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Harnessing an Integrative In Silico Approach to Engage Highly Immunogenic Peptides in an Antigen Design Against Epsilon Toxin (ETX) of *Clostridium perfringens*

Mahdieh Mahboobi[1] · Hamid Sedighian[1] · Ehsan Malekara[1] · Saeed Khalili[2] · Mohammad Reza Rahbar[3] · Kobra Ahmadi Zanoos[4] · Raheleh Halabian[1] · Abolfazl Jahangiri[1]

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Abstract

Epsilon toxin (ETX) is one of four lethal toxins of *Clostridium perfringens* produced by types B and D of the pathogen. This pore-forming toxin is one of the most potent bioterrorism agents with economic importance. Although an effective vaccine and an equine antitoxin are available to protect livestock against ETX, no approved vaccine or antitoxin is available for humans. In the current study, an integrative, simple, fast, and reliable approach is availed to design a safe and minimized construct based on preexisting experimental linear B cell epitopes. This guideline is designed based on the surface accessibility, flexibility, hydrophilicity, content of beta-turn structure, and antigenicity of potential epitopes. Experimental linear B-cell epitopes were analyzed with respect to antigenicity. Two antigenic regions were introduced based on the distribution of epitopes. Moreover, three constructs were designed based on top ranking epitopes. These constructs were evaluated for their antigenicity, surface accessibility, flexibility, hydrophilicity, and beta-turn content and were compared to the ETX sequence. The 114-aa construct with an antigenicity score of 0.8592 was the most stable, antigenic, hydrophilic, flexible, and surface accessible antigen within the ETX sequence, the selected regions, and the designed constructs. Although the performed in silico analyses revealed that the designed construct could serve as a safe antigen triggering highly reactive and neutralizing anti-ETX antibodies, it should be verified by experimental assays in future studies.

Keywords Vaccine · Epitope · In silico · Bioinformatics · Immunoinformatics

Introduction

Clostridium perfringens is an anaerobic, Gram-positive, rod-shaped, and spore-forming bacterium, which could produce up to 17 exotoxins (Schlatter et al. 2019; Shrestha et al. 2019). This pathogen is classified into five toxigenic types based on the production profile of four lethal toxins (alpha, beta, epsilon, and iota) (Rumah et al. 2017). *C. perfringens* types B and D produce the ETX, which belongs to the pore-forming toxins of the aerolysin family (Kang et al. 2017). ETX is a secreted pro-toxin (33-kDa) which is converted to an active form (28.6-kDa) via an enzymatic cleavage process. The active form of the enzyme is endowed with approximately > 1000-fold higher toxicity (Khalili et al. 2017). ETX could cause enterotoxemia in domestic ruminants (Yao et al. 2016). In addition to economic importance, ETX is one of the most potent bioterrorism agents classified in category B by the U.S Centers for Disease Control (CDC) (Koo et al. 2020).

Mahdieh Mahboobi and Hamid sedighian have contributed equally to this work.

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✉ Abolfazl Jahangiri
jahangiri.fazel@gmail.com; fazel.jahangiri@yahoo.com

[1] Applied Microbiology Research Center, System Biology and Poisoning Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St., P.O. Box 19395-5487, Tehran, Iran

[2] Department of Biology Sciences, Shahid Rajaee Teacher Training University, Tehran, Iran

