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Research Article

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Immunoinformatics approach for the determination of antigenic epitope candidates against *Burkholderia cenocepacia*

Çiğdem Yılmaz[1*], Ahsen Morva Yılmaz[1], Duygu Aydın Tekdaş[1], Altan Kara[1]

[1]*Genetic Engineering and Biotechnology Institute, TUBITAK, Gebze, Kocaeli*

[*]**Corresponding author**

Dr. Çiğdem Yılmaz, Genetic Engineering and Biotechnology Institute, TUBITAK Gen Mühendisliği ve Biyoteknoloji Enstitüsü, Gebze, Kocaeli, 41470.

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Abstract

Cystic fibrosis (CF) is a genetic disorder that affects mainly the lungs but also the liver, intestine, and kidneys. It causes an increase in dehydrated mucus which results in the proliferation of numerous pathobionts. Lung inflammation in CF patients is commonly observed due to the opportunistic infection of the Burkholderia cepacia complex (Bcc) that includes one of the deadly pathogens named B. cenocepacia. Although a low incidence rate is present, B. cenocepacia infection is still a major problem for the CF community. In the study, it was aimed to uncover T and B cell epitopes of novel antigenic proteins of B. cenocepacia that can be potential targets for vaccine development studies. The whole proteome of B. cenocepacia ATCC BAA-245 was first analyzed to eliminate the non-redundant proteins which were then subjected to Vaxign analysis for the determination of vaccine candidates. After subcellular localization analysis, the shortlisted proteins were directed to the antigenicity, virulence, and allergenicity analyses, resulting in nine antigenic, virulent, and non-allergenic candidates. The epitope prediction was then carried out for these candidates and seven proteins containing four TonB-dependent receptors, ornibactin receptor, phospholipase C, and putative outer membrane usher protein were found to contain common T and B cell epitopes. Besides, the epitopes of the three candidates had the best exposed topology that can be used to construct multi-epitope vaccines against Bcc.

Keywords: Burkholderia Cenocepacia, Vaccine, Proteome Mining, Reverse Vaccinology

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease that the deficiency in the *cystic fibrosis transmembrane conductance regulator* (CTFR) causes an increase in dehydrated mucus in the various organs, including the lungs and gut, which, in turn, leads to the expansion of numerous opportunistic pathogens [1]. Inflammation of lungs and decrease in lung function are commonly observed in CF patients and caused by the complex polymicrobial communities like the *Burkholderia cepacia complex* (Bcc). *Burkholderia cenocepacia* is one of the deadly pathogens in Bcc, treatment of which is particularly challenging due to its intrinsic high level of resistance to different antibiotic classes [2]. A small percentage of CF patients is colonized with this pathogen, resulting in little investment in the development of new treatment options against *B. cenocepacia*, but it should be considered that the pathogen causes about a 2-fold decrease in patient's life expectancy compared to *B. cenocepacia*-negative patients [3]. Although limited interest is available for *B. cenocepacia* due to the low incidence rate, this deadly pathogen is still a serious problem and worth investigating to help the CF community, especially in the era of antibiotic resistance.

Limited antibiotics are effective against *B. cenocepacia* infection as it poses various intrinsic resistance factors such as reduced cell-wall permeability and enhanced efflux pump activity like other *Burkholderia species* [2]. Thus, alternative approaches have been studied to tackle *Burkholderia spp*. including antimicrobial peptides that can block biofilm formation and quorum sensing molecules [4, 5]. Besides new biotherapeutic agents, the development of Bcc vaccines is also critical as the most efficient approach and these vaccines must also consider the inclusion of *B. cenocepacia* antigenic proteins for wide range protection against the complex. Although no vaccine is available up to now, many studies have been conducted by using various virulence factors of *B. cenocepacia* including outer membrane proteins, linocin, and flagellin [6,

7, 8].

Besides known virulence factors, immunoproteomics approaches have been applied to identify novel immunogenic proteins to develop protective vaccines [9, 10, 11]. In an immunoproteomics screening, four immunogenic proteins were identified by using a patient serum: 38 kDa porin, DNA-directed RNA polymerase, GroEL, and Ef-Tu that can be potential factors to be used in multi-component vaccine development [9]. In a recent study, LC/MS and bioinformatic tools allowed to identify 55 surface-exposed proteins, sixteen of which were predicted as immunogenic after the shaving approach. Among them, two immunogenic proteins provided high antibody response when the sera of CF patients were used [10]. Another study revealed that the immunoproteomic approach provided the identification of 19 proteins as immunogenic for the first time and ten proteins were estimated as extracytoplasmic [11].

