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ORIGINAL ARTICLE

Bioinformatic Analysis of the Structural Features of the H106P/N137A Mutant as a Potential Vaccine Candidate Against *Clostridium perfringens*

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SUMMARY

Background: **The complex and expensive processes involved in the production of current clostridial toxoid vaccines require the design of new methods. The aim of this study was to introduce single and combined nontoxic mutants of *Clostridium perfringens* epsilon toxin (ETX) into the vaccine production.**
Methods: **Antigenic properties, amino acid sequence, physicochemical characteristics, stability, and protein structure of individual and combined ETX mutants were investigated with bioinformatics software. Spatial structure prediction was done by using a homologous modeling method based on the structure of wild type ETX (1UYJ1A.pdb) and the validity of the drawn spatial model was evaluated by estimating the quality and accuracy of the spatial chemistry of the modeled mutant proteins by Ramachandran plot.**
Results: **The highest instability index was observed in $H_{106}P/N_{137}A$ mutant. The results of the homology modeling did not show a clear structural change in any of the mutants compared to the wild ETX. The percentages of amino acids in the favored regions, allowed regions, and outlier regions were 92.81%, 5.03%, and 2.16%, respectively, which indicate the desirability of the proposed model for the three-dimensional structure of the $H_{106}P/N_{137}A$ hybrid mutant.**
Conclusions: **Recombinant $H_{106}P/N_{137}A$ mutant of ETX can be considered a suitable candidate for the production of epsilon genetic toxoid, and this requires extensive laboratory evaluations.**
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KEYWORDS

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INTRODUCTION

Clostridium perfringens (*C. perfringens*), an anaerobic and spore-forming bacillus, is classified into seven toxinotypes: A, B, C, D, E, F, and G. This classification is based on the production of one or more of six exotoxins. The primary exotoxins include alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), and necrotic enteritis B-like (NetB) [1]. Type D strains are known to produce CPA and ETX, two potent toxins, with ETX being the predominant virulence factor within this toxinotype. Experimental research using various animal models has successfully fulfilled molecular Koch postulates, confirming the role of ETX in the pathogenicity of Type D strains. ETX is generated during the exponential growth phase of the bacterium, in the form of a protoxin, which is activated by trypsin, α-chymotrypsin, and λ-protease secreted by the bacterium itself. Once activated, the toxin exerts its toxic effects [2,3]; the activated protein is a deadly toxin that can cause skin necrosis, edema, cerebral swelling, and death [4].

One of the main challenges for livestock farmers is preventing enterotoxemia in ruminant livestock. The current clostridial vaccines are in the form of toxoids, which require complex, time-consuming, and costly production processes [5]. One alternative method is to produce genetic toxoids [6]. For the first time, researchers produced mutant toxoids from ETX by performing a direct mutation in the ETX coding sequence of *C. perfringens*. They caused the substitution of an amino acid in three different positions of the ETX by creating mutation, and the best ETX mutant was histidine 106 (H_{106}) substituted with proline, which created suitable protective immunity in mice [7]. Researchers reported the H_{106}P mutant of ETX on the surface of *Lactobacillus casei* using the PT1NX vector and successfully confirmed its non-toxicity and immunogenicity [6]. Moreover, the H_{149}A mutation of *C. perfringens* ETX has been examined as a platform for receptor binding studies [8]. The utilization of a combined mutant model (Y_{30}A-Y_{196}A) has resulted in a more effective immune response, and this type of mutant may be a promising candidate for an enterotoxaemia vaccine [9].

Additionally, another combined mutant (Y_{30}A-Y_{196}A-A_{168}F) has been proposed as a vaccine candidate by simultaneously performing multiple mutations on the ETX [10]. The identification of crucial amino acids in the ETX, that act as receptors in its attachment to the host cells (Y_{29}, Y_{30}, Y_{36}, and Y_{196}), has also been confirmed [11]. Studies have demonstrated that tyrosine 71 (Y_{71}) is an important amino acid for the toxin's activity against MDCK cells [12]. Moreover, the non-toxic Y_{71}A mutant has the ability to create protective immunity in mice and sheep [13]. The non-combined mutant Y_{196}E (rEtx-Y_{196}E-C) has, due to the evaluation of its immunogenicity characteristics [14], along with the F_{199}E mutant of ETX, also been suggested as potential vaccine candidates against enterotoxemia [15].