[3] Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

[4] Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran

Contemporary, formalin-inactivated ETX is used as an effective vaccine to protect livestock (McClain and Cover 2007; Babele et al. 2020). Moreover, an equine antitoxin is available to be administrated for unvaccinated animals in enterotoxemia outbreaks (McClain and Cover 2007). However, to the best of our knowledge, no approved vaccine or antitoxin is available for humans. Cytotoxicity of this toxin is the main obstacle ahead of its approval for human administration. Hence, introducing a safe and highly antigenic immunogen is imperative to develop effective ETX-based vaccines and antitoxins. In this regard, rational design strategies such as 'antigen minimization' could play a pivotal role. This strategy relies on the preservation of appropriate immunogenic regions such as antigenic determinants and removal of junk regions (Jahangiri et al. 2018b, c). Therefore, localizing the position of existing B-cell epitopes is a critical step in the vaccine design procedure. Recently, Alves et al. have mapped the positions for linear B-cell epitopes of ETX type D. They have revealed that more than 40 overlapping and non-overlapping 15-meric linear B-cell epitopes are scattered throughout the ETX sequence with various reactivity (Alves et al. 2017). Although highly reactive epitopes could be considered as regions of interest for vaccine design, there is not a direct correlation between epitope reactivity and protectivity (Jahangiri et al. 2019). In the case of toxins, elicitation of neutralizing antibodies could confer protection against their toxicity (Cole et al. 2019). Therefore, accommodation of appropriate epitopes within the designed immunogen is pivotal to trigger neutralizing antibodies. In this regard, bioinformatics could be harnessed to select epitopes of interest (Mahboobi et al. 2017). Nowadays, bioinformatics tools are widely employed in various fields of biological researches such as vaccine design and development, structure and function prediction, and drug discovery (Moghaddam et al. 2017; Jahangiri et al. 2018a, b, c; Khalili et al. 2018; Sedighian et al. 2018; Moghadam et al. 2019; Rahbar et al. 2019a, b; Rezaie et al. 2019; Mahmoudi et al. 2020; Neshani et al. 2020; Payandeh et al. 2020; Rasooli et al. 2020; Sefidi-Heris et al. 2020). Harnessing bioinformatic tools turns to be inevitable in rational antigen design efforts; however, various available tools with different specificity and sensitivity encourage researchers to adapt integrative approaches in which tools are employed in consensus or combined manners. Recently, an integrative approach is introduced to design vaccines based on OMPs sequences (Jahangiri et al. 2018b, c). In this approach, exposed antigenic B-cell epitopes are assigned based on topology, antigenicity and linear B-cell epitope predictions. However, the suggested guideline is not suitable for globular antigens (Jahangiri et al. 2018b, c). This study is conducted aiming at the design of a novel, minimized, and safe antigen consisting of highly immunogenic peptides of ETX which are engaged by an innovative, simple, fast, and reliable approach.

Methods

Protein Sequence and Structure Retrieval

A reference sequence of ETX type D with entry identifier A0A140F7X0 was retrieved from the UniProt knowledge base at http://www.uniprot.org in FASTA format. FASTA format is a standard text-based format used in bioinformatics to represent protein and DNA sequences, in which amino acids or nucleotides are represented as single-letter codes (Jahangiri et al. 2018b, c). In this research, all analyses were carried out on this sequence.

Linear B Cell Epitope Predictions

Linear B-cell epitopes determined experimentally via epitope mapping (Alves et al. 2017) along with two neutralizing epitopes were considered (McClain and Cover 2007; Féraudet-Tarisse et al. 2017) as controls for B-cell epitope predictions. The antigenicity of these epitopes was examined by VaxiJen v2.0 at http://www.ddgapharmfac.net/vaxijen/VaxiJen/VaxiJen.html. VaxiJen is a free online alignment tool to predict the antigenicity of protein sequences with an accuracy of 80–90%; the VaxiJen approach is based on physicochemical properties of queried proteins (Doytchinova and Flower 2007).

In order to improve the prediction accuracy, linear B-cell epitopes of ETX were predicted by various servers including Ellipro (Ponomarenko et al. 2008), BepiPred (Larsen et al. 2006), BepiPred 2 (Jespersen et al. 2017), SVMTriP (Yao et al. 2012) and LBtope (Singh et al. 2013). The threshold and specificity of all servers were kept as default. All available different length options of the epitopes in SVMTrip were set for the ETX sequence. Predicted linear B-cell epitopes with a length of >8 amino acids (aa) were considered as positive results.

Physicochemical Properties

Some physicochemical properties that are important for B-cell epitope prediction (beta-turn, flexibility, surface accessibility, and hydrophilicity) were analyzed. The analyses were carried out by the tools (Chou and Fasman 1978; Emini et al. 1985; Karplus and Schulz 1985; Parker et al. 1986) available in IEDB at http://tools.iedb.org/bcell/.

Construct Designs

Two main approaches were considered in the selection and design strategies: (1) selection of appropriate regions as

subunit vaccine candidates; (2) engineering and design of novel immunogenic constructs.

Based on the location of the experimentally defined neutralizing and highly reactive epitopes, the immunogenic regions of ETX were selected. The criteria for selection were as follow: the region should be 110–150 aa in length, the region should contain at least one neutralizing epitope and it should include epitopes with experimental reactivity of > 3.

To design the construct, we have employed an integrated strategy to retain all experimental epitopes with the reactivity of ≥ 3, the antigenic (VaxiJen score of > 0.4) experimental epitopes with the reactivity of ≥ 1.5, and the neutralizing epitopes (Doytchinova and Flower 2007). If the length of the obtained construct failed to comply with the length criteria, the peptides with the lowest reactivity score were removed from the sequence. Another construct was derived from the first one. It was designed to only include the antigenic (VaxiJen score of > 0.4) experimental epitopes with the reactivity of ≥ 3 and the neutralizing ones.

