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In silico Analysis of Non-Synonymous Single Nucleotide Polymorphisms in α-Toxin of *Clostridium perfringens* Toxinotype B Isolated from Lamb Dysentery Cases in Pakistan

Mian Muhammad Khubaib Sattar[1,4], Aftab Ahmad Anjum[1]*, Yung Fu Chang[2], Tahir Yaqub[1] and Asim Aslam[3]

[1]*Institute of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan.*
[2]*Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, 14853 Ithaca, New York, United States of America.*
[3]*Department of Pathology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan.*
[4]*Department of Microbiology, Faculty of Veterinary and Animal Science, The Islamia University of Bahawalpur, 63100 Bahawalpur, Pakistan.*

ABSTRACT

This study delves into the *in-silico* analysis of non-synonymous single nucleotide polymorphisms (SNPs) in the α-toxin of *Clostridium perfringens* toxinotype B strains causing lamb dysentery and provide insights for future therapeutic interventions. The SNP annotations / mutations at T4S, G31V, E37A, Y39T, A60S, T62I and A83S obstructed the normal function of the α-toxin as indicated by PolyPhen-2 PhDSNP, PROVEAN, SIFT, MUpro and I-MUTANT. In terms of polarity, 40% amino acids are polar, 33% small non polar and 11% hydrophobic. Whereas, 32.26% amino acids were intermediately accessible to solvents. I-TASSER analysis indicated a secondary structure composition with 59.14% helix, 36.56% coil, and 4.30% strand, while PSIPRED showed a distribution of 64.52% helix and 35.48% coil. The 3D model had C-score 0.11 with estimated TM score 0.73±0.11. The α-toxin exhibited a binding site for Calcium with the highest C-score 0.21 followed by binding site for 1,3-dihydroxypropan-2-yl octadecenoate (2JT), Nucleic Acids (NUC) and Tribromomethane (MBR). I-TASSER GO terms indicated that the molecular functions of the toxin include binding to ions, vitamin B6, carboxylic acid, D- or L-enantiomers of glucose, and adenosine 5'-triphosphate as well as catalysis of phospholipids, redox reaction of 2-oxoglutarate and pyrophosphate bond hydrolysis. The α-toxin is also predicted to be involve in several biological processes such as maintenance of an internal metal ions balance, pathways involving weakly basic organic compounds, chemical reactions with carboxylic acid, mammary gland development over time, reproductive function of multicellular organisms, the directed movement of glucose into a cell or organelle, regulated fluid release by cells, mitochondrial morphogenesis and the regulation of glucose transport across membranes. InterProScan GO terms indicated hydrolase, action on ester bonds, phospholipase C activity and zinc ion binding as prominent molecular functions of α-toxin. These findings underscore the importance of computational approaches in elucidating the molecular basis of pathogenesis and guiding preventive strategies against this infectious disease.

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MMKS conducted the research and written the initial draft while AAA, YFC, TY and AA (members of supervisory committee) evaluated the results the edited the final version of the manuscript.

Key words
Annotations, Bioinformatics, *Clostridium perfringens* Toxinotype B, *In silico*, I-TASSER, PSIPRED

INTRODUCTION

Clostridium perfringens is a Gram-positive, anaerobe recognized as one of the major etiological agents of infectious diseases in both humans and animals (Fu *et al*., 2022). Among its numerous toxins, α-toxin (also known as phospholipase C or CPA) has been identified as a pivotal virulence factor in *C. perfringens* infections (Camargo *et al*., 2024). Lamb dysentery, a severe enteric disease primarily affecting young lambs, is caused by *C. perfringens* toxinotype B strains carrying the NetB toxin

* Corresponding author: aftab.anjum@uvas.edu.pk
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gene, in addition to α-toxin (Mehdizadeh-Gohari *et al*., 2021). In *C. perfringens* there is a two component (VirR/VirS) system which regulate the expression of many toxin genes including α-toxin (plc) and various other virulence factors (Shimizu *et al*., 2002).