Utilizing bioinformatics techniques to generate non-toxic, non-combined mutants from epsilon toxin has the potential to achieve significant goals in the development of new *Clostridial* vaccines. In this particular study, we concentrated on single mutations, namely H_{106}P and N_{137}A, as well as a combined mutation, H_{106}P/N_{137}A, in the epsilon toxin of *C. perfringens*. The structural antigenic properties, amino acid sequences, physicochemical characteristics, stability, and secondary and tertiary protein structures of these mutants were analyzed by using cutting-edge bioinformatics software tools.

MATERIALS AND METHODS

Nucleotide sequence retrieval

To obtain the nucleotide sequence, the researchers retrieved the ETX gene sequence with the accession number AY858558 from the NCBI database (http://ncbi.nlm.nih.gov) for use in their bioinformatics studies.

Design and selection of epsilon mutants

From previous research and findings, it has been established that H_{106} plays a crucial role in the toxicity of ETX. Point mutations at this amino acid site, such as its conversion to proline, have been shown to be significant in creating a non-toxic ETX mutant [7]. Additionally, the amino acid N_{137}, which has a cross-link to H106 in adjacent sequences, plays an important role in the formation of the three-dimensional structure of ETX. Thus, these two mutants were chosen for bioinformatics analyses, both individually and in combination, to determine their respective advantages, disadvantages, and potential usefulness in the genetic toxoid production of ETX.

Analysis of the structure of ETX mutants

The primary structure analysis of the H_{106}P, N_{137}A, and H_{106}P/N_{137}A mutants of ETX involved predicting various physicochemical parameters such as molecular weight, theoretical isoelectric point (pI), total number of positive and negative amino acids, half-life, instability index, aliphatic index, and average hydrophobicity. This was accomplished through the use of online tools such as ProtParam and the Expasy web portal (http://expasy.org/tools/protparam.html). The protein solubility of each ETX mutant during expression in *E. coli* was determined by using the Oklahoma web portal (http://biotech.ou.edu/). To design and optimize the secondary protein structures of the mutants, including alpha helices and beta sheets, the PSIPRED web portal (http://bioinf.cs.ucl.ac.uk/psipred/) was utilized. For the evaluation and prediction of the tertiary protein structures of the ETX mutants, the Phyre2 online program was used. The complete sequence of each mutated protein was obtained, and several templates were selected for modeling the ETX mutants based on maximum confidence, detection percentage, and alignment coverage.

Prediction of the spatial structure of ETX mutants using the homology modeling method

To carry out comparative or homology modeling, the three-dimensional structures of each ETX mutant ($H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$) were predicted by utilizing the crystal structure of wild-type epsilon toxin (1UYJ1A.pdb) as a template. This was accomplished through the utilization of the SWISS-MODEL workspace and Expasy web portal (http://swissmodel.expasy.org). A sample search was performed by using Blast and HHblits in the SWISS-MODEL model archive. The workflow for this study consisted of several steps, including pattern search, model building, quality assessment, ligand modeling, and preservation of the oligomeric structure. To initiate the process, the target sequence was searched against the primary amino acid sequence in SMTL by using BLAST, which led to the identification of three templates. A HHblits profile was generated by using the HH-suite method for the initial profile searching, followed by one iteration of HHblits in Uniclust30. The resulting profile was then utilized to search all SMTL profiles to identify potential patterns. Subsequently, models were built based on the template-target alignment using ProMod3. Finally, the quality of the models was evaluated, and the oligomeric structure was preserved [16]. The conserved regions between the target and template were reconstructed by implementing a fragment reconstruction library, which involved making necessary insertions and deletions to the model. Subsequently, the side chains were rebuilt before optimizing the resulting model geometry via a force field. The quality of the model for each amino acid residue and its overall quality were assessed by using the Qualitative Model Energy Analysis (QMEAN) scoring process [17].

