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In Silico Analyses of Extracellular Proteins of *Acinetobacter baumannii* as Immunogenic Candidates

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Abstract

Background: *Acinetobacter baumannii* is an important nosocomial pathogen causing high morbidity and mortality in immunocompromised patients with prolonged hospitalization. Multidrug-resistant *A. baumannii* infections are on the rise worldwide. Therefore, the discovery of an effective vaccine against this bacterium seems necessary as a cost-effective and preventive strategy.
Methods: In this present study, 35 genomes of *A. baumannii* strains were considered, and the extracellular proteins were selected, maximally having one transmembrane helix with high adhesion probability and no similarity to host proteins, as immunogenic candidates using the web tool Vaxign. Subsequently, the role of these selected proteins in bacterial pathogenesis was investigated using VICMpred. Then, the major histocompatibility complex class II, linear B-cell epitopes, and conservation of epitopes were identified using the Immune Epitope Database, BepiPred, and Epitope Conservancy Analysis, respectively. Finally, the B-cell discontinuous epitopes of each protein were predicted using ElliPro and plotted on the three-dimensional structure (3D) of the proteins. The role of the unknown proteins was predicted using the STRING database.
Results: In this study, eight acceptable immunogenic candidates, including FilF, FimA, putative acid phosphatase, putative exported protein, subtilisin-like serine protease, and three uncharacterized proteins, were identified in *A. baumannii*.
Conclusions: The results of the STRING database showed that these three uncharacterized proteins play a role in nutrition (heme utilization), peptide bond cleavage (serine peptidases), and cellular processes (MlaD protein). Extracellular proteins might play a catalyst role in the outer membrane protein-based vaccine of *A. baumannii*. Furthermore, this study proposed a list of potent immunogenic candidates of extracellular proteins.

Keywords: Nosocomial Pathogen, Reverse Vaccinology, Fimbriae, Pilus Assembly, Protein-Protein Interaction

1. Background

Acinetobacter baumannii, as a nosocomial pathogen, causes various infections in different anatomical sites of the human body, such as skin, bloodstream, and urinary tract, with high mortality and morbidity rates (1, 2). This bacterium has a high incidence in immunocompromised individuals, especially those who have had a prolonged ($\geq$ 90 days) hospital stay (3). Although this Gram-negative coccobacillus was considered a low-grade pathogen in the past, it has become a major public health concern nowadays due to its high resistance to most clinically available antibiotics. In recent years, various strategies, including vaccination (4), monoclonal antibodies (5), phage therapy (6), iron chelators, and gallium nitrate (7), have been developed to control infections with this bacterium. Of all the aforementioned strategies, vaccination might be the most effective approach to prevent infections associated with this bacterium, especially in target groups, such as the elderly, soldiers, and immunocompromised patients (8).

Multicomponent antigens and purified recombinant proteins are the two main vaccine strategies against *A. baumannii*. Although these immunogenic candidates showed acceptable results in animal models, they are not yet satisfactory for human injection (9, 10). Less attention has been paid to the extracellular proteins of *A. baumannii* as potential immunogenic candidates. According to the results from different bioinformatics databases and studies, this group of proteins plays an important role in the pathogenesis of *A. baumannii* (11).

With the advances in bioinformatics and the presence

of the whole genome sequence of bacteria in various bioinformatics databases, such as Vaxign, the ideal promising immunogenic candidates can be found by reverse vaccinology method and in silico approach. In this context, it is worth mentioning that *Meningococci* group B represents the first example of the successful application of reverse vaccinology (12). With the consideration of all the above-mentioned information, this study investigated a dataset of *A. baumannii* whole-genomes to analyze extracellular or secreted proteins as putative immunogenic candidates.

