of both type B and D strains. It was ranked as the third most potent Clostridium-derived toxin after botulinum and tetanus toxins (Alves et al., 2014). Enterotoxaemia can cause acute or super acute disease, with the sudden death of the affected animals. It provokes huge economic losses when large numbers of livestock are affected (Souzaet al., 2010). Considering the high lethality levels of these pathogens, efficient vaccination is highly desirable. Different vaccines were introduced to prevent the disease. Traditional vaccines are composed of live attenuated or fixed whole pathogens and carry native protein antigens. Although, these vaccines prevent the disease, some challenges such as the variable levels of the immune- and inflammatory responses induction following their application remains unsolved (Adhikari et al., 2012).

The latest progress in the evolution of vaccine formulations is the development of epitope-based vaccines (Palatnik-de-Sousa et al., 2018). The main benefit of immunization with these vaccines is the ability to effective stimulation of immune system with minimal structure of antigen and without any undesirable effects (Oscherwitz, 2016).

Previous studies confirmed that antibodies act more specifically when they are in contact with the epitopes of an antigen rather than the whole antigen (Watts, 2004). Hence, in the present study, we detected the antigenic regions of *C. novyi* alpha-toxin and *C. perfringens* epsilon-toxin to design a fusion peptide. In the chimeric protein these fragments were fused by a properly designed linker

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sequence. During *in vivo* experiments, the immunogenic capacity of the new peptide was compared with native alpha and epsilon toxins.

Fusion of different antigens from two or more pathogenic sources is a technology which is proposed for concurrent induction of the immunity against different pathogens. Putting both antigens in just one protein, not only makes the immunization faster and cheaper, but also in some cases makes it more effective (Schmidt, 2009). Moreover, the combination of vaccines is economically preferred when we consider its direct and indirect

consequences such as costs of extra injections, delayed or missed vaccinations, and additional handling and storage steps (Chandran et al., 2010). Epitope-based vaccines have been demonstrated to induce protection against many infectious diseases. For example, Pilechian et al designed a fusion protein of beta end epsilon toxins derived from type B and D of *C.perfringens* (Pilechian et al., 2011). In another study, a recombinant protein vaccine from *Pseudomonas aeruginosa* A and B flagellin epitopes was produced which caused less inflammation and infection in mice compared to the control samples (Weimer et al., 2009).

Materials and Methods

Bioinformatics

The most complete gene sequence of the alpha-toxin was introduced as Z48636.1 *C.novyi* in NCBI database with 6836 bps length. The gene sequence of epsilon toxin was obtained from NCBI with accession number of pCP8533etx and 1098 bps length.

The gene sequence of alpha toxin was obtained from NCBI with accession number of Z48636.1 and 6836 bps length and for epsilon toxin,

To determine the antigenicity of alpha and epsilon-toxins, their secondary structures and some other essential parameters were obtained from CLC Main Workbench 5 offline software. Reverse translation of the above mentioned gene sequences were applied as the input data to obtain the secondary structures of the proteins. Primer pairs and the linker sequence were selected according to our previous results obtained via bioinformatics (Noshahri et al, 2016, Mehrvarz et al, 2020). The designed construct was subjected to CLC Main Workbench and bioinformatics tools analysis to reveal its binding affinity with the major histocompatibility complex II (MHC-II) DRBI polymorphism in *Ovis*. Docking analysis was

Table 1. The first 10 highest scored MHC-II peptide binding results according to IEDB algorithm and similarity level of the sequences with previously identified sequences of the DRBI allele.

Allele	Start	End	length	peptide	IEDB rank
DRBI	108	122	15	GLQKISDKYYFNDN	76
DRBI	150	164	15	WFNNNKERYYFDSEG	66
DRBI	321	335	15	SDTVNKSDLNEDGIT	63
DRBI	36	50	15	KKGYQEIEGERYYFN	58
DRBI	5	19	15	HYKNIPGDTEFEYGW	51
DRBI	374	388	15	EKSNDSNIVKYRSLS	50
DRBI	271	284	15	PANTTVEVIAYLKKV	46
DRBI	250	264	15	TNTNTNSKEITHNVP	43
DRBI	160	174	15	FDSEGRLLTGYQVIG	41
DRBI	86	100	15	YTGWLTIDGNKYYFQ	35

performed using Pydock online software (https://life.bsc.es/servlet/pydock/).