To transfer the ligands from the template structure to the model, homology modeling was used and relevant indices were obtained. The fourth structure interpretation of the template was utilized to model the oligomeric shape of the target sequence in order to estimate the conservation of the oligomeric structure. This method is based on a supervised machine learning algorithm called Support Vector Machines (SVM) that combines conserved connections, structural clustering, and other aspects of the template to calculate the Quaternary Structure Quality Estimate (QSQE) score. The QSQE score is a numerical value ranging from 0 to 1, which indicates the expected accuracy of inter-chain connections in a model built from the template alignment. Higher scores indicate a greater reliability. In addition, the QSQE score complements the Global Model Quality Estimation (GMQE) score, which estimates the accuracy of the third structure of a model. Finally, homology modeling was utilized to design the three-dimensional structure of each mutant based on the crystal structure of wild-type ETX (1uyj.1.A.pdb).

Validation and quality assessment of the depicted spatial model of ETX mutants

In order to evaluate the accuracy and quality of the homology models generated for the $H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$ mutants, a Ramachandran plot was utilized to estimate the spatial chemistry of the ETX mutants. Typically, this plot is drawn after predicting the third structure of a protein in order to assess the structural parameters of both the template and the target proteins, thus indicating the allowable regions for each angle within protein structures. Each amino acid is assigned to a set of two angle values, and like fingerprints, no two amino acids have the exact same role.

By using the PROCHECK web portal (https://www.ebi.ac.uk/thorntonsrv/software/PROCHECK), the optimized stoichiometric results of each ETX mutant were evaluated by plotting a Ramachandran diagram [5].

RESULTS

Primary sequence analysis of ETX mutants

An analysis of the primary structure of ETX mutants, $H_{106}P$, $N_{137}A$, and the combined mutant $H_{106}P/N_{137}A$, was conducted. Physiochemical parameters were predicted by using ProtParam, an online software. The results revealed no significant differences between the examined mutants and the wild-type ETX in terms of their primary analyses. All the ETX mutants shared an isoelectric point of 8.75, which is similar to that of the wild-type ETX. The absorbance coefficient at 280 nanometers was determined to be 32,320 for all examined mutants. However, the instability index of the mutants was higher than that of the wild-type ETX (93.21), with the combined mutant $H_{106}P/N_{137}A$ displaying the highest value (42.22). The ProtParam analysis classified the ETX mutants as unstable proteins based on their instability index, which for stable proteins is typically less than 40.

The aliphatic index (AI) was measured for all ETX mutants and was found to be above 75. This index reflects the mutants' stability over a wide temperature range. Each of the $H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$ mutants had an AI of 23.82, 53.82, and 53.82, respectively. The primary structure analysis of the ETX mutants indicated their stability with a half-life of over 10 hours under laboratory conditions in *E. coli*, and all the examined mutants showed 100% solubility during expression in *E. coli* at an unusually high rate. The calculated grand average of hydropathicity (GRAVY) for the mutants was approximately -0.5. GRAVY value is determined by dividing the sum of the hydropathicity values of all amino acids by the number of amino acid residues in the protein sequence. The negative values of GRAVY calculated for all the examined mutants indicate their non-polarity.

Secondary and tertiary protein structure of ETX mutants

The PSIPRED bioinformatics server was used to improve the secondary structure, including alpha helices

Table 1. Secondary structure evaluation results of the epsilon toxin mutants using Phyre2 online program.

Mutant	$H_{106}P/N_{137}A$	$H_{106}P$	$N_{137}A$
Number of identified patterns based on sequence alignment	68	68	68
Top pattern	d1uyja	d1uyja	d1uyja
Maximum confidence (%)	100	100	100
Detection percentage (%)	99	100	100
Overlap level (%)	80	81	80
Number of amino acids similar to the pattern	272	272	272

Table 2. Homology modeling results of $H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$ mutants of the epsilon toxin from *Clostridium perfringens* using the Expasy website.