Single nucleotide polymorphisms (SNPs) are communal genetic variations which arise when a single nucleotide is altered in the DNA sequence. Particularly, non-synonymous single nucleotide polymorphisms (NS-SNPs) results in change of the encoded protein by exchange of amino acids which potentially change the protein structure and its function along with pathogenicity (Al-Shuhaib, 2024). Considering the impact of non-synonymous SNP's on the α-toxin of *C. perfringens* toxinotype B associated with lamb dysentery is crucial for explicating the molecular basis of the pathogenesis and devising effective preventive and therapeutic interventions.

In silico analysis, now a days, has appeared as a valued mean for discovering the functional magnitudes of genetic variations without executing the essential strenuous experimental procedures. By using detailed computational drills, the probable effects of missense mutations on function and structure of protein is possibly projected which provide substantial insights into the phenotypical variations and pathogenic mechanism of different strains (Gerasimavicius *et al*., 2020).

This research focuses on the *in-silico* analysis of NS-SNPs in the α-toxin of *C. perfringens* toxinotypes B strains specifically those isolated from lamb dysentery cases. By employing advanced bioinformatics tools and databases, the study aims to evaluate how these SNPs may affect the structure, stability and enzymatic activity of the α-toxin. Furthermore, the research seeks to investigate the link between specific SNPs and the molecular function and biological processes involving the α-toxin ultimately providing deeper insights into the genetic factors contributing to its pathogenesis. The findings from this *in silico* analysis lay the groundwork for future experimental research and could contribute to the development of new therapeutic strategies to reduce the impact of this disease.

MATERIALS AND METHODS

The α-toxin (*cpa*) gene of *Clostridium perfringens* toxinotype B was amplified (Sattar *et al*., 2023) (Supplementary File). Non-synonymous SNPs were selected for application of bioinformatics tools. Using a range of bioinformatics tools allows for a comprehensive analysis of the NS-SNPs. Various tools have diverse algorithms and approaches that help in cross validation of results and ensure robustness in the assessment. Specific algorithms are designed to evaluate how amino acids altering SNPs affect protein structure and functions.

Predicting impact on α-toxin function

Various tools such as PolyPhen 2, Phd SNP, PROVEN, SIFT were used to prediet impact on α-toxin functions. PolyPhen2 proceeds with an inexperienced Bayesian classifier to foresee the functional impact of alleles using structural and functional characteristics. It involves sequence alignment, feature extraction, machine learning model training, and prediction. The PolyPhen2 score ranges from 0 to 1, representing the probability of a mutation being probably damaging, possibly damaging or benign (Adzhubei *et al*., 2010; Zhao *et al*., 2023).

PhD-SNP is a web-based tool utilized for annotating non-synonymous SNPs. The approach ensured systematic annotation and reliable prediction of the functional impact of SNPs. It includes evidence about the new amino acids and wild-type amino acid, indicating whether the SNP is considered neutral or deleterious (Alzahrani *et al*., 2020; Capriotti and Fariselli, 2017).

PROVEAN is an online tool which utilizes BLAST to search for homologs of the query sequence against the NCBI database. The PROVEAN cutoff value is set at -2.5, where amino acid substitutions with scores exceeding this threshold are reflected as deleterious (Choi and Chan, 2015; Dutta *et al*., 2022).

Sorting Intolerance from Tolerance (SIFT) is an online device which utilizes the PSI-BLAST protein catalog to collect functionally related protein sequences. Through homologous alignment, SIFT determines the likelihood of an amino acid existing at a specific site. A score below 0.05 indicates intolerance, while scores above 0.05 suggest tolerance to the amino acid substitution (Janani *et al*., 2019; Vaser *et al*., 2016).