Construct Analyses

The selected regions, as well as the designed construct, were analyzed with respect to epitope density, antigenicity, and physicochemical properties.

Some physicochemical properties (e.g. molecular weight, pI, etc.) of the selected regions and the designed constructs were analyzed by ProtParam at https://web.expasy.org/protparam/. The antigenicity of these sequences was estimated by VaxiJen. Flexibility, beta-turn, surface accessibility, and hydrophilicity of the sequences were predicted by tools available at IEDB. All previously employed B-cell epitope prediction tools were harnessed to retrieve the original B-cell epitopes within the construct sequences.

Data Validation

In the selection regions of interest and construct designs, an integrative method was harnessed to engage highly antigenic peptides of ETX experimentally validated as B-cell epitopes.

Moreover, to enhance the results accuracy, robust servers and tools with heavily cited articles were employed for performed predictions. Retrieval of original B-cell epitopes of ETX in the context of designed constructs was also carried out to enhance validity of the prediction.

Results

Linear B Cell Epitopes

Experimental linear B cell epitopes and their reactivity scores (Alves et al. 2017) along with VaxiJen scores are shown in Table 1. The minimum score was − 0.4297 assigned to "YDNVDTLIEKGRYNT" peptide and the maximum score was 1.3311 assigned to

Table 1 Experimental linear B cell epitopes and their VaxiJen scores

Experimental epitope Sequence	Position	Experimental Score (reactivity)	VaxiJen
YSIVNIVSPTNVIAK	19-33	1.5	-0.1275
IAKEISNTVSNEMSK	31-45	1.5	0.4971
KASYDNVDTLIEKGR	46-60	1.0	-0.2902
YDNVDTLIEKGRYNT	49-63	1.5	-0.4297
VDTLIEKGRYNTKYN	52-66	3	0.3261
LIEKGRYNTKYNYLK	55-69	4	0.1567
KGRYNTKYNYLKRME	58-72	4	0.6974
YNTKYNYLKRMEKYY	61-75	4	0.1485
KYNYLKRMEKYYPNA	64-78	3	0.0863
YLKRMEKYYPNAMAY	67-81	3	0.0597
RMEKYYPNAMAYFDK	70-84	3	-0.1225
KYYPNAMAYFDKVTI	73-87	3	-0.1585
PNAMAYFDKVTINPQ	76-90	1.5	0.2522
MNYLEDVYVGKALLT	109-123	2.5	-0.0112
LEDVYVGKALLTNDT	112-126	2.5	0.0555
GKALLTNDTQQEQKL	118-132	4	0.4896
QQEQKLKSQSFTSKN	126-141	1	1.1006
KSQSFTSKNTDTVTA	132-147	1	0.9681
SKNTDTVTATTTHTV	139-153	1	1.2898
TTTHTVGTSIQATAK	148-162	1.5	1.3311
HTVGTSIQATAKFTV	151-165	1.5	1.0805
GTSIQATAKFTVPFN	154-168	2	0.9296
LVPANTTVEVIAYLK	202-216	1.5	0.0263

Table 1 (continued)

ANTTVEVIAYLKKVN	205-219	2	0.1116
TVEVIAYLKKVNVKG	208-222	3	0.4768
VIAYLKKVNVKGNVK	211-225	3.5	0.6548
YLKKVNVKGNVKLVG	214-228	3.5	0.7942
KVNVKGNVKLVGQVS	217-231	1	0.7728
GSEWGEIPSYLAFPR	232-246	2	-0.1543
WGEIPSYLAFPRDGY	235-249	1.5	-0.2367
TVNKSDLNEDGTINI	256-270	5	0.6741
KSDLNEDGTININGK	259-273	5	0.9360
LNEDGTININGKGNY	262-276	5	0.9651
SAVMGDELIVKVRNL	277-291	2.5	0.5602
MGDELIVKVRNLNTN	280-294	2.5	0.8533
ELIVKVRNLNTNNVQ	283-297	3	0.7841
VKVRNLNTNNVQEYV	286-300	2.5	1.0025
RNLNTNNVQEYVIPV	289-303	2	0.7199
NTNNVQEYVIPVDKK	292-306	1	0.1436
DKKEKSNDSNIVKYR	304-318	3	1.2864
NDSNIVKYRSLSIKA	310-324	1	0.8373
NIVKYRSLSIKAPGI	313-327	1	0.6810
IVKYRSLSIKAPGIk	314-328	1.5	0.9704
SFANTNTNTNSK	179-190	Neutralizing	1.3230
LLTNDTQQ	121-128	Neutralizing	-0.0573