Besides traditional methods, in silico approaches can also contribute to discovering novel antigens in a fast manner through the mining of genomes or proteomes of pathogens. In this sense, this study aims to uncover antigenic proteins of *B. cenocepacia* through a proteome-mining approach and reveal their T and B cell epitopes that can be used in the design of a multi-epitope vaccine against Bcc infections.

Methodology

Data retrievel and selection of non-reduntant proteins

The whole proteome of *B. cenocepacia* ATCC BAA-245 was downloaded from the UNIPROT database and analyzed through the CD-HIT server to identify non-redundant proteins [12]. The cut-off value was set at 60% sequence identity and proteins with less than 100 amino acids in length were also excluded [13].

Sorting Novel Vaccine Candidates Through Vaxign

Non-redundant proteins were sorted by Vaxign (http://www.violinet.org/vaxign2) for the identification of putative vaccine candidates based on the parameters defined as: adhesion probability > 0.51, Vaxign-ML score ≥ 90, and the number of transmembrane helices ≤ 1. In addition, the proteins having homology with human or mouse proteins were excluded [14].

Subcellular Localization Prediction

Vaxign server provides a prediction of subcellular localization using PSORTb as a tool. The proteins stated as 'unknown' were subjected to CELLO v2.5 (http://cello.life.nctu.edu.tw/) to uncover the proteins' locations [15].

Identification of Antigenic and Virulent Protein Candidates

After the prediction of subcellular localization, outer membrane and extracellular proteins were analyzed using the VaxiJen v2.0 database (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/) to reveal the antigenic and virulent proteins, respectively [16, 17]. The VFDB analysis was based on the parameters including BLAST score ≥ 100 and e value 0.0001, while the threshold value was set at ≥ 0.6 for VaxiJen search. The remaining proteins were analyzed by using Expasy ProtParam and sorted according to their molecular weights to select the proteins having molecular weight ≤110 kDa.

Allergenicity Evaluation

AllerTOP v.2.0 (https://www.ddg-pharmfac.net/AllerTOP/method.html) and Allergen FP v.1.0 (http://ddg-pharmfac.net/AllergenFP/) servers were used to assess the allergenicity of the vaccine candidates [18, 19]. While AllerTOP determines allergens better than non-allergens, AllergenFP provides balanced predictions of both non-allergens and allergens [19].

Prediction of Mhc I and Mhc Ii Binding Sites

Immune Epitope Database (IEDB) server was used to scan selected antigen sequences for amino acid patterns to determine potent T cell epitopes (http://tools.iedb.org/main/tcell/). Antigenic sequences indicative of *Major Histocompatibility Complex* (MHC) class I-specific binding were predicted using the *Stabilized Matrix Method* (SMM) incorporating solely frequently occurring alleles into the analysis (http://tools.iedb.org/mhci/) [20]. The prediction output was determined according to IC50 values. The cut-off value was accepted as IC50 < 50 nM. Peptides with IC50 < 50 nM indicate high binding capacity. Intermediate and low binding capacity were referred to IC50 < 500 Nm and < 5000 nM, respectively. Antigenic sequences were also evaluated to predict the immunogenicity for T cell MHC I binding epitopes (http://tools.iedb.org/immunogenicity/). Epitopes with positive value were accepted as immunogenic. Finally, immunogenic epitopes with high binding capacity were taken into consideration as potent epitopes. MHC II binding prediction was performed using the SMM-align (NetMHCII 1.1) method predicting peptide:MHC II binding affinity values [21]. The analysis covered only human HLA-DR locus (http://tools.iedb.org/mhcii/). Peptide is composed of 15 amino acids as a default value proposed by the server. Peptides with IC50 < 50nM were sorted for the next analysis.

Prediction of B Cell Epitopes

To determine linear B cell epitopes, Bepipred Linear Epitope Prediction 2.0 was used (http://tools.iedb.org/bcell/) [22]. The predictive method is based on the use of antigenic sequence characteristics using a Random Forest algorithm. Amino acid residues with > 0.5 default threshold (Sensitivity=0.58564, Specificity=0.57158) were considered to be part of a B cell epitope.