Evaluation of ALE linear and structure-based epitopes was performed by Ellipro and Discotope online softwares, respectively (Table 1 and Figure 1). For both Pydock and IEDB (www.iedb.org) softwares, the PDB files of the fragments and proteins were obtained from I-TASSER online software (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

common phenol-chloroform method. The quality and quantity of DNA samples were determined by agarose gel electrophoresis and Nanodrop spectrophotometric method (260 nm), respectively.

The polymerase chain reaction (PCR) experiments were performed as follows: Initial denaturation (94°C, 5 min), denaturation (94 °C, 30 sec), annealing (53 °C and 55°C, 30 sec for epsilon and alpha toxin, respectively), extension (72 °C, 30 sec), and final extension (72 °C, 5 min). Primer sequences are shown in the Table 2. Fragments which were amplified by PCR, were cloned into pTZ57R vector by TA cloning system (CloneJET PCR Cloning Kit.,

Table 2. Primer sequences applied to perform PCR experiment. The linker sequence is also shown

Primer name	Sequence (5´-3´)
AHEF*-Forward	AATGGAGAGCTTCATTACAAAAAT
AHEF-Reverse	AAGAAGGGTTAAAGGTAAATATAA
EHEF*-Forward	AAAAATACTGATACAGTAACTGCAACTACTAC
EHEF-Reverse	AAAATAAGGACCACGGAATTATCTTTCTGAGG
Linker	GGATCCGGCAACTACAACCTGAAGAGCAACCAATATGAAGCTGAGCTC

*Abbreviations: AHEF (alpha high epitopic region), EHEF (epsilon high epitopic region)

Bacterial culture and molecular studies

C. perfringens (CN409) and *C. novyi* (CN804) bacterial strains were obtained from Razi Vaccine and Serum Research Institute, Mashhad, Iran. Both bacteria were cultured anaerobically in the liver extract medium prepared by Anomax instrument (Mart® microbiology B.V., Netherlands) at 37°C, pH 7.5 for 16 h. Bacterial DNA was extracted by the

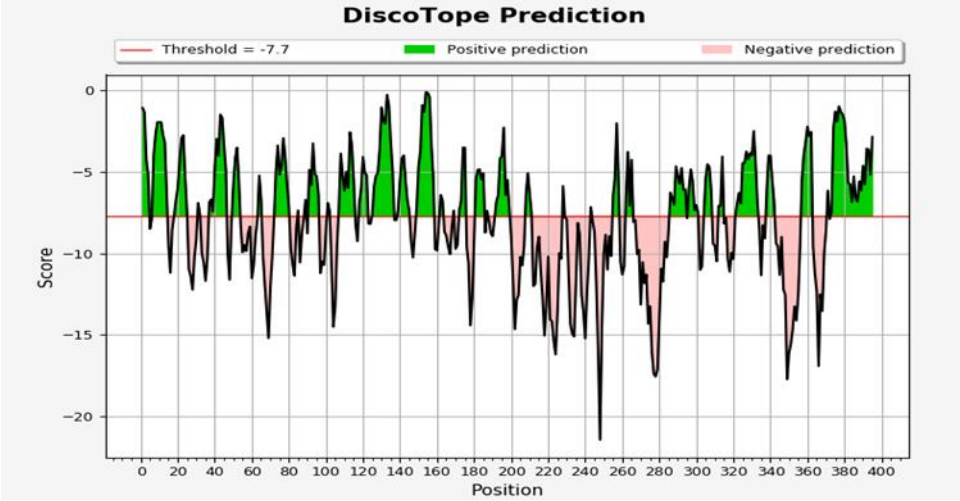

Figure 1. Structure-based antibody prediction for ALE fusion protein epitopes by Discotope according to Discotope score. The score is calculated through combining the contact numbers with propensity scores (PS). DiscoTope scores above the threshold (green regions) indicates positive predictions and that below the threshold (pink regions) indicates negative predictions .