Underlined epitopes are those with the reactivity of > 2.5

Highlighted epitopes are antigenic epitopes (VaxiJen > 0.4) with reactivity of > 1.5

"TTTHTVGTSIQATAK". No direct correlation was found between the antigenicity score and reactivity score of the epitopes.

Ellipro predicted 7 epitope regions within the ETX sequence among which "RNLNTNNVQ" (position 257–265) was the best. BepiPred 1.0 assigned 10 peptides as linear B-cell epitopes. The highest scored residues were located within the "PVDKKEKSNDSN" peptide sequence at position 302–313. BepiPred 2.0 assigned 7 peptides as linear B-cell epitopes. SVMTriP determined 10 (12-meric), 7 (14-meric), 9 (16-meric), 7 (18-meric) and 5 (20-meric) epitopes. "KSLAIASAVISI", "NYSAVMGDELIVKV", "NYSAVMGDELIVKVRN", "NYSAVMGDELIVKVRNLN" and "PKVELDGEPSMNYLEDVYVG" were assigned as the highest scored 12, 14, 16, 18 and 20-meric epitopes respectively. Two peptides were predicted as epitopes by LBtope ("ELDGEPSMNYL" and "MGDELIVKVRNLNTNNVQEYV") (Fig. 1).

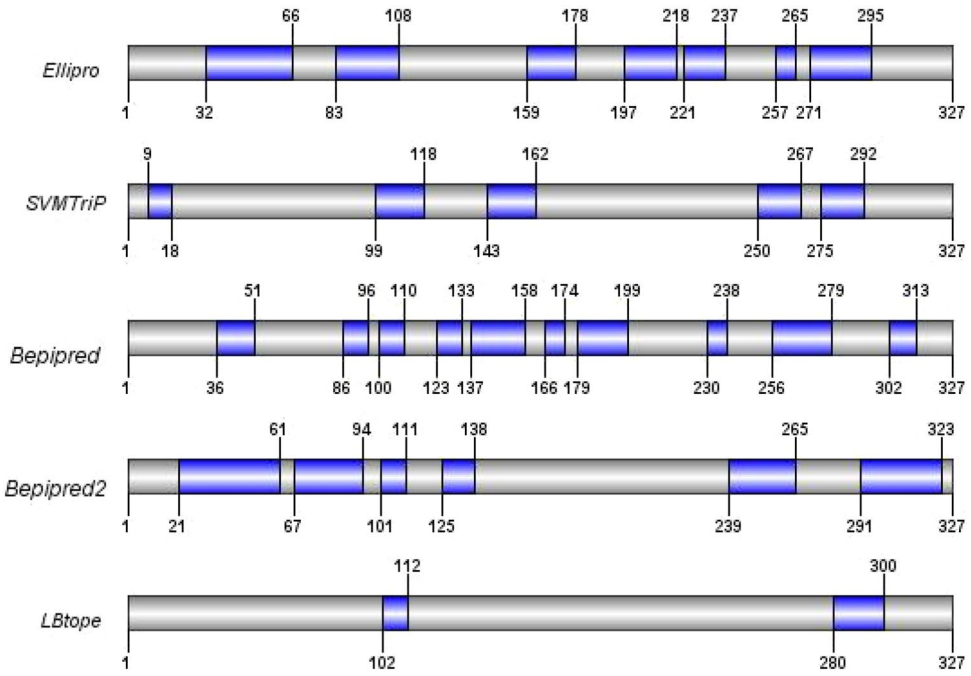
Physicochemical Properties

The average score of beta-turn for the ETX sequence was 1.017. I_{212} with 0.657 and G_{274} with 1.403 were respectively the residues with the minimum and maximum scores. The average flexibility score of the ETX sequence was 1.010. A_{80} (0.894) and Q_{128} (1.127) were the least and the most flexible residues, respectively. The average surface accessibility score of the ETX sequence was 1.000. S_{13} (0.091) and K_{306} (5.65) were the least and the most surface accessible residues respectively. The average hydrophilicity score of the ETX sequence was 2.123. Y_{241} (− 2.514) and N_{310} (7.214) were the least and the most hydrophilic residues, respectively.