After the identification of T and B cell epitopes, all predicted residues were cross-checked to reveal common epitopes of the antigenic candidates that can bind both T and B cell receptors.

Structural Modeling

The 3D structures of the selected antigenic proteins were modeled by using Phyre2 and refined by the Protein Preparation Wizard of Schrödinger package with default settings [23]. Maestro v12.4 was used for the visualization of the exo-topology of the shortlisted common epitopes [24].

Results and Discussion

Substractive Proteome for The Identification Vaccine Candidates

The whole proteome of *B. cenocepacia* ATCC BAA-245 containing 6994 proteins was subjected to CD-HIT analysis to eliminate

redundant proteins with > 60% sequence similarity as they are likely to represent similar structure and functions. The analysis resulted in a total of 6282 proteins which were then directed to the Vaxign server for the identification of putative vaccine candidates (Table S1). Vaxign is a vaccine target prediction pipeline that computes the possibilities of the proteins as targets through reverse vaccinology approach. The pipeline consists of several criteria including subcellular localization, topology (transmembrane helices), adhesin probability, and the similarity to human and mouse proteins. For the prediction of these criteria, open-source software or databases are used, including PSORTb, TMHMM, SPAAN, and BLAST. Moreover, Vaxign assigns a Vaxign-ML score to each protein which represents an estimation of protegenicity for the induction of immunity [25]. After the Vaxign analysis, a total of 423 proteins were identified as novel candidates (Table S2). The subcellular localization of the proteins identified as 'unknown' in Vaxign was estimated with the CELLO2 server. B. cenocepacia is a Gram-negative bacterium and thus, the proteins can be classified into five possible locations; 1) cytoplasmic, 2) cytoplasmic (inner) membrane, 3) outer membrane, 4) extracellular, and 5) periplasmic. While 12 proteins were assigned as cytoplasmic, the location of 27 proteins was estimated as cytoplasmic membrane and periplasmic localization was proposed for 172 proteins. In addition, 108 outer membrane and 104 extracellular proteins were estimated (Figure 1).

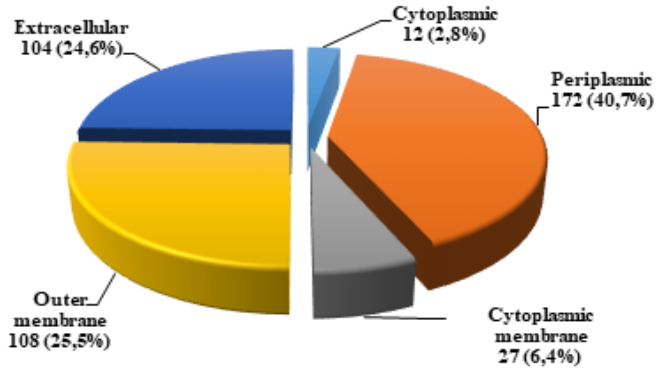

Figure 1: Predicted subcellular localization of the putative vaccine candidates.

It is well-known that the proteins located in the outer membrane or secreted outside the cell are more likely to induce immune responses as they can easily be recognized by immune cells. Therefore, 104 extracellular and 108 outer membrane proteins were further analyzed for their antigenicity, virulence, and allergenicity properties.

Evaluation of Antigenicity, Virulence, And Allergenicity of Vaccine Candidates

The antigenicity of the vaccine candidates was assessed by using the VaxiJen server which is the first server for the prediction of protective antigens without sequence alignment. The prediction is solely dependent on the physicochemical features of proteins as alignment-dependent approaches have limitations [16]. The VFDB database was used to evaluate the virulence potential of the candidates. VFDB contains virulence factors of the well-characterized bacterial pathogens and it provides the comprehensive search of sequence similarity to identify potential virulence factors. The allergenicity of vaccine candidates was estimated by using AllerTOP v.2.0 and AllergenFP v.1.0 servers. Both servers use auto-cross covariance (ACC) transformation to transform the amino acid sequences of the proteins into uniform equal-length vectors and the proteins are classified according to a training set consisting of 2427 known allergens from different species and 2427 non-allergens. Finally, the molecular weight of the candidates was estimated via Expasy ProtParam and the proteins having molecular weight ≤ 110 kDa were respected as studying small candidates is less complicated for vaccine development [26]. All these analyses resulted in nine vaccine candidates (Table 1). TonB-dependent receptors, as well as ornibactin (siderophore) receptors, have significant potential as vaccine candidates due to their wide distribution on the membrane and surface exposure [27]. Also, flagellar hook-associated proteins and metalloproteinases have been found as novel vaccine candidates in other pathogens including *Pseudomonas aeruginosa*, *Edwardsiella tarda*, and *Staphylococcus aureus* [28, 29, 30]. Moreover, bacterial *phospholipases C* can be also used in vaccine design as Mycobacterium abscessus *phospholipase C* demonstrated protective action against the infection caused M. abscessus that is an emerging CF pathogen [31].