K1231, Thermo scientific, USA). *Escherichia coli* DH5a competent cells were prepared and transformed by the recombinant plasmids using methods described by Sambrook (Sambrook et al., 2012).

To select cells containing recombinant plasmids,

they were cultured in the presence of ampicillin (100 µg/ml), IPTG (Isopropyl β- d-1-thiogalactopyranoside), and X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside). White colonies, which are supposed to carry recombinant plasmids, were cultured. Then, their plasmids were extracted to confirm the validity of the cloning based on PCR experiments for epsilon and alpha toxin sequences. Other rounds of PCR studies were performed using M13 forward and reverse primers (specific to PTZ57R) to make sure that the fragments are properly placed in plasmids in the correct direction. Selected recombinant clones were cultured and plasmid contents were extracted by Roche plasmid extraction kit.

Digestion of plasmids was performed using the mentioned enzymes in Figure 2. Desirable fragments were isolated from low melting agarose gel (Thermofisher, 16520-050, USA) using Roche gel extraction kit (Roche, 11696505001, Germany). Ligation of fragments was performed using a designed linker and then directly cloned into poly histidine tagged vector: pET28a (+) (Merck, 69864, Germany). Competent cells (*E. coli*, DH5a) were prepared and transformed by the recombinant plasmid pET28a+ containing a fusion sequence of AHEF-Linker-EHEF (ALE) (AHEF: alpha high epitopic region; EHEF: epsilon high epitopic region). Plasmid extraction was performed for the clones with desirable properties. Then, *E. coli* BL21 (DE3) host cells were transformed with these plasmids. To express high amounts of the recombinant proteins, validated clones were inoculated in 10 ml of the LB medium supplemented with kanamycin (50µg/ml) and IPTG (100uM), and incubated at 37ºC for 16-20 hours. Recombinant proteins were released to the culture medium by repeated rounds of freeze (liquid nitrogen, 5 min) and thawing (boiling water, 5 min). Then, the supernatants were used for indirect enzyme-linked immunosorbent assay (ELISA) against histidine tag. Colonies with the highest level of ALE expression were selected for downstream applications.

Fusion protein expression and purification

The selected colony in each experimental group was cultured overnight at 37°C in 500 ml LB medium containing 50µg/ml kanamycin and 100µM IPTG. The cultures were centrifuged and the pellets were collected and cell lysis was performed based on the freeze/thaw method. Briefly, cell pellets were resuspended in the lysis buffer (10 mM Imidazole, 50 mM NaH_2PO_4, 250 mM NaCl, pH=8), Lysozyme was added to the final concentration of 500 ug/ml. Following 40 min incubation on ice, cells were disrupted by sonication (Hielscher, Germany). Subsequently, in each case, 1.5 ml of Ni-NTA agarose (Qiagen, 30210, USA) was added to 10 ml of the lysate and mixed while shaking on ice for 60 min. To purify the target protein, the mixture was loaded on the column with the bottom outlet capped. Then, recombinant proteins were washed with 4 ml of washing buffer (250 mM NaCl, 20 mM Imidazole, 50 mM NaH_2PO_4, pH=8). The 6xHis-tagged recombinant proteins were eluted 4 times with 1 ml of elution buffer (250 mM NaCl, 250 mM Imidazole, 50 mM NaH_2PO_4, pH=8) per column and were collected in 4 separate tubes. Protein Slysates