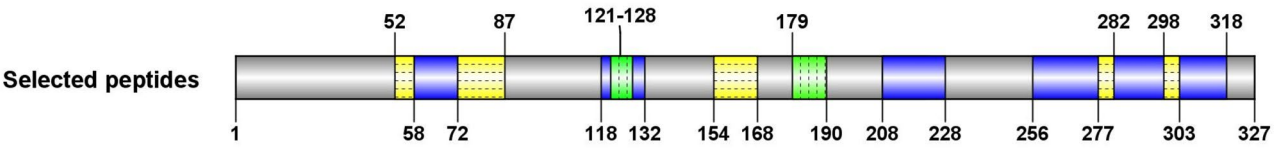
Selected Regions and Constructs

Among the 44 reactive peptides, 18 peptides had an experimental score of ≥ 3. Amongst, 9 peptides were predicted to be antigenic by VaxiJen. These peptides were the candidates to be elongated within the sequence. Moreover, the two neutralizing epitopes were also considered in this regard (Fig. 2).

Two regions (52–190 and 190–318) could be introduced as appropriate immunogenic regions containing highly reactive and neutralizing epitopes (Fig. 2). The first region, RETX-1, comprises 139 residues and the other one, RETX-2, is containing 140 residues. VaxiJen score of the V52-K190 and S179-R318 regions were predicted as 0.6757 and 0.6731, respectively. The highest reactive (with a score of 5) experimental epitope (256–276) was located in the second region. The V52-K190 region contained both neutralizing epitopes while the S179-R318 region harbors one neutralizing epitope. Table 2 shows the antigenicity and physicochemical properties of ETX, selected regions, and the designed constructs in detail. Details of flexibility, surface accessibility, hydrophilicity, and beta-turn secondary structure of these sequences are shown in Supplementary Fig. 1.



(a)



(b)

Fig. 1 Predicted epitopes by various tools

Fig. 2 Schematic illustration of the selected peptides and regions. **a** The selected regions; **b** the selected peptides. 121–128 and 179–190 are neutralizing epitopes, speckled regions are antigenic peptides with reactivity of 3 > peptide > 1.5 or non-antigenic peptides with reactivity of > 2.5

Discussion

Antigen minimization is an immunogen design approach in which key epitopes of the original antigen are retained while the rest regions are removed (Kulp and Schief 2013). This strategy could be used in toxin-based antigen designs aiming at eliminating the toxicity of the antigen. Moreover, this strategy could be employed to avoid non-protective and antibody-dependent enhancement (ADE) epitopes. ADE had been reported for in vitro cytotoxicity of *Bacillus anthracis* lethal toxin (Little et al. 2011). Among 73 monoclonal antibodies (mAbs) against the protective antigens, 17 mAbs enhanced cytotoxicity of lethal toxin at suboptimal concentrations (Little et al. 2011). Although the possibility of ADE is not studied for ETX, it could not be ruled out for occurrence of this phenomenon is neutralizing of this toxin. Linear epitopes of ETX type D have already been mapped on its sequence. The information offered by the epitope mapping could be used

Table 2 Antigenicity and physicochemical properties of ETX, selected regions, and the constructs

	ETX	Region 1	Region 2	Construct 1	Construct 1.1	Construct 2
Number of amino acids	327	139	140	162	147	114
Molecular weight	36191.78	15703.42	15452.26	18259.72	16695.94	12957.62
Theoretical pI	8.57	6.76	6.55	9.48	9.42	9.81
The instability index (II)	22.22	13.81	16.97	8.19	7.12	2.20
Aliphatic index	82.48	57.48	86.14	81.11	83.40	78.51
Grand average of hydropathicity (GRAVY):	− 0.442	− 0.740	− 0.536	− 0.635	− 0.714	− 1.012
VaxiJen score (antigenicity)	0.5955	0.6757	0.6731	0.6085	0.5867	0.8592
The average score of beta-turn	1.017	1.027	1.041	1.013	1.019	1.040
Average flexibility score	1.010	1.014	1.017	1.014	1.016	1.029
Percentage of surface accessible regions	30.58%	27.33%	31.42%	32.71%	34.01%	36.84%
Average hydrophilicity score	2.123	2.449	2.308	2.278	2.359	2.787