2. Methods

2.1. Identification and Selection of Putative Immunogenic Candidates

In the first step, Vaxign (vaccine design webserver), a vaccine target prediction and analysis system based on the principle of reverse vaccinology, was used to select ideal putative immunogenic candidates. A total of 35 genome sequences of *A. baumannii* strains were selected from this database. Subsequently, the *A. baumannii* strain ACICU (belonging to ST2Pas) was determined as a reference strain. In the next step, the analysis was restricted to only extracellular proteins present in all genomes. In addition, other criteria, such as the number of transmembrane helices ≤ 1 and no similarity to human and mouse proteins, were considered (Figure 1) (13).

2.2. Antigenicity Measurement and Function Prediction of Putative Immunogenic Candidates

The VaxiJen database is a helpful tool to predict protective antigens and subunit vaccines. This database was used to measure the antigenicity of putative immunogenic candidates with a cut-off $\geq$ 0.3 (14). The VICMpred is a useful database for the prediction of functional classes (e.g., virulence factors, cellular processes, and metabolism molecules). The amino acid sequence of each putative immunogenic candidate was submitted to this database to predict their functions and scores (15).

2.3. Identification of Major Histocompatibility Complex Class II Binding Sites

The prediction of $CD4^+$ T cell immunogenicity was performed using the Immune Epitope Database (IEDB) Analysis Resource. In this study, the maximum combined score threshold was 50%, and a combined score $\geq$ 45 was acceptable. In the next step, the number of major histocompatibility complex class II (MHC-II) epitope binding sites was divided by the number of amino acids for each protein and expressed as ratios (16, 17).

2.4. Identification of Continuous and Discontinuous B-Cell Epitopes

BepiPred predicts the location of continuous B-cell epitopes from amino acid sequence using a Hidden Markov Model. For this purpose, the sequence of each protein in FASTA format with a threshold of $\geq$ 0.65 was submitted (18). In this part, similar to the MHC- II epitopes, the number of continuous B-cell epitopes was divided into the number of amino acids for each immunogenic candidate and reported as ratios.

ElliPro is another database for antibody epitope prediction. This database was used to predict the B-cell discontinuous epitopes of each putative immunogenic candidate with a threshold of $\geq$ 0.8 (19). The three-dimensional (3D) structure prediction files were obtained using Robetta servers and refined using the ModRefiner server. In the next step, their quality was checked using QMEAN Swiss Model. The Protein Data Bank (PDB) files of the putative immunogenic candidates were shown in the Jmol software (version 14) for illustration. This software is an open-source Java viewer for chemical structures in three dimensions (20). In the next step, the B-cell discontinuous epitopes of each immunogenic candidate were displayed on the surface of the proteins with different colors.

2.5. Evaluation of MHC-II and B-Cell Epitopes Conservancy among Different Strains of Acinetobacter baumannii

In this part, the MHC-II and B-cell epitopes were transmitted to Epitope Conservancy Analysis in IEDB Analysis Resource, and their conservation among different strains of *A. baumannii* was analyzed (21).

2.6. Identification of Conserved Domains in Selected Proteins

Conserved Domain Database is a part of NCBI's Entrez query that provides an annotation of protein sequences with the location of the conserved domain. This database was used to predict the conserved domain of each putative immunogenic candidate (22).

2.7. Protein-Protein Interaction Network

The STRING is a helpful database for understanding how proteins interact with each other. This database contains information from numerous sources, including experimental data, computational prediction methods, and public texts. In this part, the STRING database was used to understand the interaction of uncharacterized proteins with other *A. baumannii* proteins. This approach can be effective for the function estimation of unknown proteins (23). The red spheres show the target proteins, and others represent other proteins in relation to the hypothetical proteins. Each interaction has a specific color (light