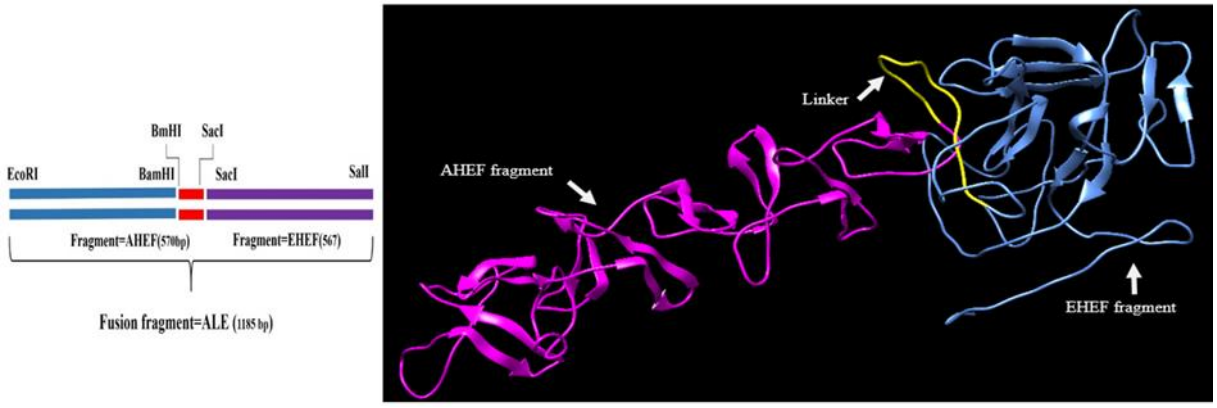

Figure 2. Schematic 3D representation of the designed fusion protein as it was drafted by the I-Tasser …. (A). Restriction map of the recombinant protein ALE (B). Different enzymes including EcoRI, BamHI, SacI, and SalI were applied to connect desirable fragments. The Linker was restricted by BamHI and SalI. In the final structure, AHEF (alpha toxin related fragment: pink-colored structure) is bounded to the EHEF (epsilon toxin related fragment: blue-colored structure) via the yellow-colored linker.

(25 µg/lane) were loaded on a 12% SDS–PAGE and transferred onto nitrocellulose membranes (BioRad Laboratories instrument, USA). The nitrocellulose membranes (Amersham, USA) were blocked with 3% BSA (Bovine serum albumin, Fraction V, Merck, A3311, Germany) and immunoblotting was performed with HRP-conjugated anti-poly Histidin (anti Rabbit, 1:1000) antibodies. The protein bands were visualized by DAB (3, 3'-Diaminobenzidine) staining (Merck, D12384, Germany).

Evaluation of rabbit immune responses against ALE fusion protein

Purified ALE fusion proteins were injected subcutaneousley to New Zealand White rabbits (female, 6 months old, 350-450g) 3 times with 2 weeks intervals. At the end, blood samples were collected and processed to prepare serum samples. Anti-alpha (*Clostridium novyi*) and anti-epsilon (*Clostridium perfringens*) toxins were obtained from Razi Vaccine and Serum Research Institute, Mashhad, Iran Immune responses of the rabbits were evaluated in case of the ALE protein and its cross-reactions with epsilon and alpha toxins. To do so related antibodies were measured based on indirect ELISA experiments.

Results

Bioinformatics studies and recombinant protein design

The best antigenic regions of alpha (EHEF) and epsilon-toxins (AHEF) were selected based on bioinformatics studies. The 3D schematic structure of the recombinant protein and the location of restriction sites are shown in Figure 2. Results from MHC-II peptide binding prediction obtained by IEDB are presented in table 3. The result from docking of ALE and the sequence of protein are being presented in figure 4. For docking studies, the 3D structure of the DRBI was applied as the ligand and the structure of the designed fusion protein was used as the receptor. The results from Pydock program indicated that the maximum affinity score of the fusion protein to DRBI was equal to -611.8, which demonstrated an acceptable level of stimulation for this protein. The binding sites of the MHC-II allele with ALE are shown in figure 4.

Bacterial strains were accurately cultured and identified and their DNA was extracted. Cloning and expression steps were successfully performed and confirmed by the mentioned methods. SDS-PAGE and Western blotting results verified the cloning and transformation steps and the proper expression of the antigenic peptides AHEF, EHEF, and fusion ALE in BL21 DE3 (Figure 3).