Mutant	$H_{106}P/N_{137}A$	$H_{106}P$	$N_{137}A$
Number of identified patterns	89	88	91
Number of filtered patterns	22	22	22
Top pattern	1uyj.1. A	1uyj.1. A	1uyj.1. A
Sequence identity similarity	99.32	99.66	99.66
Oligo-state	homo-trimer	homo-trimer	homo-trimer
GMQE of top pattern	0.81	0.82	0.82
QSQE of top pattern	0.46	0.47	0.45
Methods	X-ray, 2.6Å	X-ray, 2.6Å	X-ray, 2.6Å
Search	Blast	Blast	Blast
Seq similarity	0.61	0.61	0.61
GMQE model	0.74	0.70	0.70
QMEAN model	-1.19	-1.22	-1.03

and beta sheets, of the $H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$ mutants based on their amino acid sequences. The Phyre2 online program was utilized to evaluate and predict the tertiary protein structure of the ETX mutants. Domain analysis was performed after predicting the secondary structure and its disturbances, and the resulting patterns were determined based on sequence alignment overlap, maximum confidence discovery, and detection percentage (Table 1). Twenty top models were designed for each mutant's three-dimensional structure, ranked based on the initial score of the sequence and secondary structure similarities, as well as insertions and deletions. The best model was selected, and its three-dimensional structure was drawn for each mutant (Figure 1). The performance and quality of the top model were evaluated by using Phyre2 Investigator, which indicated that the $H_{106}P$ mutation (histidine to proline) is a significant mutation leading to local structural changes in ETX. We previously, through *in vitro* and *in vivo* evaluations, verified that $H_{106}P$ is a non-toxic mutant [6].

Homological modeling of ETX mutants

The SWISS-MODEL Template Library (SMTL version 02-17-2021, PDB release 02-12-2021) was utilized with Blast and HHblits to identify structures related to the amino acid sequence of each of the $H_{106}P$, $N_{137}A$, and $H_{106}P/N_{137}A$ mutants. The top model, which was a homologous sequence, was selected, and its protein structure was visualized (Table 2). Local structural changes were detected in the $H_{106}P$ mutant compared to the wild-type ETX, while no significant structural changes were observed in the $N_{137}A$ mutant compared to the wild-type ETX. When evaluated from different angles, the three-dimensional structures designed for each of the ETX mutants using homology modeling showed no significant differences from the wild-type ETX (Figure 2).

