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Structural modeling of leukotoxin A secreted by *Aggregatibacter actinomycetemcomitans* as a target for biopharmaceutical applications

Maryam Pourhajibagher[1], Samira Gharesi[2] and Roghayeh Ghorbanzadeh[3]*

[1]*Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[2]*Department of Genetics, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran*
[3]*Private Practices, Tehran, Iran*

ABSTRACT

Aggregatibacter actinomycetemcomitans is a major putative periodontopathic microorganism that produces a repeats-in-toxin (RTX) leukotoxin (LtxA) that selectively kills human immune cells. Systemically administered antibiotics are recommended for removal of this bacterium from infected periodontium. But recently, the use of conventional antibiotics is limited due to the emergence of antibiotic resistance among A. actinomycetemcomitans isolates. Consequently, it is imperative that new drugs used for the treatment of A. actinomycetemcomitans infections. In this study, using bioinformatic tools and computer simulation molecular modelling, we introduced the LtxA protein of A. actinomycetemcomitans as a new drug target for treatment infections caused by A. actinomycetemcomitans. Hydropathy analysis of LtxA protein (114194.6 dalton; 1050 amino acids) predicts four domains: N-terminal region, the central region, the repeat region and the C-terminal region. Protein interaction network have shown the association among LtxA with ten functional partners. Our findings suggest that LtxA is a stable protein that was concentrated outside of the cells. Computational approach has facilitated the search for potential drug targets. More detailed analysis of biochemical pathways of LtxA set high hopes and provide insight into the development of new antibiotics for appropriate treatments of A. actinomycetemcomitans infections.

Keywords: *Aggregatibacter actinomycetemcomitans*, leukotoxin, periodontal- endodontic disease, in silico**.**

INTRODUCTION

Periodontal diseases result from a polymicrobial infection of the subgingival crevice [1] which may be categorized a pocket debridement, gingivitis, and aggressive periodontitis [2]. Living conditions for periodontal pathogens are special and these microorganisms are found in dental plaque, in periodontal pockets, and buccal mucosa [3]. One of the pathogens involved in the periodontal infection is *Aggregatibacter actinomycetemcomitans* [4]. *A. actinomycetemcomitans* is a gram-negative, facultative anaerobic coccobacillus that can be found in aggressive periodontitis, chronic periodontitis and periodontally healthy individuals [5-7]. *A. actinomycetemcomitans* is found in subgingival plaque from combined periodontal-endodontic lesions due to of bacteria from the root canal system to the periodontal pocket [7]. *A. actinomycetemcomitans* possesses a large number of virulence factors such as collagenase, endotoxin, production of immunosuppressive factors, secretion of proteases, which cleave immunoglobulin G (IgG), and a fibroblast-inhibiting factor [8]. It can colonize and persist in the oral cavity through adhesins, bacteriocins, invasins and antibiotic resistance [9].

A. actinomycetemcomitans secretes two protein toxins, leukotoxin (LtxA) and cytolethal distending toxin (CDT) that are thought to play a role in immune evasion [10]. It was shown that leukotoxic activity of *A. actinomycetemcomitans* was due to its LtxA [11] which belongs to the repeat in toxin family (RTX) [12]. LtxA can affect human polymorphonuclears (PMNs), monocytes, lymphocytes and erythrocytes from human and animal origin [13, 14]. Using different antibiotics is one way to deal with *A. actinomycetemcomitans*, but antibiotics use have been limited to limiting drugs because *A. actinomycetemcomitans* has potential which can lead to increased resistance and infection in the pocket and root canal system [15]. Therefore, it is important to identify new potential targets with appropriate antibiotic sensitivity. In this study we have specifically analyzed the characteristics of LtxA through *in silico* analysis tools to can be introduced it as a target for drug design against *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Retrieval of the target sequence

Amino acid sequences of LtxA from *A. actinomycetemcomitans* were obtained from NCBI (http://www.ncbi.nlm.nih.gov), and a BLAST alignment was performed against Protein Data Bank (PDB) entries to retrieve identity and similarity percentages by pairwise alignment. LtxA sequences were aligned in T-COFFEE (http://tcoffee.crg.cat/apps/tcoffee).

Domain and protein family membership

Protein families, domains and functional sites of LtxA were analysed by Interpro server (http://www.ebi.ac.uk/interpro).