Table 3. Predicted B Cell Linear epitopes of the ALE fusion protein identified using Ellipro

Number	Start	End	length	Score*	Peptide sequence
1	1	57	57	0.801	NGELHYKNIPGDTFEYGWINIDSRWYFFDSINLIAKKGYQEIEGERYYFNPNTGVQE
2	371	395	25	0.766	DKKEKSNDSNIVKYRSLSIKAPGIK
3	290	333	44	0.732	NVKLVGQVSGSEWGEIPSYLAFPRDGYKFSLSDTVNKSDLNEDG
4	355	365	11	0.659	VRNLNTNNVQE
5	71	88	18	0.655	TNKHASSKRWGRAINYTG
6	262	276	15	0.624	NVPSQDILVPANTTV
7	100	105	6	0.599	QSNSKA
8	377	353	17	0.596	INGKGNYSAVMGDELIV
9	241	246	6	0.567	LTTSYS
10	194	199	6	0.502	NYNLKS

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* ElliPro gives each predicted epitope with a score, defined as a PI (Protrusion Index) value averaged over epitope residues. In this method, the protein's 3D shape is approximated by a number of ellipsoids for each residue, a PI value is defined based on each residue's center of mass placed outside the largest possible ellipsoid

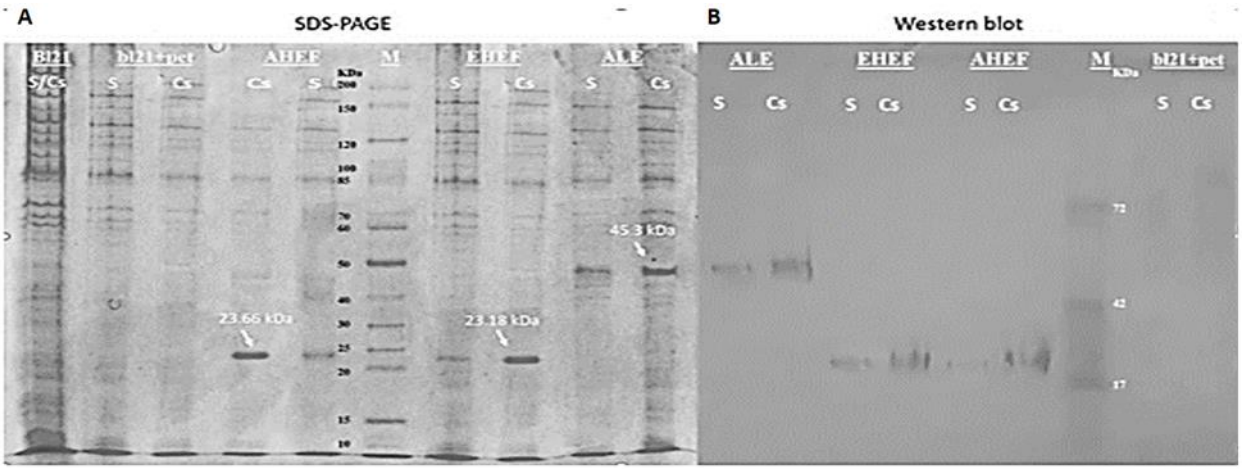


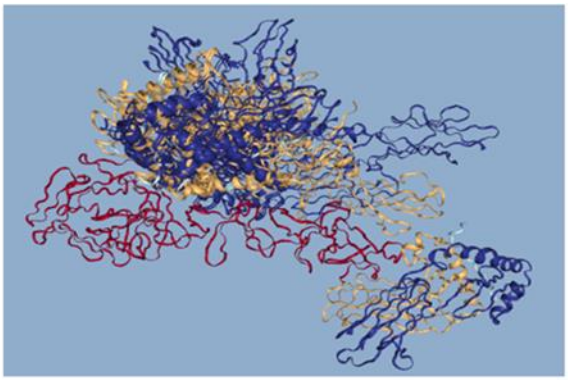
Figure 3. SDS-PAGE (A) and Western blotting (B) results of expressed AHEF (alpha related), EHEF (epsilon related) and ALE fusion fragments. (S: culture supernatant, Cs: cell lysate supernatant).