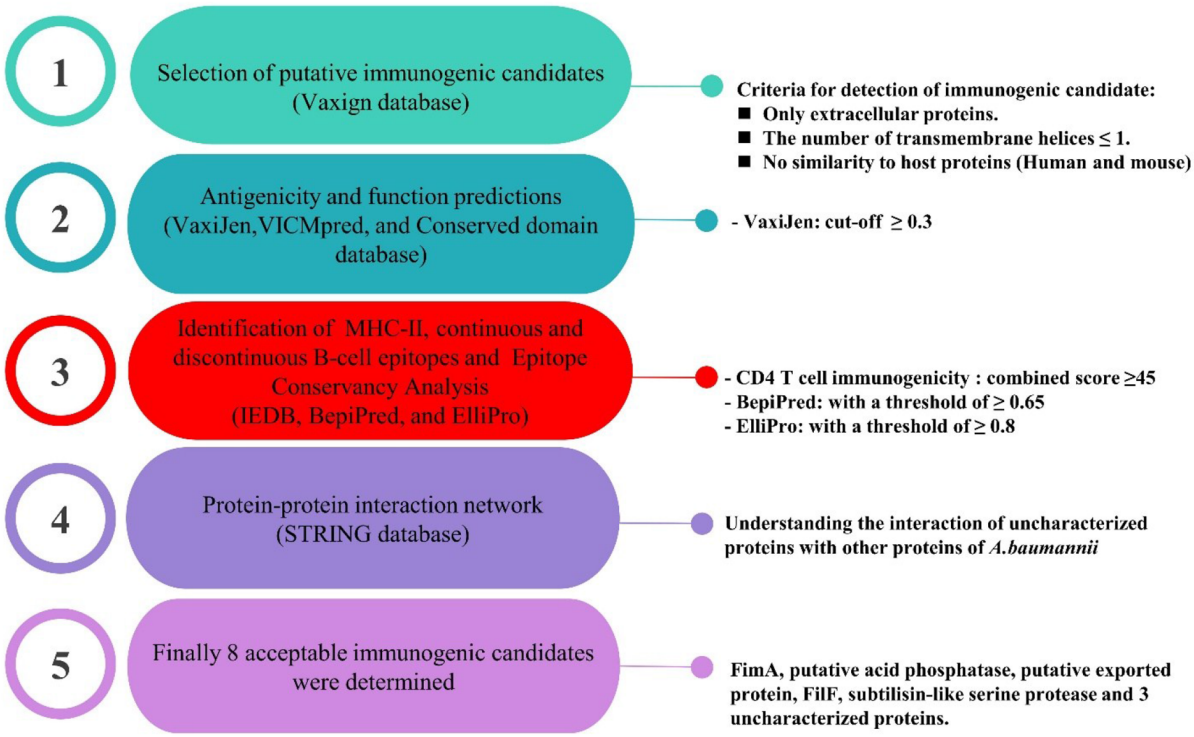

Figure 1. Flowchart for Identification of New Putative Immunogenic Candidates against *Acinetobacter baumannii* by Reverse Vaccinology Method (Containing All Databases, Tools, and Thresholds)

blue: from curated databases, violet: experimentally determined, green: gene neighborhood, red: gene fusions, blue: gene co-occurrence, yellow: text mining, black: coexpression, grey: protein homology).

3. Results

3.1. Identification and Characterization of Putative Immunogenic Candidates

In this study, 19 extracellular proteins were discovered in the Vaxign database. The accession numbers and other characteristics of all 19 extracellular proteins are shown in Appendix 1 (see Supplementary File). All of these proteins have a ≤ 1 transmembrane helix. The proteins that have similarities to host proteins were excluded. The next step was to measure the antigenicity of the remaining proteins, and the proteins with the highest antigenicity value were considered potential immunogenic candidates. These proteins were FilF (ADX02311.1), FimA (ADX03638.1), putative acid phosphatase (ADX03936.1), putative exported protein (ADX04185.1), subtilisin-like serine protease (ADX04983.1), and three uncharacterized proteins (i.e., ADX02417.1, ADX04125.1, and ADX05009.1). According to VICMpred prediction, the aforementioned proteins are categorized as virulence factors, metabolic molecules, and cellular processes. Table 1 shows the complete results of this investigation.