Molecular Modeling and functional characterization of LtxA

Physicochemical properties of LtxA were carried in at a glance using UniProt (http://expasy.org) and Expasy ProtParam server (http://www.expasy.org/cgi-bin/protpraram). The secondary structure of LtxA protein was obtained using the protein homology/analogy recognition engine V 2.0 (Phyre 2) (www.sbg.bio.ic.ac.uk/phyre2). Using the OCTOPUS server, we predicted the membrane protein topology, signal peptides, network value and subcellular localization of LtxA. Three dimensional structure of LtxA protein was achieved through the SWISS-MODEL Workspace (http://swissmodel.expasy.org). To assess the quality of three-dimensional structure, it was compared with a large collection of structures determined by NMR or X-ray crystallography in ProsaWeb server (https://prosa.services.came.sbg.ac.at/prosa.php).

Protein interaction network analysis

Protein interaction has been done to find out the most potential metabolic functional associations among all identified choke point proteins through protein interaction database STRING (http://string-db.org). To select the appropriate region of the protein sequence as a target for drugs, SignalP4.1 server (http://www.cbs.dtu.dk/services/SignalP) was used to identify the signal peptide.

RESULTS

Protein sequence identity analysis at the NCBI GenBank database showed that LtxA has 1050 amino acids that its estimated structure weight was 114194.6 dalton. Basic information obtained from LtxA is shown in Table 1 and domain and proteien family membership is displayed in Table 2. Multiple sequence alignment of template and target has been performed by T-COFFEE software for modelling (Fig. 1).

Table 1. Basic information of LtxA protein

Protein	Gene	Organism	Template PDB code	Original Database ID	Accession No. NCBI	Accession No. Uniprot
Leukotoxin	LtxA	*Aggregatibacter actinomycetemcomitans*	5CXLA	gi 126355	AAA21922.1	P16462.1

Table 2. Domains of LtxA protein

Domain	Interpro No.
RTX N-terminal	IPR018504
Serralysin-like metalloprotease, C-terminal	IPR011049
RTX C-terminal	IPR013550
Haemolysin-type calcium-binding repeat	IPR001343
Hemolysin-type calcium-binding conserved site	IPR018511
RTX toxin determinant A	IPR003995

Fig 1. Alignment of target and template proteins by T-COFFEE

Initial structure prediction showed that LtxA had 137 positively charged residues (Arg + Lys) and 115 negatively charged residues (Asp + Glu). The very high aliphatic index was 93.30 and instability index (II) was computed to be 17.58. Its theoretical isoelectric point (PI), extinction coefficient, and grand average of hydropathicity value were 9.20, 85860, and -0.366 respectively. There results show that LtxA is a stable protein. In modelled structure (Fig. 2), alpha helices (40%, 420 residues) has dominance over other secondary structure i.e.beta-sheet (22%, 234 residues) and coil (38%, 396 residues) and LtxA is present outside of the cell (Fig. 3). Three dimensional structures of LtxA have been generated by SWISS-MODEL Workspace and its quality was confirmed with ProsaWeb server (Fig. 4). Seven useable PDB homologs found such as: 5CXLA, e-value= 1.0E-14; 2AGMA, e-value= 1.0E-10; 1K7IA, e-value= 9.0E-10; 1SAT, e-value= 6.0E-9; 1KAPP, e-value= 1.0E-8; 1G9KA, e-value= 2.0E-8 that among them query sequence has 37% homology with PDB entry 5CXLA of Chain A, crystal structure of Rtx domain block V of adenylate cyclase toxin from *Bordetella pertussis*.