Antibody responses and cross-reactions analysis

Functional analysis based on ELISA experiments indicated that recombinant AHEF, and EHEF peptides were recognized by anti-native alpha and epsilon-toxin antibodies, respectively (Figure 5). Additionally, the ALE recombinant peptide reacted with both anti-native toxin antibodies due to the presence of AHEF and EHEF fragments in its structure. Cross reactions among anti-ALE antibodies, prepared by injection of the ALE purified peptides to the rabbit, and rabbit anti-alpha and anti-epsilon toxins with various antigens including the alpha-toxin (AT), epsilon-toxin (ET), AHEF, EHEF, and ALE are shown in the Figure 6. The protein content of the *E. coli* BL21 (DE3) containing pET28a (+) with no insertion was injected subcutaneously into a rabbit (150ug/injection). Then, antiserum was isolated and applied as the negative control.

Discussion

Antigenic epitopes are responsible for the interactions between antigens and their specific antibodies. To design effective vaccines against a wide range of pathogens, these epitopic regions, usually identified based on bioinformatics studies, can be utilized. The concurrent application of different algorithms to predict antigenic regions of peptides is a more reliable approach which ensures us about the final results. In the present study to predict highly immunogenic epitopes of both *Clostridium novyi* alpha toxin and *Clostridium perfringens* epsilon toxin, a set of different online and offline bioinformatics softwares were applied. These algorithms use different parameters such as hydrophobicity, hydrophilicity, rate of surface exposure, and etc. To be more efficient instead of



A

NGELHYKNIPGDTFEYGWINIDSRWYFFDSI
NLIAKKGYQEIEGERYYFNPNTGVQESGVFL
TPNGLEYFTNKHASSKRWGRAINYTGWLTL
DGNKYYFQSNSKAVTGLQKISDKYYYFNDN
GQMQIKWQIINNNKYYFDGNTGEAIIGWFNN
NKERYYFDSEGRLLTGYQVIGDKSYYFSDNI
NGNWEEGSGNYNLKSNQYEAELKNTDTVT
ATTTHTVGTSIQATAKFTVPFNETGVSLTTSY
SFANTNTNTNSKEITHNVPSQDILVPANTTVE
VIAYLKKVNVKGNVKLVGQVSGSEWGEIPSY
LAFPRDGYKFSLSDTVNKSDLNEDGTININGK
GNYSAVMGDELIVKVRNLNTNNVQEYVIPVD
KKEKSNDSNIVKYRSLSIKAPGIK

B

Figure 4. A) Results from the molecular docking of DRBI and ALE fusion protein. The output gives the 3D coordinates of two interacting fragments. B) The amino acid sequence of the ALE peptide. Blue: epsilon related fragment (EHEF), Yellow: alpha related fragment and red colored part represents the linker.

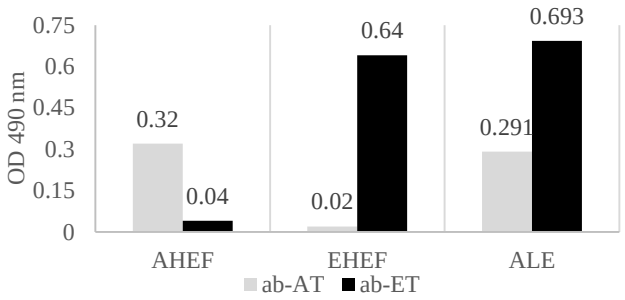


Figure 5. Immunologic assesment of the recombinant EHEF, AHEF, and ALE peptides reactions with specific anti-native alpha and epsilon toxin antibodies, as they were obtained by ELISA experiments (Wavelenght: 490 nm).

using one single epitope of each toxin a fragment which contains the highest scored epitopic region was selected from each structure to build a larger and more immunogenic multi-epitopic fragment. This removes the need for the simultaneous application of the adjuvants or repeated rounds of the injection (Majidi et al., 2020).