Table 1. Putative vaccine candidates evaluated in terms of antigenicity, virulence, and allergenicity through the VaxiJen, VFDB, AllerTOP, and AllergenFP. The estimated molecular wieght of the proteins was calculated via Expasy ProtParam.

Uniprot ID	Protein name	Location	Vaxign-ML score	Adhesin probability	VaxiJen score	Molecular weight (kDa)
B4E8V0	Putative outer membrane usher protein	Outer membrane	93.2	0.791	0.6515	88.35
B4EKY9	Phospholipase C	Extracellular	93.5	0.636	0.6422	84.53
B4E998	Ornibactin receptor	Outer membrane	97	0.876	0.6508	81.70
B4EK02	TonB-dependent receptor	Outer membrane	95.9	0.697	0.6475	79.99
B4EGU2	TonB-dependent siderophore receptor	Outer membrane	98	0.864	0.6349	79.83
B4EJZ3	TonB-dependent receptor	Outer membrane	98.5	0.706	0.6402	76.80
B4ENB0	TonB-dependent receptor	Outer membrane	94.8	0.685	0.6484	76.13
B4ENF7	Neutral metalloproteinase	Extracellular	95	0.850	0.6513	59.90
B4E8L9	Flagellar hook-associated protein 3 (HAP3)	Extracellular	94.3	0.846	0.6630	42.13

Sorting T and B Cell Epitopes of Vaccine Candidates

T and B cells are the essential elements of the immune system that play roles in cell-mediated and humoral immunity through pathogen-specific response, respectively. The pathogen-specific response is provided by the recognition of the epitopes of the antigenic proteins by T or B cell receptors. For the recognition by T cell receptors, antigens are processed and presented by MHC I or MHC II molecules expressed on the cell surface of antigen-presenting cells. Therefore, T cell epitopes represent the peptide sequences that can be bound by MHC molecules. While MHC I molecules recognize short peptides with the length between 8 and 11 amino acids, MHC II molecules bind the residues that extend from 9 to 22 in length [32]. On the other hand, the receptors on B cells can directly bind to the epitopes of solvent-exposed antigens which can result in the production of antibodies. Finally, B cells act equally as professional antigen-presenting cells and activate cognate T cells by presenting peptidic antigens bound on B cell receptors. B cell epitopes are grouped as linear (continuous) and conformational (discontinuous) where linear epitopes contain sequential residues while conformational epitopes consist of disordered patches of peptides. The linear epitopes are considered in the case of a vaccine design as they can be used for immunizations to produce antibodies [32, 33].

Antigenic, virulent, and non-allergenic nine vaccine candidates were subjected to the IEDB server for the identification of putative MHC I and MHC II binding epitopes as well as linear B cell epitopes. Moreover, the immunogenicity of MHC I binding epitopes was also evaluated via the server. After the identification of MHC II binding epitopes, they were cross-checked with immunogenic MHC I binding epitopes to reveal common T cell epitopes. Finally, the shortlisted T cell epitopes were then cross-checked with linear B cell epitopes to identify common peptides that can trigger the activation of both T and B cells. The analyses resulted in various common T and B cell epitopes in seven proteins of nine vaccine candidates (Table S3).

For the effective pathogen-specific response, the epitopes should be efficiently recognized by immune cells and thus, their topology has critical importance that they should not get folded inside protein structure for proper exposure. The shortlisted common epitopes were first listed according to their IC50 scores as the lowest one represents the strong binding of epitopes to MHC molecules and B cell receptors [34]. Then, seven vaccine candidates were modeled by using Phyre2 and, for simple demonstration, the MHC I binding epitopes were visualized on the structures via Maestro v12.4 to determine their exo-topology. Among seven candidates, the epitopes of three proteins had the best epitope exposure, suggesting their potential use for the design of a multi-epitope vaccine against Bcc infections (Table 2) (Figure 2).