Predicting impact on α-toxin stability

MUpro and I-MUTANT 3.0 were used for predicting impact on α-toxin stabilities. MUpro is an online software that utilizes SVM to envisage the outcome of single point mutations on protein stability. It employs sequence and structure information, taking into account protein sequences, mutation positions, and the original and substituted amino acids as input for analysis. It provides a concise and effective means of evaluating the effect of single point mutations on stability of protein (Alizadehmohajer *et al*., 2023; Khan and Vihinen, 2010).

I-MUTANT 3.0 utilizes information from the ProTherm database, which provides experimentally determined free energy changes associated with alterations in amino acids. I-MUTANT 3.0 calculates the corresponding free energy change (Gromiha, 2007; Patnaik *et al*., 2023).

Predicting structure based functional impact on α-toxin

For this purpose, we utilized a two-step approach to create a precise and reliable structure and function for the α-toxin from *C. perfringens* toxinotype B. Firstly, we retrieved the α-toxin's protein sequence from the Expasy Swiss Bioinformatics Resource Portal. PSIPRED method was used to predict protein secondary structure elements of α-toxin of *C. perfringens*. PSIPRED utilizes a machine learning approach, combining a neural network and a hidden Markov model, to generate accurate predictions for α--helices, beta-sheets, and coil regions (Asadollahi and Kalani, 2024; Buchan and Jones, 2019). Protein sequences were submitted to InterProScan for functional analysis. The tool performed a comprehensive search against databases such as Pfam, PROSITE, and SMART to identify functional domains and motifs. The annotations were validated against known experimental structures (Jones *et al*., 2014; Kumar *et al*., 2023). I-TASSER, was used for 3D structure prediction, which utilized the LOMETS to identify structural patterns. The template with the greater score was selected for generating the 3D model of the α-toxin. This enabled us to overcome the lack of an experimentally determined structure and obtain a predictive model, providing valuable insights into the protein's properties, such as ligand binding affinities and the effects of mutations (Yang and Zhang, 2015; Zheng *et al*., 2021; Zhou *et al*., 2022).

RESULTS

NS-SNPs in α-toxin

The nucleotide sequenced data for α-toxin of *C. perfringens* was used for identification of variations by blastx. Among all mutations, there were nine (09) NS-SNPs within α-toxin. According to Poly Phen-2, 66.67 % mutations were classified as possibly damaging while 33.33 % were considered benign. On the other hand, PhD SNP indicated 22.22% mutations were associated with diseased while 77.78% mutations were neutral. PROVEAN declared 55.56% of the mutations as deleterious and 44.44% as neutral. Additionally, SIFT predicted that 44.44% of the mutations affected function of α-toxin while 55.56% were tolerated. Moreover, both MUpro and I-MUTANT indicated that all mutations had decreased the stability of α-toxin of *C. perfringens* (Table I).

Polarity of α-toxin

The polarity of α-toxin of *C. perfringens* toxinotype B was predicted by PSIPRED tool indicating small nonpolar amino acids as orange color, hydrophobic amino acids presented as green, polar amino acids presented as red color and aromatics plus cysteine containing amino acids indicated as blue color. The α-toxin sequence had the 33% small nonpolar amino acids, 11% hydrophobic amino acids, 40% polar amino acids and 16% aromatics plus cysteine containing amino acids (Fig. 1).

The distribution of these amino acids suggests that the α-toxin of *C. perfringens* toxinotypes B has a complex structural and functional profile. The significant proportion of polar amino acid indicates that α-toxin may have extensive interaction with its environment and other molecules which could be relevant for its pathogenic mechanisms.

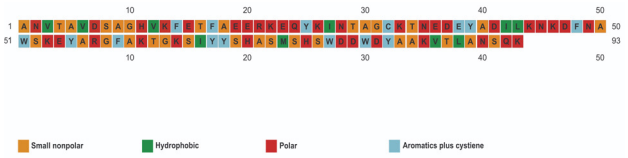

Fig. 1. Polarity prediction of the *Clostridium perfringens* α-toxin as analyzed using the PSIPRED tool.