Antigenic regions are probably found with a higher frequency in C-terminal regions of the peptides in comparison to the N-terminal sequences with a higher number of beta-sheet structures. It was reported previously based on studies performed using Kolaskar and Willing algorithms and CLC5.5 software (Attasi, 1984). The antigenic fragment of the alpha-toxin (from amino acid 1731 to 1921) was introduced by Noshahri and colleagues (Noshari et al., 2016). Also, in a recent experiment, the epitopic region of the epsilon toxin (aa 531-1098) was reported based on the scores calculated by the Discotope and graphs which were obtained from different softwares (Mehrvarz et al., 2020). By designing multi-epitopic fragments with both B-cell- and T-cell-specific receptors, it would be possible to produce more potent proteins with the capacity for concurrent stimulation of cellular and humoral immune systems. However, direct fusion of different functional domains may lead to many undesirable outcomes such as misfoldings, low yields of protein production, and impaired bioactivities. Hence, designing proper linkers to connect individual fragments is a crucial step since they should have non-polar amino acids which kept the secondary structures, biological activities, and antigenic properties of all fragments, while they are conserving the ORF intact (Chen et al., 2011). Considering all above mentioned criteria a 48 bps linker was designed in which unique BamHI (5´) and SacI (3´) restriction sites were applied. Results from ELISA assay confirmed the desirable properties of the linker, as the ALE fusion protein was recognized with both anti-alpha and anti-epsilon antibodies. This confirms that both fragments of the protein maintained their natural secondary structures, biological activities, and antigenic capacities. Furthermore, according to results obtained during docking experiments, ALE can be recognized and interact with the DRB1 Ovis MHC-II allele.

Cross ELISA assay with anti-epsilon-toxin antibody showed quite noticeable differences between the reactions of the anti-epsilon antibody with alpha-toxin and AHEF compared to its reactions towards EHEF, ALE, and epsilon-toxin. This is an indicative that the anti-epsilon antibody can recognize selected epitopic regions of this toxin

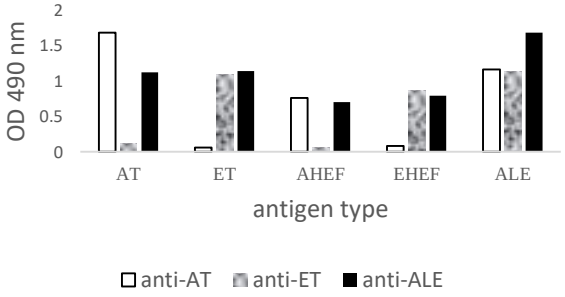


Figure 6. cross reactions of prepared antibodies against AT, ET and ALE with whole toxins and fragments.

only in native epsilon-toxin, EHEF, and ALE. Similarly, the anti-alpha-toxin antibody could only have noticeable reactions with alpha toxin, AHEF, and ALE but not epsilon-toxin and EHEF. This points out the specificity of antibodies for the epitopic areas of their corresponding toxins in alpha toxin, epsilon-toxin, AHEF, EHEF, and ALE. These results support our bioinformatics studies indicating that our antigenic fragments consist of most epitopic regions of the initial toxins which enables them to be recognized by the antibodies produced against their native toxins.

Rabbits immunized with ALE fusion protein were resulted in high production of related antibody. Results of ELISA assay of the anti-ALE fusion protein with different antigens showed that this antibody could recognize Alpha toxin, Epsilon-toxin, AHEF, EHEF and ALE itself indicating that our designed ALE fusion protein is capable of eliciting an immune response against epitopes of alpha and epsilon-toxins. So this could be a promising strategy to produce multi-purpose vaccines against enterotoxaemia diseases, which to our knowledge has not been reported previously.

In a similar study, a fusion protein of non-toxic regions of A and B toxins of *Clostridium difficile* (*C. diff*) was produced with a proper capacity for strong immune response induction. This recombinant protein protects the animal models against *Clostridium difficile* spores (Karczewski et al., 2014). In another report, non-pathogenic areas of the *Clostridium perfringens* type D epsilon-toxin were cloned and expressed in inclusion bodies of *E. coli* BL21 cells and their immunogenicity were evaluated in the animal models. It was confirmed that the anti-recombinant epsilon antibody could successfully recognize the recombinant peptide as well as the native epsilon-toxin (Souza et al., 2010). In addition, in another study by Uppalapati antibody against their designed fusion protein was specifically against alpha-toxin (one part of the their designed fusion protein) and the fusion protein was studied using cross ELISA method (Uppalapati et al., 2012). The results of our study confirmed the high specificity of the recombinant fusion protein (ALE) for anti-epsilon and anti-alpha toxin antibodies, which makes it a proper candidate for future vaccine examinations against enterotoxaemia and the black disease.

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