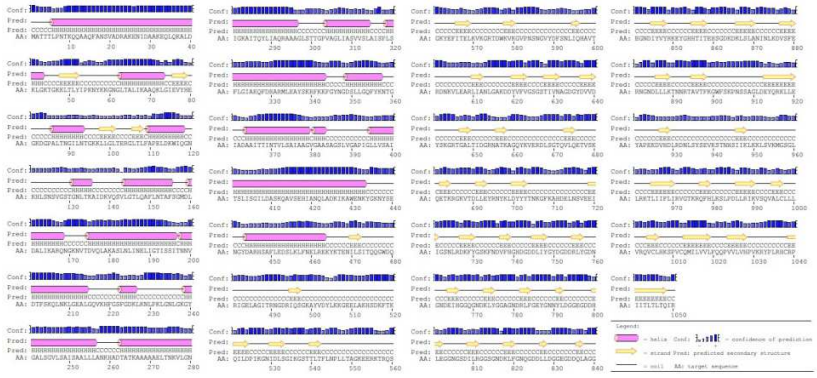

Fig 2. Secondary structure prediction of LtxA protein by Phyre 2 server

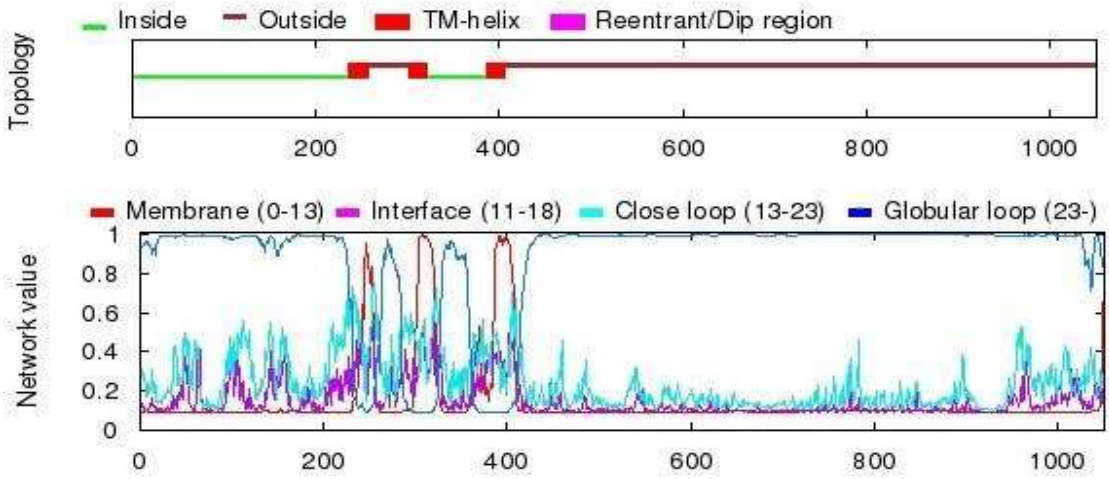

Fig 3. Prediction of membrane protein topology, network value and subcellular localization of LtxA protein by OCTOPUS server

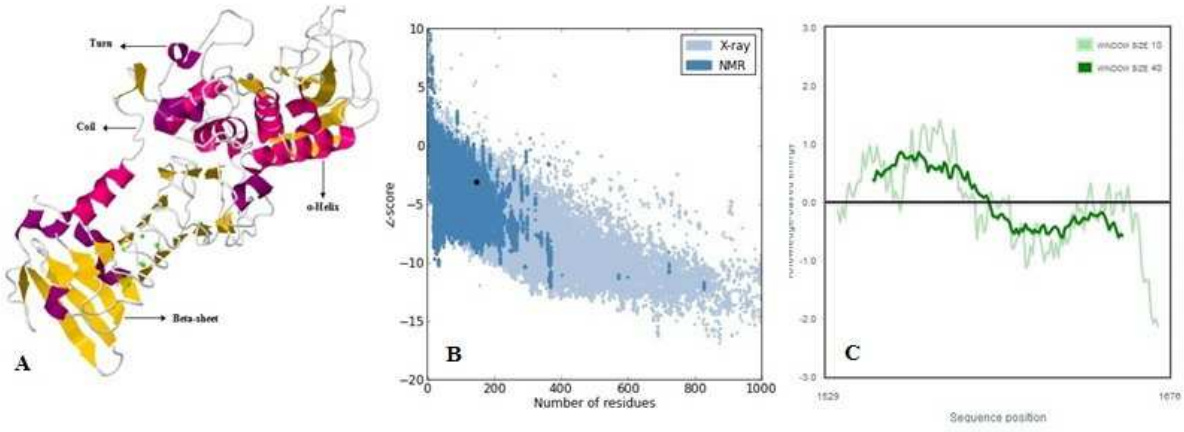

Fig 4 A) Three dimensional visualization of the modelled structure of LtxA protein by SWISS-MODEL Workspace. B) Overall model quality with ProsaWeb server. C) Local model quality with ProsaWeb server

Protein interaction network have shown the association among highly interacting proteins with together. In figure 5, proteins and their length and type of relationship with LtxA protein has been shown. It has several predicted functional partners such as hemolysin- activating lysine- acyltransferase HlyC, hemolysin secretion protein HlyB, hemolysin D, and serine hydroxymethyltransferase. The results of the identification signal peptide is shown in Figure 6. Cleavage site is located at 25 amino acid (value: 0.108) and signal peptide is located at 2 amino acid (value: 0.22).