3.2. Identification of MHC-II and Continuous and Discontinuous B-Cell Epitopes of Putative Immunogenic Candidates

Table 1 shows a list of the ratios of MHC-II and linear B-cell epitopes of each immunogenic candidate. In the case of discontinuous B-cell epitopes, the predicted epitopes were plotted on a 3D structure of the putative immunogenic candidates in Figure 2 (the full results of discontinuous B-cell epitopes prediction were listed in Appendix 2).

3.3. Evaluation of MHC-II and B-Cell Epitopes Conservancy among Different Strains of Acinetobacter baumannii

Table 2 shows a summary of the results of MHC-II and B-cell epitopes conservancy among different strains of *A. baumannii* with the percentage of conservation. FimA, putative exported protein, and uncharacterized proteins, including ADX04125.1 and ADX05009.1, had high conserved epitopes.

Table 1. Characterization of Extracellular Proteins of *Acinetobacter baumannii* as Putative Immunogenic Candidates

No.	Protein Name	Protein Domain	Subcellular Localization (PSORTb)	MHC-II 15-mer Epitopes Sites (CS $\geq$ 45)	T-Cell Epitope Ratio	B-cell Epitopes (T = 0.65)	B-Cell Epitope Ratio	VaxiJen Score	VICMpred
1	FilF	No domain	Extracellular	20	0.030	10 aa	0.0154	0.64	Virulence factors (2.18)
2	Putative uncharacterized protein	No domain	Extracellular	7	0.014	18 aa	0.036	0.72	Virulence factors (2.6)
3	FimA	Fimbrial superfamily protein	Extracellular	9	0.026	6 aa	0.017	0.66	Virulence factors (0.86)
4	Putative acid phosphatase	Polyphosphatase and 5'/3'-nucleotidase SurE	Extracellular	7	0.021	20 aa	0.061	0.62	Metabolism molecule (2.57)
5	Putative uncharacterized protein	No domain	Extracellular	14	0.027	8 aa	0.015	0.54	Virulence factors (0.32)
6	Putative exported protein	TolA-binding protein	Extracellular	8	0.027	37 aa	0.12	0.68	Virulence factors (1.50)
7	Uncharacterized protein	MlaD protein superfamily	Extracellular	7	0.030	30 aa	0.13	0.73	Cellular process (1.54)
8	Subtilisin-like serine protease	Peptidase S8 family	Extracellular	11	0.025	3 aa	0.007	0.57	Metabolism molecule (2.17)

Abbreviation: MHC-II, major histocompatibility complex class II.