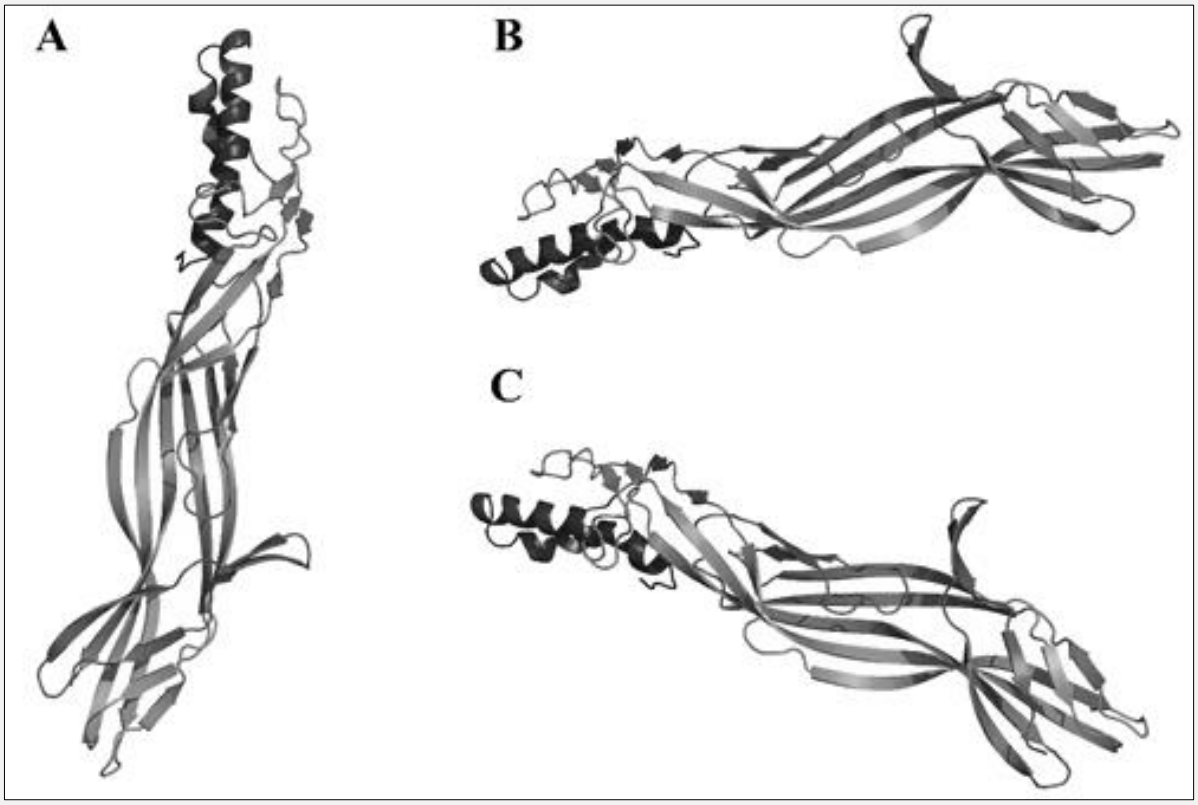

Figure 1. Three-dimensional (3D) model designed for $H_{106}P$ (A), $N_{137}A$ (B), and $H_{106}P/N_{137}A$ (C) mutants of the epsilon toxin from *Clostridium perfringens* based on the sequence alignment with the top pattern (d1uyja).

Validation and evaluation of predictive models for ETX mutants

The stereochemical quality and accuracy of the predicted models for each of the ETX mutants in *C. perfringens* were assessed by using Ramachandran plot. The analysis of the Ramachandran plot for the $N_{137}A$ mutant revealed that 93.50% of the amino acids were in the favored region, 4.69% were in the allowed region, and 1.81% were in the disallowed region. For the $H_{106}P/N_{137}A$ and $H_{106}P$ mutants, the percentages of amino acids in the favored, allowed, and disallowed regions were found to be 92.81%, 3.57%, and 16.20% and 81.92%, 5.03%, and 16.24% in the desired region, respectively. The sum of the percentages of the amino acids in the desired and allowed regions was determined to be 97.84% for the $H_{106}P/N_{137}A$ mutant, indicating suitability of the proposed model for the three-dimensional structure of the combined $H_{106}P/N_{137}A$ mutant of ETX from *C. perfringens* (Figure 3).

DISCUSSION

Enterotoxemia is a disease that has spread globally among sheep and goats and that carries significant economic importance due to its high mortality rate of up to 30% in herds. *Clostridial* toxoid vaccine production can be an expensive and time-consuming process, which is why producing non-toxic recombinant vaccines is a crucial alternative. Achieving this goal requires a deep understanding of the precise structure of ETX as well as the structural and functional properties of each constituent amino acid through bioinformatic and laboratory evaluations. Extensive studies have been conducted on cloning, expression, and analyzing the properties of ETX. Domain identification, critical amino acids, and the design of various ETX mutants have also been explored in this regard [18,19].

Tryptophan and tyrosine amino acids are crucial for epsilon toxin's binding to target cells. Furthermore, histidine amino acids at positions 106 and 149 are necessary for the toxin's structure and activity [20]. Researchers have introduced mutations by altering amino acids