Table I. Annotations of the functional and stability impacts of SNPs on the *Clostridium perfringens* α-toxin.

Mutations	Functional impact on toxin				Stability of toxin	
	PolyPhen-2	PhD SNP	Provean	SIFT	MUpro	I-Mutant
T4S	Possibly damaging	Neutral	Deleterious	Affect protein function	Decrease	Decrease
A30V	Benign	Neutral	Neutral	Tolerated	Decrease	Decrease
G31V	Possibly damaging	Disease	Deleterious	Affect protein function	Decrease	Decrease
E36N	Benign	Neutral	Neutral	Tolerated	Decrease	Decrease
E37A	Benign	Neutral	Deleterious	Tolerated	Decrease	Decrease
Y39T	Possibly damaging	Neutral	Deleterious	Affect protein function	Decrease	Decrease
A60S	Possibly damaging	Disease	Deleterious	Affect protein function	Decrease	Decrease
T62I	Possibly damaging	Neutral	Neutral	Tolerated	Decrease	Decrease
A83S	Possibly damaging	Neutral	Neutral	Tolerated	Decrease	Decrease

Solvent accessibility of α-toxin

The solvent accessibility of α-toxin of *C. perfringens* toxinotype B was predicted by I-TASSER. The values range from 0 indicating buried residues to 9 indicating highly exposed residues. The predictions indicated that 2.15% residues were highly buried with score 0 followed by 13.97% fairly buried residues with score 1. Whereas, 9.68% residues were moderately buried with score 2 and 22.58% residues were slightly buried with a score of 3. Moreover, 32.26% residues were placed at intermediate state with score 4 and 7.53% slightly exposed residue with score 5. Similarly, another 7.53% residues were moderately exposed with a score 6. Furthermore, 2.15% of each residue were fairly exposed and highly exposed with scores of 7 and 8, respectively (Fig. 2).

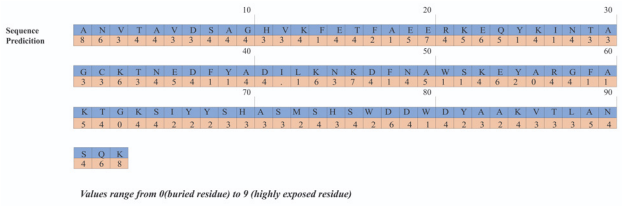

Fig. 2. Predicted solvent accessibility of the *Clostridium perfringens* α-toxin, as analyzed using the I-TASSER tool.

A substantial percentage of α-toxin had the solvent accessibility score of 4 signifying that these residues are in an intermediate state and play roles in both structural and functional aspects. This specifies that they may be intricated in forming functional domain or interacting with cellular targets. Whereas, another notable percentage of the α-toxin had a solvent accessibility score of 3, which infers that these residues are part of flexible regions tangled in structural transition or communication with external molecules. These residues are vital for binding to target cells and undergoing the structural changes necessary for biological activity.

Secondary structure of α-toxin

Secondary structure of α-toxin of *Clostridium perfringens* toxinotype B was formed by PSIPRED and I-TASSER web tools. Both tools verified that the submitted partial codons of α-toxin was comprised of 93 amino acids. I-TASSER predicted that the α-toxin of *C. perfringens* toxinotype B had the secondary structure comprised of coil (36.56%), helix (59.14%) and strand (4.30%) with a confidence score ranging from 0 indicating lowest score to 9 indicating the highest score (Fig. 3A). Whereas, PSIPRED presented that the secondary structure of α-toxin was consisted of coil (35.48%) and helix (64.52%) only (Fig. 3B).