Conclusion

Bcc bacteria, particularly *B. cenocepacia*, have intrinsic resistance to various antibiotics and are able to adapt to environmental changes. In fact, transcriptional reprogramming is developed during long-term colonization of *B. cenocepacia* which, together with stated features, makes the clearance of the pathogen challenging [2]. In recent years, many studies have been conducted to develop an effective treatment regimen against Bcc infections, but no standard therapy is present up to date. Moreover, there is no vaccine in clinical use against Bcc infections. Due to the complex nature of Bcc, vaccine initiatives should consider various bacterial species for efficient protection. Therefore, multi-epitope vaccines containing different epitopes of diverse antigens from Bcc bacteria

can serve as a potential therapeutic agent. In this sense, the predicted common epitopes of putative vaccine candidates from *B. cenocepacia* can be used to design a multi-epitope vaccine against Bcc infections.

Table 2: Common T and B cell epitopes of putative vaccine candidates with the best exo-topology. They were evaluated through the IEDB and BepiPred servers.

Uniprot ID	Protein name	MHC I peptide	IC_{50} score	MHC II peptide	IC50 score	B cell peptide	BepiPred score
B4E8V0	Putative outer membrane usher protein	PNQLQLEGT	22.71	CTIAALPNQLQLEGT	46.00	NQLQLEG	0.53
B4EKY9	Phospholipase C	SRLAGARAP	16,02	RFTGDLSRLAGARAP	10.00	GDLSRLAG	0.57
B4ENB0	TonB-dependent receptor	PQFARATTV	7,10	QPQFARATTVGVSAG	43.00	PQFARA	0.53

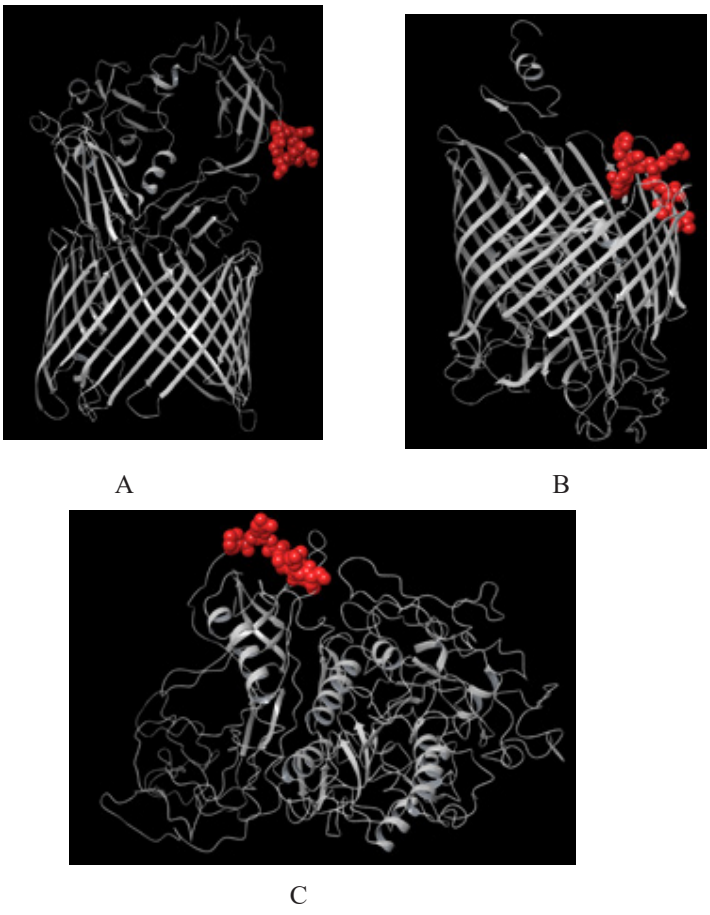

Figure 2: Topological view of the three vaccine candidates (in gray) with their corresponding epitopes (in red). A) Putative outer membrane usher protein (B4E8V0) with its epitope 'PNQLQLEGT', B) TonB-dependent receptor (B4ENB0) with its epitope 'PQFARATTV', and C) Phospholipase C (B4EKY9) with its epitopes 'SRLAGARAP'.

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