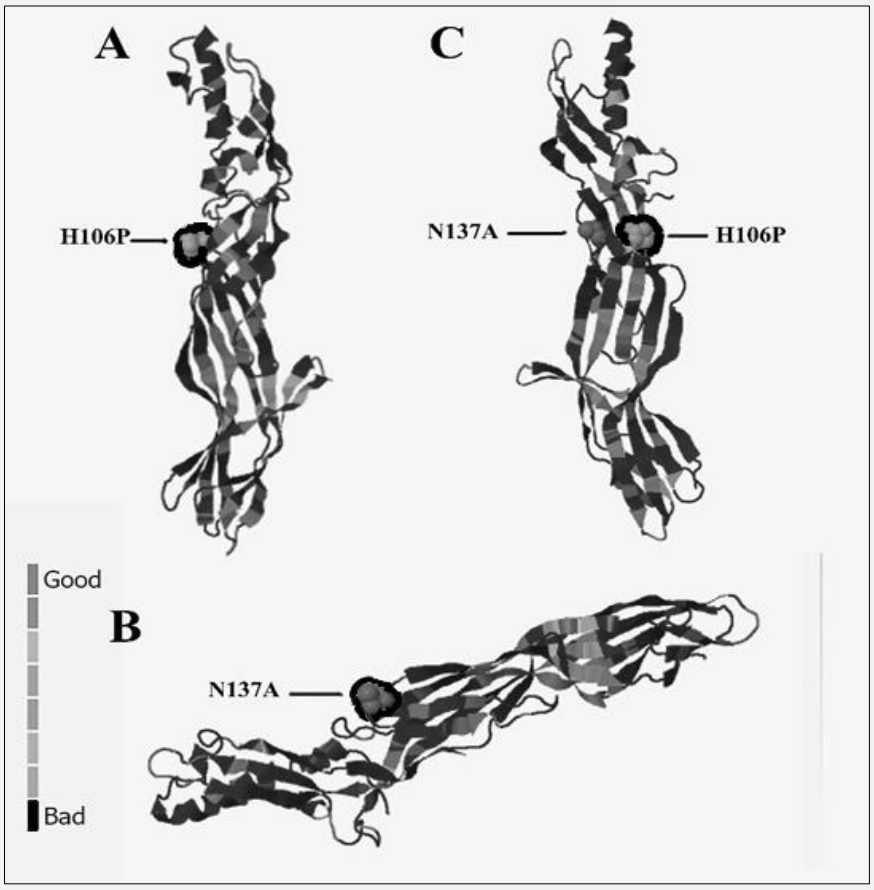

Figure 2. Homology modeling performed for $H_{106}P$ (A), $N_{137}A$ (B), and $H_{106}P/N_{137}A$ (C) mutants of the epsilon toxin from *Clostridium perfringens*.

The location of the mutated amino acid in each mutant has been identified.

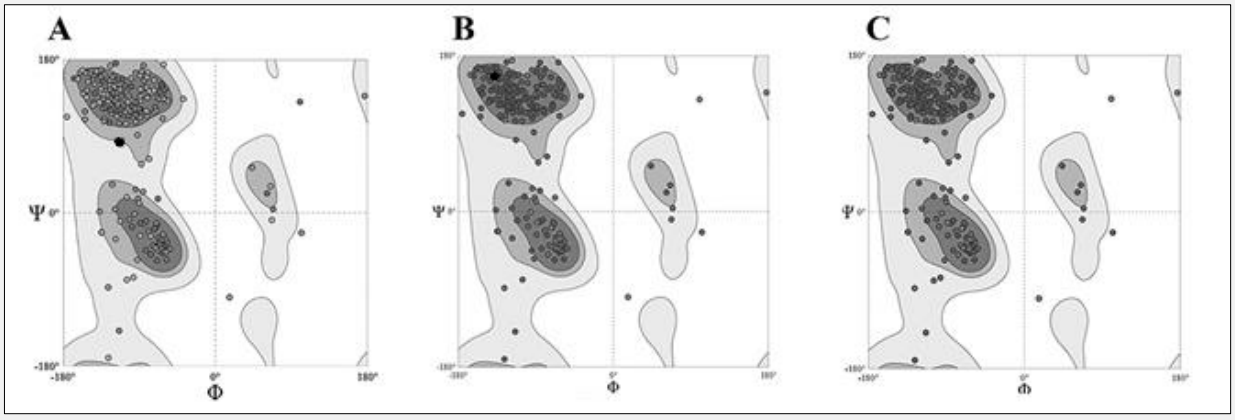

Figure 3. The Ramachandran map of $H_{106}P$ (A), $N_{137}A$ (B), and $H_{106}P/N_{137}A$ (C) mutants of the epsilon toxin from *Clostridium perfringens*.