to design a non-toxic ETX-based antigen. Previous studies have revealed that the denatured antigens could elicit protective antibodies against the corresponding pathogens (Toobak et al. 2013; Jahangiri et al. 2019). These antibodies are attributed to linear B-cell epitopes which could be recognized in native and denatured forms of the antigens (Toobak et al. 2013; Jahangiri et al. 2019). Since keeping the native protein structure is suggested to be dispensable for linear B-cell epitope recognition, the immunogenic antigens based on these epitopes could be designed without artificial linkers (Jahangiri et al. 2018b, c). Such an antigen design strategy in which the epitopes are tandemly fused could be employed for the design of minimized antigens with high epitope density. To integrate antigen minimization and incorporation of maximum numbers of immunogenic epitopes within the construct, length of 110–150 aa was considered. This size also ensures immunogenicity of the designed antigen in regard of molecular weight (> 10,000 Da). Linear B-cell epitope prediction was performed by various servers to compare the prediction results with the results obtained from the experimental epitope mapping. None of the employed B-cell epitope predictors assigned all of the experimentally approved epitopes. These results imply that harnessing one or two prediction tools alone is not sufficient for highly accurate and successful B-cell epitope predictions. In this study, linear B-cell epitope predictors were not quite successful to predict the majority of highly reactive epitopes in a consensus manner. Moreover, only Bepipred predicted neutralizing epitopes of which 121–128 region was partially assigned. Although, the employed B-cell epitope predictors are among powerful tools, they performed unreliable in case of ETX. Bepipred, Ellipro, and Bepipred2 were more successful in recognizing top-ranking (score > 3.5) experimental epitopes. However, Ellipro needs a PDB ID or a PDB file as an input for B-cell epitope prediction. The neutralizing and top-ranking (score > 3.5) experimental epitopes were not successfully predicted by LBtope and SVMTrip; in the light of these observations, LBtope and SVMTrip tools along with Ellipro were ignored for B-cell epitope retrieval analyses of the designed construct. Two out of four regions with highest surface accessibility score (58–76, 124–133, 183–192 and 304–310) are located in both neutralizing epitopes. The experimental reactivity score of the 58–75 region is 4 (Alves et al. 2017). The 304–310 residues are involved in an epitope with an experimental reactivity score of 3. Hence, surface accessibility prediction could be considered as an appropriate criterion to select protective B-cell epitopes. In addition, the majority of the residues of the neutralizing epitopes are among the four hydrophilic and flexible regions with highest scores. One of these neutralizing epitopes (179–190) forms a beta-turn secondary structure. The majority of the experimentally defined epitopes with highest reactivity score are hydrophilic and flexible regions with beta-turn secondary structure. Hence, the predicted physicochemical parameters could be used in the design of an antigenic construct. The first construct that was designed based on all highly reactive epitopes (> 2.5) as well as moderately reactive antigenic ones (3 > epitope > 1.5) did not meet the allowed sequence length criteria. Moreover, this construct was not superior to the selected regions' in terms of antigenicity, flexibility, hydrophilicity, and beta-turn structure. Removal of "GTSIQATAKFTVPFN" resulted in increased stability, surface accessibility, flexibility, hydrophilicity, and beta-turn content of the construct. However, the obtained scores were not satisfactory to be considered as an optimized construct design. Moreover, the antigenicity score of the construct was reduced to the lowest score among the antigenic regions, constructs, and ETX sequences. The reduced antigenicity could be attributed to the non-antigenic epitopes embedded within the construct. Hence, in order to minimize the antigen and improve its antigenicity, a stringent approach

was followed in which non-overlapping residues of non-antigenic epitopes and antigenic epitopes with moderate reactivity (3 > epitope > 1.5) were trimmed from the construct. Among the selected regions, designed constructs, and the full-length ETX, the designed construct was associated with the minimized sequence length with the highest antigenicity, stability and physicochemical property scores involved in B-cell epitope assignment. Since the pore-forming activity of ETX highly relies on the structure of the toxin and the interaction of its monomers, this function is expected to be hampered in the minimized construct. This property would render the designed construct as safe with respect to cytotoxicity.

Based on the obtained results, surface accessibility, flexibility, hydrophilicity, and beta-turn structure of a given protein sequence along with antigenicity of overlapping peptides are reliable criteria to engage highly immunogenic peptides in design of an antigen with minimized length against ETX. Although the current in silico study demonstrated that the designed construct is a safe antigen triggering highly reactive and neutralizing anti-ETX antibodies, it should be verified by experimental assays in future studies.

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Compliance with Ethical Standards

Competing interest The authors declare no conflict of interest.

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