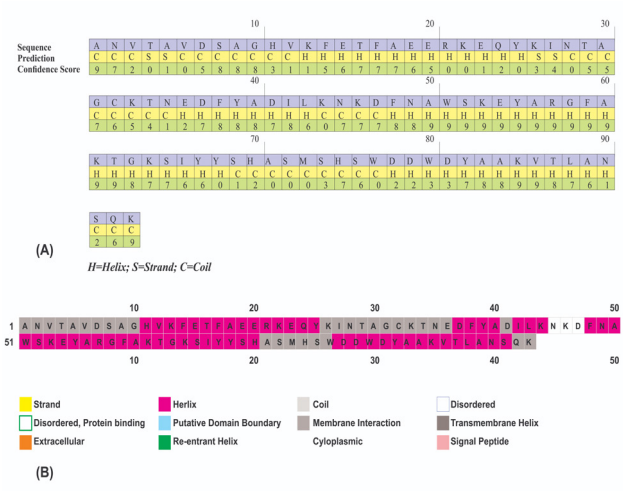

Fig. 3. Predicted secondary structure of the *Clostridium perfringens* α-toxin as determined by (A) I-TASSER and (B) PSIPRED.

The higher helical content prophesied by both tools suggests that these regions are critical for the toxin's functionality including receptor binding and pore formation in host cells. Coil regions which are stretchy, may enable the toxin to adjust its structure upon interaction with receptors, enhancing its pathogenicity. The low beta-strand content designates that the toxin relies more on helical and coil structures for stability and function, which is important for its interaction with the target.

3D structure of α-toxin

The 3D structure of α-toxin was designed by I-TASSER software. It provided output in the form of five (n=5) different structure with C-score which usually range from 2 to -5 and the greater C-score indicates that the model has higher assurance. The model of α-toxin which has been selected among five had the C-score=0.11 with TM score (estimated)=0.73±0.11 and RMSD (estimated)=3.6±2.5Å (Fig. 4).

By employing I-TASSER, binding site for ligand in α-toxin was investigated as these are significantly vital for functional annotation. If mutation occurs at this point, then it can interrupt the communication among transmembrane proteins and their ligands. The ligand named Calcium (II) Cation (CA) have the highest C-score (0.21) and possible binding sites are 2,3,4,5,6 and 7 (Table II).

Reorganized gene ontology classifications

The most frequently occurring term in each of the three functional aspects including molecular function, biological process and cellular components were reconciled with consensus GO term. For molecular function,

Table II. Predicted Ligand binding sites for the *Clostridium perfringens* α-toxin.

Rank	C Score	Cluster size	PDB Hit	Ligand Name and code	Ligand Binding site residues
1	0.21	481	4eoyA	Calcium (II) Cation (CA)	2,3,4,5,6,7
2	0.19	407	4nb5D	1,3-dihydroxypropan-2-yl octadecenoate (2JT)	3,4,6,7,10,11
3	0.12	261	2xqcD	Nucleic Acids (NUC)	9,10,13,14
4	0.10	234	4qikA	Nucleic Acids (NUC)	4,7,8,11,12
5	0.06	121	4hfdE	Tribromomethane (MBR)	8,11,12,15

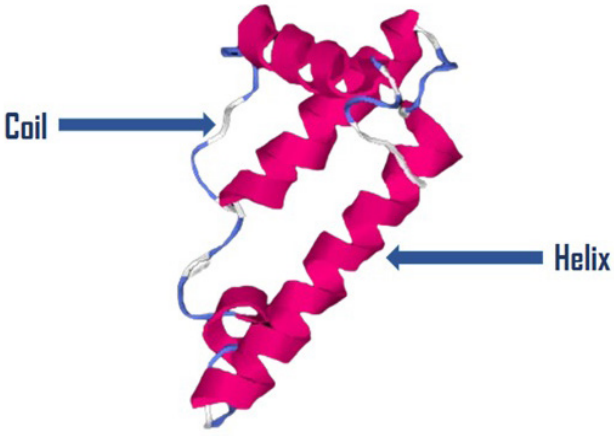

Fig. 4. Visualization of the 3D structure of the *Clostridium perfringens* α-toxin as predicted by I-TASSER, highlighting the helical region involved in receptor binding and the coil region associated with conformational changes necessary for binding specific receptors.