in three distinct positions of the ETX (histidine 106, histidine 149, and tryptophan 190). To achieve this, histidine amino acids at positions 106 and 149 were substituted with serine, proline, or alanine, while tryptophan 190 was replaced with tyrosine, phenylalanine, or leucine [21]. The present study demonstrated that H_{106} is indispensable for the biological activity of the toxin, whereas histidine 149 and tryptophan 190 play a role in maintaining the protein structure and are not involved in the biological activity of the toxin. The most effective ETX mutant identified in this study was the substitution of H_{106} with proline ($H_{106}P$). This mutation conferred a good protective immunity in mice against 1,000 MLD of wild-type ETX and was also verified to be non-toxic in mice [22,23]. In another investigation, it was found that the substitution of H_{106} with proline results in a fundamental change in the protein, but its activity and secretion remain preserved [7]. Mutants $W_{190}K$ and $H_{149}P$, similar to the non-toxic mutant $H_{106}P$, are also non-toxic. However, the instability of mutant $W_{190}K$ and the inability of mutant $H_{149}P$ to be secreted into the periplasmic space make these mutants less efficient candidates for selection in the genetic toxoid production [8, 20]. Therefore, based on the patterns and findings of previous studies, H_{106} amino acid was selected as a suitable candidate for bioinformatic evaluations in this study. H_{106} plays a critical role in the creation of a non-toxic epsilon mutant, which is known as an epsilon genetic toxoid. It is previously confirmed by Alimolaei et al. that $H_{106}P$ mutant is able to induce both cellular and humoral immune responses through T and B cells, respectively [6].

In addition, amino acid N_{137} was also considered as a candidate for creating a non-toxic epsilon mutant. This is because N_{137} forms a cross-link to H_{106} in the adjacent chains during the formation of the ETX heptamer pore on the cell wall. However, single mutants pose a significant risk of complete toxin activity reinstatement due to only one codon change. Therefore, vaccine production should consider at least two mutations. Two single mutants, $H_{106}P$ and $N_{137}A$, along with a double mutant, $H_{106}P/N_{137}A$, were selected for bioinformatic analyses to investigate their potential advantages and disadvantages in producing genetic ETX toxoids. The results of the bioinformatic analyses demonstrated that substituting amino acids in the selected positions causes local structural changes in ETX, which enables a satisfactory expression and secretion of the mutant protein into the periplasmic space of bacteria. It should be noted that if a single mutation is used, there is a possibility of mutation reversal, as the toxin´s toxicity may be lost. Therefore, it is recommended that, similar to this study, two mutants be simultaneously targeted to reduce the likelihood of mutation reversal. On the other hand, the response obtained from the results of the bioinformatics research should be further investigated and confirmed in more extensive laboratory and *in vitro* studies.

CONCLUSION

Based on the results of bioinformatic studies, the combined mutant $H_{106}P/N_{137}A$ exhibited superior physicochemical properties compared to other mutants. Homology modeling evaluations did not reveal any significant structural changes in any of the mutants, when compared to wild-type ETX. The Ramachandran map analysis of the $H_{106}P/N_{137}A$ mutant suggests that the proposed model is suitable for the three-dimensional structure of the $H_{106}P/N_{137}A$ combined mutant related to the ETX of *C. perfringens*. The non-toxicity of the $H_{106}P$ mutant was previously confirmed by us, and our results showed that it can stimulate the humoral and cellular immune responses [6], where the protective immune responses were confirmed by challenge of the immunized mice. So, the non-toxic combined mutant of ETX ($H_{106}P/N_{137}A$) can be considered a desirable candidate for genetic toxoid production, although proving this assertion requires laboratory evaluations at a broader level.

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Declaration of Interest:
The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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