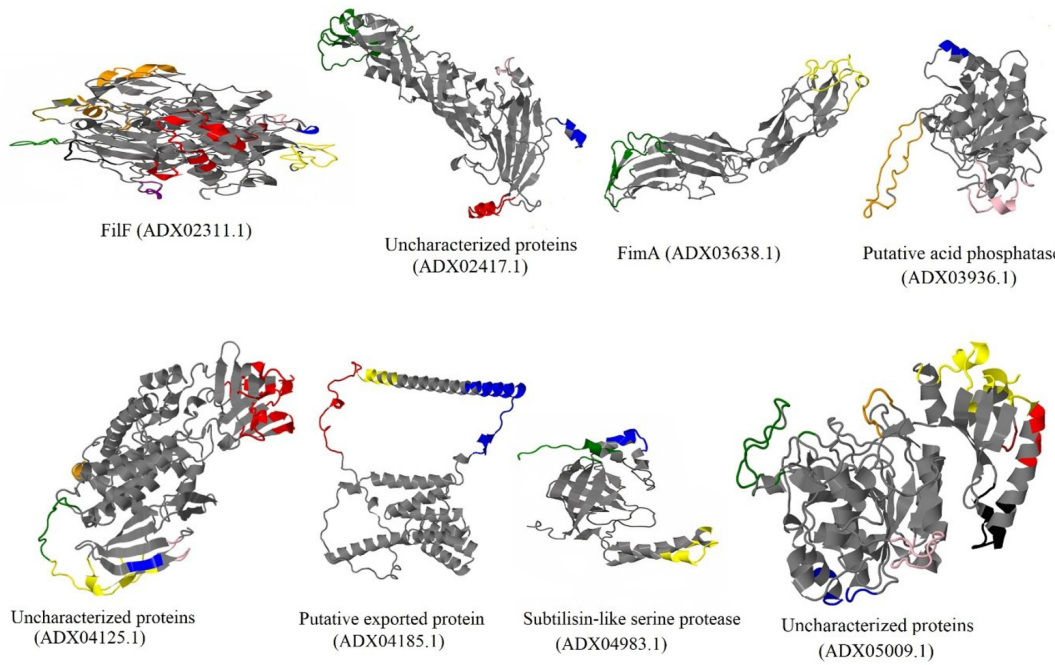

Figure 2. Predicted discontinuous B-cell epitopes of putative immunogenic candidates against *Acinetobacter baumannii* shown on a three-dimensional structure of proteins; FilF, uncharacterized protein (ADX02417.1), FimA, putative acid phosphatase, uncharacterized protein (ADX04125.1), putative exported protein, subtilisin-like serine protease, and uncharacterized proteins (ADX05009.1) with 10, 4, 2, 3, 7, 3, 3, and eight conformational epitopes, respectively.

3.4. Protein-Protein Interaction Network

According to the results from the STRING database, the uncharacterized protein with accession number ADX02417.1 had co-occurrence and neighborhood interactions with heme utilization protein. Another uncharacterized protein with accession number ADX04125.1 had neighborhood interactions with ClpA, ClpP, and ClpS. The CLP protease family is a family of serine peptidases. This class of enzymes cleaves peptide bonds in proteins, with serine serving as the nucleophilic amino acid at the (enzyme) active site (24). The last uncharacterized protein, ADX05009, belongs to the MlaD protein superfamily. This family of proteins contains MlaD, which is a component of the Mla pathway, an ATP-binding cassette transport system that maintains the asymmetry of the outer membrane (OM) (Figure 3) (25).

4. Discussion

Acinetobacter baumannii is an emerging opportunistic pathogen responsible for healthcare-associated infections and has become a global problem (26). Due to the frequent outbreaks caused by multidrug-resistant *A. baumannii*, the development of a safe and effective vaccine appears necessary. Despite all efforts to date, there is still no licensed vaccine, and studies continue to find an ideal vaccine candidate (27). In terms of vaccinology, an ideal or promising immunogenic candidate should be exposed on the bacterial surface (OM or extracellular proteins) for host immune system induction, have a high antigenicity value, be conserved across circulating strains, and be expressed during bacterial infection. In addition, the ideal immunogenic candidates should have an important role in bacterial pathogenesis (28). In the case of *A. baumannii*, most studies focused on outer membrane proteins (OMPs), such as OmpA, OmpW, and Omp22 (29), and extracellular proteins were underestimated. However, studies showed that the consideration of OMPs as immunogenic candidates had some obstacles and limitations. For example, OmpA is the most abundant OMP in *A. baumannii* and is involved in host-cell recognition, biofilm formation, and antibiotic resistance. Nevertheless, the coexistence of different OmpA variants and subvariants in the *A. baumannii* population can severely limit the efficacy of a vaccine (30).

However, the present study focused on the extracellular proteins of *A. baumannii* and selected the best of them according to the defined criteria, such as transmembrane helix, antigenicity score, and high adhesion probability. The current study showed that extracellular proteins, such as FilF and FimA, can be considered putative immunogenic candidates. In line with the results of the present study, Singh et al. presented FilF protein as a putative vaccine candidate in an in vivo experiment. The aforementioned study showed that immunization with FilF can produce a high antibody titer and provide 50% protection against a standard lethal dose of *A. baumannii* (31). The expression of FimA, a type 1 fimbrial protein, increases during the bacterial infection process and leads to the association of bacteria to biofilm formation. In this context, the efficacy of FimA and FimH for vaccine development was previously investigated (32).