the highest GO-score (0.43) was for GO:0043167 (ion binding) while the lowest GO-score (0.32) was for GO:0005524 (binding to ATP), GO:0005536 (binding glucose) and GO:0004340 (glucokinase activity). For Biological process, the highest GO-score (0.43) were for GO:0006875 (metal ion homeostasis in the cell) and GO:0055072 (iron ion balance). Whereas, the lowest GO-score (0.37) were for GO:0030879 (mammary gland development), GO:0048609 (multicellular organism reproductive process), GO:0046323 (glucose import), GO:0007589 (body fluid secretion), GO:0007005 (mitochondrion association) and GO:0010827 (controlling glucose transport across the membrane) (Table III).

The InterProScan predicted the gene ontology terms for molecular function of α-toxin denoted by GO:0016788 (hydrolase activity, action on ester bonds) along with GO:0004629 (phospholipase C activity) and GO:0008270 (zinc ion binding).

Domain and homologous superfamily characteristics

InterProScan predicted that α-toxin belong to IPR001531 domain encoded for zinc dependent phospholipase C along with PS51346 domain which encoded for prokaryotic zinc dependent phospholipase C domain profile. InterProScan also predicted that α-toxin belong to IPR008947 homologous superfamily encoded for Phospholipase C/P1nuclease domain superfamily along with G3DSA:1.10.575.10 homologous superfamily encoded for P1 nuclease and SSF48537 homologous superfamily encoded for Phospholipase C/P1 nuclease (Fig. 5).

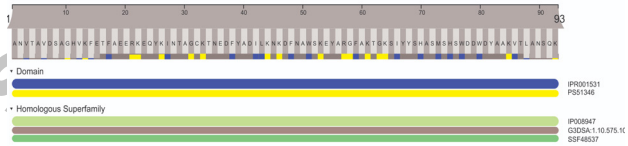

Fig. 5. Domains and homologous superfamily of the *Clostridium perfringens* α-toxin as predicted by InterProScan®.

DISCUSSION

The gene for α-toxin and its expression is rarely missing in *C. perfringens* as it is one of the vital criteria for phenotypical and genotypical identification of isolates moreover it is located in house-keeping region (Sattar *et al*., 2023; Tariq *et al*., 2022; Yanagimoto and Haramoto, 2021). α-toxin contains N-terminal domain which is responsible for enzymatic activity (amino acid 1-246) and C-terminal domain accountable for membrane binding and hemolytic activity (amino acid 256-370) (Nagahama *et al*., 2019). In this study it was revealed that T4S, G31V, E37A, Y39T, A60S, T62I and A83S were the annotations which impacted the functionality of the α-toxin as predicted by either of the tools used. Whereas A30V and E36N were the predicted annotations with no functional impact on the α-toxin. It was also predicted that all of these annotations had decreased the stability for the α-toxin of *C. perfringens*. An *in-vivo* study, also revealed a *plc* (α-toxin) variant containing internal 834-bp insertion with normal expression and functional α-toxin isolated from chicken

Table III. Gene ontology terms for the functional aspects of the *Clostridium perfringens* α-toxin, as predicted by I-TASSER.