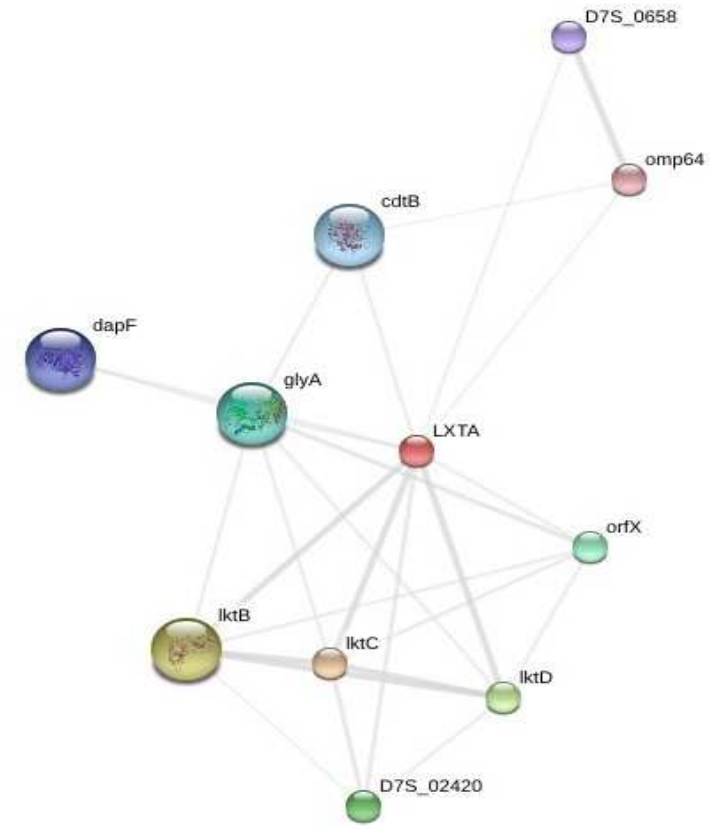

Fig 5. Interaction network of LtxA protein by protein interaction database STRING

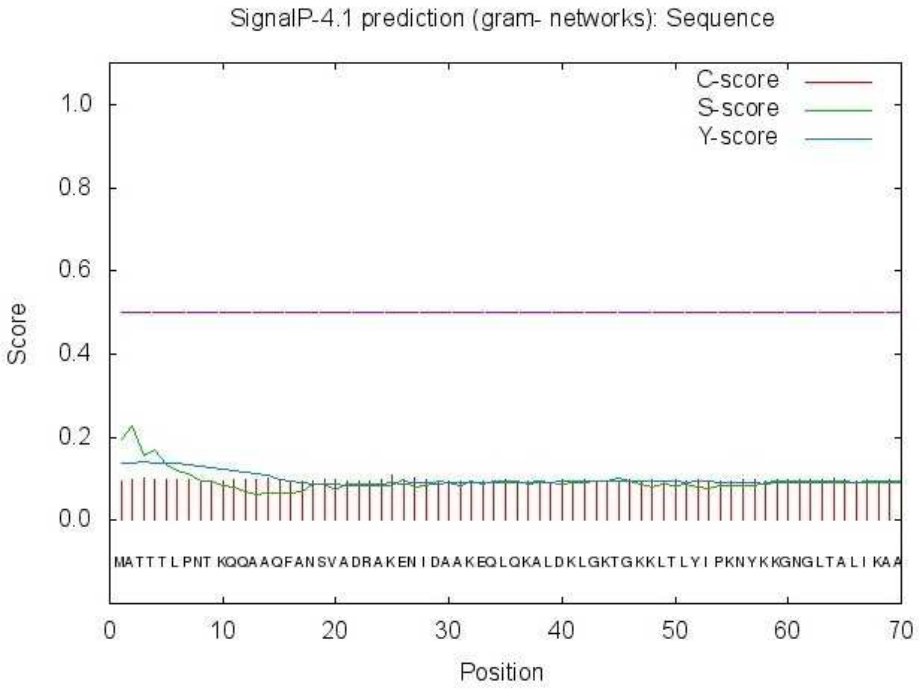

Fig 6. Identification signal peptide for LtxA protein by signalP server

DISCUSSION

A vast number of pathogens potentially have been reported to contribute in pulpitis, apical periodontitis and endodontic disease [16]. Periodontal disease may affect the pulp through dentinal tubules, lateral canals or from apical foramen. Migration due to presence of communication between the internal and external spaces of periodontal pocket, or remnants of residual microbiota due to insufficient cleaning and shaping can cause presence of *A. actinomycetemcomitans* in periodontal-endodontic disease [17].