In line with the results of the current study, an in silico study conducted by Zadeh Hosseingholi et al. showed that proteins involved in pilus assembly and four hypothetical proteins were likely immunogenic candidates. The aforementioned results underscored the importance of the pilus assembly system in the pathogenesis and immunogenicity of *A. baumannii*. Furthermore, the aforementioned data suggest that in silico approaches might be helpful in discovering likely drug and immunogenic candidates that have been ignored up to now (33).

Moreover, putative acid phosphatase, putative exported protein, and subtilisin-like serine protease were determined as desirable immunogenic candidates. The acid phosphatase enzyme is able to dephosphorylate various organic substances (34). This enzyme plays a fundamental role in Gram-negative bacteria (35). The putative acid phosphatase has a SurE domain which is expressed when bacterial cells are under environmental stress and in the steady growth phase of bacteria (36). The putative exported protein has a TolA-binding domain which has a vital role in the import mechanisms for the uptake of bacteriocins and the deoxyribonucleic acid of filamentous bacteriophage (37). The subtilisin-like serine proteases are widespread enzymes among bacteria. For example, Mycosin-1, a subtilisin-like serine protease in *Mycobacterium tuberculosis*, is expressed after macrophages infection and is submitted to proteolytic processing (38).

This study identified three uncharacterized proteins as potential immunogenic candidates according to the defined criteria. The obtained results from the STRING database demonstrated that these proteins had a role in nutrition (heme utilization), peptide bond cleavage (serine peptidases), and cellular processes (MlaD protein), respectively. Another in silico approach by Mobarak Qamsari et al. indicated that the ZnuD protein is a potentially suitable antigen for subunit vaccine development. This protein belongs to TonB-dependent receptors and is involved in zinc acquisition (39). Among the aforementioned proteins, the uncharacterized protein with the MlaD protein superfamily domain is more desirable for further investigation due to its high score of MHC-II and B-cell epitopes ratios. MlaD stands for the maintenance of OM lipid asymme-