GO-term code	GO-term name/ description	Go-score
Molecular function		
GO:0043167	Binding to an ion, a charged atoms or groups of atoms	0.43
GO:0070279	Binding to a vitamin B6 compound: pyridoxal, pyridoxamine, pyridoxine, or the active form, pyridoxal phosphate	0.39
GO:0004629	Catalysis of the reaction: a phospholipid + H2O = 1,2-diacylglycerol + a phosphatidate	0.37
GO:0031406	Binding to a carboxylic acid, an organic acid containing one or more carboxyl (COOH) groups or anions (COO-)	0.36
GO:0050498	Catalysis of an oxidation-reduction (redox) reaction in which hydrogen or electrons are transferred from 2-oxo-glutarate and one other donor, and the latter donor is dehydrogenated	0.36
GO:0016462	Catalysis of the hydrolysis of a pyrophosphate bond between two phosphate groups, leaving one phosphate on each of the two fragments.	0.34
GO:0042802	Binding to an identical protein or proteins	0.34
GO:0004340	Catalysis of the reaction: ATP + D-glucose = ADP + D-glucose-6-Phosphate	0.32
GO:0005536	Binding to D- or L-enantiomers of glucose	0.32
GO:0005524	Binding to ATP, adenosine 5'-triphosphate, a universally important coenzyme and enzyme regulator	0.32
Biological process		
GO:0006875	Any process involved in the maintenance of an internal steady state of metal ions at the level of a cell	0.43
GO:0055072	Any process involved in the maintenance of an internal steady state of iron ions within an organism or cell	0.43
GO:0044106	The chemical reactions and pathways involving any organic compound that is weakly basic in character and contains an amino or a substituted amino group, as carried out by individual cells	0.39
GO:0019752	The chemical reactions and pathways involving carboxylic acids, any organic acid containing one or more carboxyl (COOH) groups or anions (COO-)	0.39
GO:0030879	The process whose specific outcome is the progression of the mammary gland over time, from its formation to the mature structure	0.37
GO:0048609	The process, occurring above the cellular level, which is pertinent to the reproductive function of a multicellular organism. This includes the integrated processes at the level of tissues and organs.	0.37
GO:0046323	The directed movement of the hexose monosaccharide glucose into a cell or organelle	0.37
GO:0007589	The controlled release of a fluid by a cell or tissue in animals	0.37
GO:0007005	A process that is carried out at the cellular level which results in the assembly, arrangement of constituent parts, or disassembly of a mitochondrion; includes mitochondrial morphogenesis and distribution, and replication of the mitochondrial genome as well as synthesis of new mitochondrial components.	0.37
GO:0010827	Any process that modulates the frequency, rate or extent of glucose transport across a membrane. Glucose transport is the directed movement of the hexose monosaccharide glucose into, out of or within a cell, or between cells, by means of some agent such as a transporter or pore	0.37

and humans (Matsuda *et al*., 2019). The annotation for polarity of *plc* partial sequences of current study revealed that α-toxin was composed of polar, hydrophobic, small non polar and aromatic cysteine containing amino acids with the majority of amino acids being polar. In current study, the solvent accessibility as predicted by I-TASSER indicated 48.38% residues as buried, 32.25% residues were intermediately buried and exposed while 19.36% residues were exposed and readily accessible to solvents. Previously published data indicated that exposed residues of α-toxin were meant for membrane binding and enzymatic activity while the buried residues had other critical toxic impact for the development of diseases (Alape-Girón *et al*., 2000).

The annotations for secondary structure as predicted by PSIPRED and I-TASSER elaborated that majority of α-toxin of *C. perfringens* toxinotypes B were helix. The three-dimensional analysis of α-toxin elaborated that there are nine tightly packed α-helix in N-domain and the catalytic site is located in N-domain. Moreover, α-toxin contains a zinc metalloenzyme structure with phospholipase C activity. Additionally, this enzyme is calcium dependent for binding with membrane (Oda *et al*.,

2015). The tertiary structure of α-toxin with best C-score 0.11 with TM score (estimated)=0.73±0.11 and estimated RMSD 3.6±2.5. the I-TASSER server prophesied models of α-toxin by merging the approaches of structural refining *ab initio* models and threading (Roy *et al*., 2010).