In addition to known microorganisms in periodontal diseases, other microbiota such as *Acinetobacter baumannii* have been identified in the pathogenesis of periodontal disease [18], however, the position of their virulence is different. *A. baumannii* has emerged as a medically important microorganism and clinical challenge in nosocomial infection and the pathogenicity of this bacterium depends on its ability to acquired resistance to antimicrobial agents [19-25]. it has been seen in periodontal-endodontic disease [18]. The main goal of periodontitis treatment is based on eliminate microorganisms and thoroughly clean the pockets around teeth to create a healthy periodontal environment [26].

Xylitol is a natural sugar found in many plants that can reduce the accumulation of unhealthy oral bacteria. It was known that xylitol is harmless to the gingival fibroblast and soft tissues. It also has improved gingival health and to the supporting structures around the teeth [27]. As well as, chemomechanical debridement and chemotherapeutic agents can remove the bulk of the supragingival and accessible subgingival microbial plaque and calculus but residual microorganisms are detectable in pockets [15]. The uses of topical antimicrobial agents usually significantly reduce decay and control microbiota and decrease the size of periodontal pockets [28]. But use of antibiotics has been limited because of the emergence of strains resistant to antibiotics. Thus, with having knowledge of the structural components of microorganisms and science of bioinformatics can be offered and designed new drugs for the treatment of periodontal-endodontic diseases [29, 30].

It was recognized that *A. actinomycetemcomitans* exhibits two different leukotoxin which one of them is LtxA [10-12]. LtxA has been detected in lipid vesicles that derived from the outer membrane of *A. actinomycetemcomitans* [31]. LtxA is a large pore-forming protein that consists of 1050 amino acids that can be divided into four regions: the N-terminal region, the central region, the repeat region and the C-terminal region. Protein interaction network of LtxA have shown the association among predicted functional partners such as hemolysin- activating lysine-acyltransferase HlyC; involved in fatty acylation of the protoxin, hemolysin secretion protein HlyB; part of the ABC transporter complex, hemolysin D; involved in the transport of the hemolysin/leukotoxin, and serine hydroxymethyltransferase.

Comparative analysis of the predicted secondary structure of LtxA showed that the alpha-helices was present predominantly, followed by beta-sheet and coil. OCTOPUS is predicted the membrane protein topology, signal peptides, and subcellular localization of LtxA. It has shown that LtxA is present outside of the cell. It is noteworthy that LtxA can be a good potential drug target against *A. actinomycetemcomitans*.

Recently, new therapeutic approaches were use for treatment of oral microbial diseases. Among them, the application of photodynamic therapy using light with a specific wavelength and a nontoxic photoactive dye (photosensitizer) has gained special attention [32-35]. In addition, nanoparticles are considered a viable alternative to antibiotics and seem to have a high potential for controlling bacterial infections [36-38]. They are as alternative treatment strategies that in the near future may be replaced instead of chemomechanical and antibiotical therapy. Literatures have been shown the potential association of Toll-like receptors (TLR) 4 polymorphisms and susceptibility to infectious diseases such as chlamydia, and cytomegalovirus as well as periodontal disease caused by *A. actinomycetemcomitans* [39-41].The heterogeneity of the detection methods and diversity of patients' immunologic responses in these infectious diseases in developing countries such as Iran [42-44] along with multiple endpoints should also be considered. TLR4 has also been shown to play an essential role in the regulation of the immune responses through the recognition the extracellular components of bacteria such as streptococcal streptokinase [45] and the pathogen-associated molecular patterns (PAMPs) such as LtxA in *A. actinomycetemcomitans* as well as the activation of immune response genes [46].

CONCLUSION

Each technique for periodontal-endodontic disinfection and cleaning of periodontal pocket has some advantages and disadvantages. New disinfectant agents and drug design necessitate policies toward the control and treatment of periodontal- endodontic disease. In silico studies by application of computer technology can analyze and integrate biological and genetic information which can then be applied to offer drug targets and design new drugs.

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