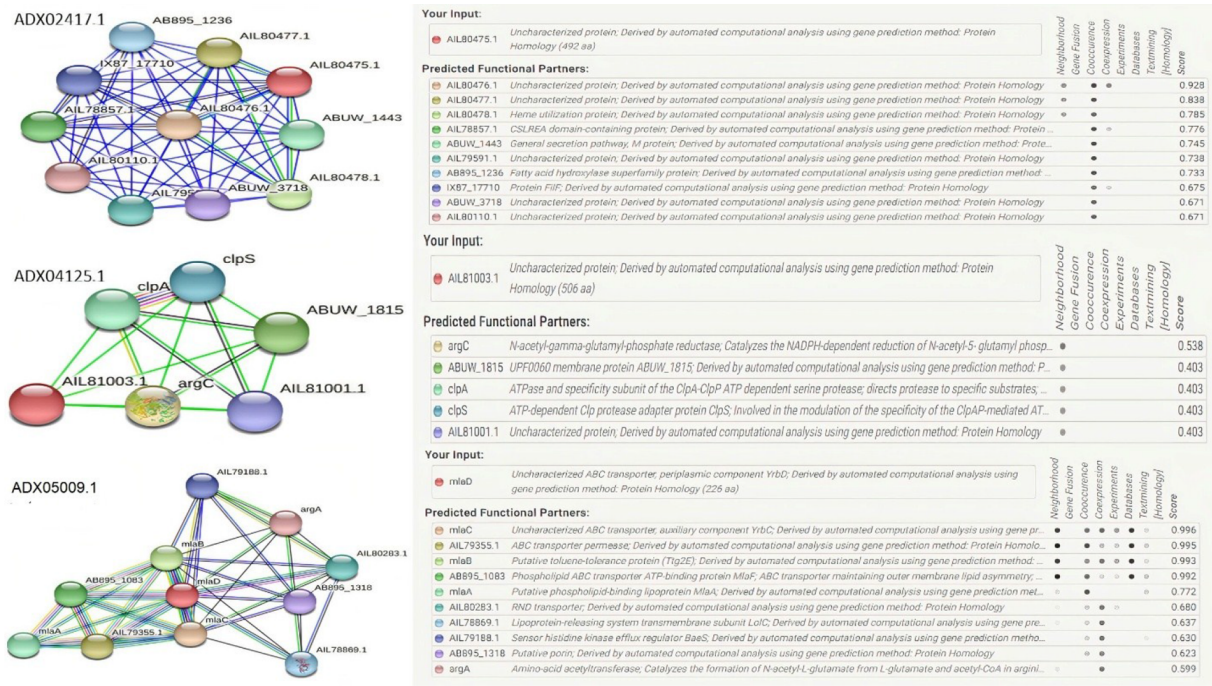

Figure 3. Protein-protein interaction network of uncharacterized proteins (ADX02417.1, ADX04125.1, and ADX05009.1) with other proteins of *Acinetobacter baumannii*; red spheres illustrating hypothetical proteins, and each of others representing other proteins in relation to hypothetical proteins.

try. In Gram-negative bacteria, the OM has an asymmetric structure, with lipopolysaccharides on the outer surface and phospholipids on the inner surface. This unique feature makes Gram-negative bacteria resistant to numerous toxic chemicals and antibiotics (40).

In the present study, in addition to the immunogenic candidates, several MHC-II and B-cell epitopes were identified. These epitopes were surface exposed and might robustly induce the host immune system. Several studies have been conducted on the antigenic epitopes of *A. baumannii*. Girija, for example, targeted the GacS protein involved in citrate utilization in *A. baumannii* and proposed five antigenic peptides as promiscuous vaccine candidates. It should be considered that these epitopes are valuable resources for multiepitope target design (41).

4.1. Conclusions

It appears that OMPs are suitable immunogenic candidates. However, due to some disadvantages of these proteins (e.g., antigenic variation), the consideration of extracellular proteins might compensate for the disadvantages and strengthen the vaccine formulation. Furthermore, this study proposed a list of potent immunogenic candidates of extracellular proteins. In addition to finding putative immunogenic candidates, this in silico approach provided new insights into the discovery of new virulence factors of *A. baumannii* that were previously undiscovered or ignored. However, discovering the effective immunogenic candidates or virulence factors requires further studies in animal models and role confirmation assays, such as site-directed mutagenesis.

4.2. Limitations

The results obtained from this study were only based on bioinformatics investigation; therefore, in vivo and in vitro investigations are needed to confirm the obtained results.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Footnotes

Conflict of Interests: The authors have no conflict of interest to declare.

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Table 2. Conservancy Analysis of Top Score Major Histocompatibility Complex Class II and B-Cell Epitopes among Different Proteomes of *Acinetobacter baumannii*