The predicted ligand binding site revealed that α-toxin had binding site for calcium, nucleic acid and tribromomethane. It was previously confirmed by crystallographic studies that C-terminal domain is architecturally comparable to phospholipid binding domain of eukaryotic protein as α-toxin interact with membrane via calcium mediated appreciation of phospholipid head group and binding of hydrophobic amino acids with phospholipid tail group (Titball *et al*., 2000). Later, it was reported that α-toxin has two conformations *viz*., open and closed form. Initially in closed form the C-domain binds with calcium ions and host cell membrane which bring about conformational change and activating the cleft of N-domain getting access to phospholipase (Uppalapati *et al*., 2013).

This study regarding in silico analysis has provided valuable insights into the potential impact of the genetic variations on the α-toxin. However, it imperative to acknowledge the limitations of this approach. Computational predictions are presumed to be powerful but may not fully cover the complexities of protein function *in vivo*. Future experimental studies, including site directed mutagenesis and functional assays are necessary to validate these findings and further elucidate the role of these SNPs in the pathogenesis of lamb dysentery.

In conclusion by using computer simulations, we studied genetic variations in the α-toxin of *C. perfringens* type B bacteria linked to lamb dysentery. This *in silico* analysis helps us grasp the influence of these variations on the structure and function of the toxin, shedding light on the development of disease. This knowledge could lead to new treatments. Further laboratory exploration is required to approve these findings.

DECLARATIONS

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Supplementary material

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Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

In silico Analysis of Non-Synonymous Single Nucleotide Polymorphisms in α-Toxin of *Clostridium perfringens* Toxinotype B Isolated from Lamb Dysentery Cases in Pakistan

Mian Muhammad Khubaib Sattar[1,4], Aftab Ahmad Anjum[1]*, Yung Fu Chang[2], Tahir Yaqub[1] and Asim Aslam[3]

[1]Institute of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan.
[2]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, 14853 Ithaca, New York, United States of America.
[3]Department of Pathology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan.
[4]Department of Microbiology, Faculty of Veterinary and Animal Science, The Islamia University of Bahawalpur, 63100 Bahawalpur, Pakistan.

SUPPLEMENTARY MATERIAL

The current study is in continuation to our previously published research, [Sattar, M.M.K., Anjum, A.A., Chang, Y.F., Yaqub, T., Aslam, A. and Ali, T., 2023. Molecular Characterization and toxins optimization of indigenous *Clostridium perfringens* Toxinotype B isolated from lamb dysentery clinical cases. *Kafkas Univ Vet Fak Derg* **29**: 79-89. https://doi.org/10.9775/kvfd.2022.28738] in this article the *in silico* gene expression analysis was conducted on the α-toxin gene of *C. perfringens* toxinotypes B.

Gene amplification and sequence for SNP annotation

The α-toxin (*cpa*) gene of *Clostridium perfringens* toxinotype B was amplified by using primer sequence CPA-F 5′-GCTAATGTTACTGCCGTTGA-3′ CPA-R 5′-CCTCTGATACATCGTGTAAG-3′ and amplified at 94°C for 10 min (initial denaturation) followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 45 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min. Gel electrophoresis was performed using 1.8% agarose with ethidium bromide 0.5µg/mL. The amplified gene product was sequenced by Sanger chain termination sequencing which retrieved following nucleotide sequence; CCTGCTGTTCTTTTTGAGAGT-TAGCTAAAGTTACCTTTGCTGCATAATCCCAAT-CATCCCAACTATGACTCATGCTAGCATGACTATA-GTATATTGATTTTCCTGTTTTAGCAAAACCTCTTG-CATATTCTTTTGACCATGCATTAAAATCTTTGT-TTTTTAAGATATCAGCATAAAAATCCTCATTAGT-TTTGCAACCTGCTGTGTTTATTTTATACTGTTCT-TTTCTTTCCTCTGCAAAAGTCTCAAACTTAACAT-GTCCTGCGCTATCAACGGCAGTAACATTAGCAAC.

* Corresponding author: aftab.anjum@uvas.edu.pk
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