Protein Name	MHC-II Epitopes	Conservation (%)	B-cell Linear Epitopes	Conservation (%)
FilF	YLERNVFKTSGNVKT	58.93	CTPTTSDNGAEDS	64.29
	REIQSTTPLRLSFSN	73.21	TAVNGKSKEINP	67.86
	YLLTTKVKPAKLNVN	69.64	KGSAIVIDNKVQN	67.86
	DKNDETIKVAIALIK	92.86	TDIKPNSTTTDMS	75.00
	YKLLVQTTNLSNAGV	69.64	AATFNDPLNGDQS	58.93
	IVIDNKVQNPYLLTT	67.86		
	FLGNVNLDTIGKFTA	92.86		
FimA	MKKMLIKSGLLITSF	90.00	LNYPVSSIGNNV	98.00
	LITSFFIYNQTYANC	100.00	DSTDFSGYYPYKRSLTPNTTYTLS	69.00
	TTYTLSPGYFVMEVI	97.00	NGGENNSGVPTSNT	97.00
	SPGYFVMEVIKTAAT	97.00	ISDTSNSQVINN	72.00
	TTTVLSSSPILIASS	97.00		
	VELLWNMNGANNPIR	92.00		
	PLTARYYQTASNVTA	94.00		
Putative acid phosphatase	ALNILLVNDDGLTSN	100.00	NDKQIAAENGCHNGAAPIGAPAAGTFTKAGY	86.00
	GRGGAIVMYSSTTIT	95.00	NTVDDKTLNNPNS	84.62
	IVMYSSTTITADNDK	95.00		
	PKNLTTSTPFAFSKI	98.00		
	YKLKFQVTKDNKGNN	73.00		
	GQEWLKIRLKSLFNK	84.00		
Putative exported protein	HSLLFIALMSTTSLY	98.00	PDSATQDDSSNATSDNTTPASAPAPQTTESNKVAAVPATQTSEQQPSAPTT	37.00
	IALMSTTSLYANIPI	99.00	AQNQSNSLEL	71.00
	TTSLYANIPIESRGL	99.00		
	IAPMQNFIKNHPNSI	93.00		
	NFIKNHPNSIYTGNA	96.00		
Subtilisin-like serine protease	GIFVIIFGIALFFLA	91.00	TQATPAQQKTMEQQLISNMNSITSIDE	30.00
	LFFLAMKVSGLVGTN	96.00	GKVAAGSSSAEEKAPASTDSS	41.00
	GRVDSITLDPVTRLA	97.00		
	MEQQLISNMNSITSI	34.00		
	DEDAYIMVATNGLLG	99.00		
	EKYLKIVPGGGLNYL	50.00		
Uncharacterized protein (ADX02417.1)	MRQFTSLQVAILALG	55	TDSANLEKLRDNSSNI	87.00
	GQALMSMSYIAPTDS	93.00	DANGNPLPMSNEDRASS	58.00
	EAELEINTNIRKLQL	87.00	GTENTTTPNGIN	61.00
	VVGLRLSAEKFIGLL	74.00	NIYVGHSSSYPVDGTVQYNAAGPHDPAYPEPTGIYTQGGK	78.00
	ISGYMKVQSDSSGLI	80.00		
	TANLNLTQSLGLIH	97.00		
	NLTQSLGLIHNLPIN	98.00		

	LGLIHNLPINSPMYL	100.00		
	SPMYLALQNQMLQWP	93.00		
	LFPQISAAVSAYLQA	51.00		
Uncharacterized protein (ADX04125.1)	ASQDVYRSLLNAFKD	91.00	SSGGSSNTPKPSEPT	100.00
	YRSLLNAFKDTNIPS	99.00	ADLQDQSHLDPIG	100.00
	TLLDIAANQKVTRDY	93.00	GDYRRAVG	100.00
	GNTSEFFRIDGAGDY	97.00	NSSSSEPNNSTQQYN	100.00
	FFRIDGAGDYRRAVG	99.00		
	CQLNFRANGQIELIK	98.00		
	IELIKGSQSYRSALS	92.00		
	QQYNFIQLHIKANKI	94.00		
	IQLHIKANKIVSAQA	94.00		
Uncharacterized protein (ADX05009.1)	LERRSYIATNKKNLQ	99.00	VVYSFDSAPDD	100.00
	AGGVQVLRDYNNLPL	97.00	VKYTSECSDS	99.00
	VLRDYNNLPLAFYRI	99.00	IGGVSWGNPVKCSDPTTAADKVACFSNG	97.00
	NDPNVKAVYPNRINR	98.00		
	TKTKIIGIDVFRKVR	96.00		
	IGIDVFRKVRSQGKW	97.00		
	SSYGTAFANARAAGV	98.00		
	IALLRANSVSPTESI	97.00		

Abbreviation: MHC-II, major histocompatibility complex class II.