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In silico and in vivo Investigations of the Immunoreactivity of *Klebsiella pneumoniae* OmpA Protein as a Vaccine Candidate

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ABSTRACT

Background: The growing threat of antibiotic resistance and *Klebsiella pneumoniae* infection in healthcare settings highlights the urgent need for innovative solutions, such as vaccines, to address these challenges. This study sought to assess the potential of using *K. pneumoniae* OmpA as a vaccine candidate through both in silico and in vivo analyses.

Methods: The study examined the OmpA protein sequence for subcellular localization, antigenicity, allergenicity, similarity to the human proteome, physicochemical properties, B-cell epitopes, MHC binding sites, tertiary structure predictions, molecular docking, and immune response simulations. The *omp*A gene was cloned into the pET-28a (+) vector, expressed, purified and confirmed using Western blotting analysis. IgG levels in the serum of the immunized mice were measured using ELISA with dilutions ranging from 1:100 to 1:6400, targeting rOmpA and *K. pneumoniae* ATCC 13883. The sensitivity and specificity of the ELISA method were also assessed.

Results: The bioinformatics analysis identified rOmpA as a promising vaccine candidate. The immunized group demonstrated significant production of specific total IgG antibodies against rOmpA and *K. pneumoniae* ATCC1 13883, as compared to the control group ($p < 0.0001$). The titers of antibodies produced in response to bacterial exposure did not show any significant difference when compared to the anti-rOmpA antibodies ($p > 0.05$). The ELISA test sensitivity was 1:3200, and the antibodies in the serum could accurately recognize *K. pneumoniae* cells.

Conclusion: This study is a significant advancement in the development of a potential vaccine against *K. pneumoniae* that relies on OmpA. Nevertheless, additional experimental analyses are required. ***DOI: 10.61186/ibj.4023***

Keywords: Klebsiella pneumoniae, Vaccine, Outer Membrane Protein

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List of Abbreviations:
3D: three-dimensional; ***A. baumannii*:** *Acinetobacter baumannii*; ***E. coli:*** *Escherichia coli*; **ESKAPE:** *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species; **HvKp:** hyypervirulent *Klebsiella pneumoniae*; **IFN-γ:** interferon gamma; **IPTG:** isopropyl-beta-thiogalactopyranoside; ***K. pneumoniae:*** *Klebsiella pneumoniae*; **LAL:** limulus amebocyte lysate; **OmpA:** outer membrane protein A; ***P. aeruginosa*:** *Pseudomonas aeruginosa*; **PBS:** phosphate buffered saline; **rOmpA:** recombinant outer membrane protein A; **SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **Th1:** type 1 T helper; **TLR:** toll-like receptor

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INTRODUCTION

Antimicrobial resistance presents a significant global health dilemma contributing to thousands of deaths each year. *K. pneumoniae*, a Gram-negative bacterium, is commonly found in hospital environments. This bacterium, along with other ESKAPE organisms, is responsible for a significant number of infections acquired in healthcare settings[1]. *K. pneumoniae* possesses a large number of antibiotic resistance genes, which play an essential role in the emergence and spread of strains resistant to multiple drugs or even extensively drug-resistant[2,3]. The slow progress in developing new antibiotics poses challenges in addressing drug-resistant bacterial strains such as *K. pneumoniae.* As a result, it becomes crucial to focus on antibacterial programs capable of circumventing bacterial drug resistance mechanisms. Vaccines play a vital role in this regard, as they offer an effective strategy of protection against drug-resistant bacterial infections[4].

In the past few decades, extensive research has been conducted to develop effective vaccines against *K. pneumoniae.* Various categories of vaccines, such as live-attenuated vaccines, inactivated whole-cell vaccines, outer membrane vesicles containing multiple virulence factors, protein-based vaccines, conjugate vaccines, and ribosomal vaccines, have been explored. However, despite these efforts, at present, there is currently no FDA-approved vaccine for *K. pneumoniae.* Therefore, there is an urgent need to design and develop a broad-spectrum vaccine that can effectively combat *K. pneumoniae*[5,6].

OmpA is a highly conserved class of proteins found in the Enterobacteriaceae family. The rOmpA from *K. pneumoniae* activates antigen-presenting cells, including macrophages and dendritic cells. It also triggers the release of cytokines by dendritic cells and induces the maturation of dendritic cells. Given the extensive presence of OmpA in the bacterial cell wall and its conservation among the Enterobacteriaceae family, OmpA holds immense importance in bacterial virulence and growth[7,8].

Advancements in bioinformatics and the availability of immunoinformatic databases have facilitated the evaluation of potential immunogenic candidates through in silico approaches. Despite various immunoinformatic studies on vaccine candidates against *K. pneumoniae*, there is limited data on the potential of OmpA to be utilized as an effective component in the development of an efficient vaccine. On the other hand, most studies have investigated the OmpA of *K. pneumoniae* in the form of DNA vaccines or conjugated formulations[9,10]. Therefore, in the present study, we investigated the OmpA protein of *K. pneumoniae* from the bioinformatics perspective, followed by evaluating its immunoreactivity as a potential vaccine candidate in a mouse model.

MATERIALS AND METHODS

Bioinformatic analyses

Protein retrieval and subcellular localization

The protein sequence of OmpA was obtained from both the NCBI (accession number: WP_004131016.1) (https://www.ncbi.nlm.nih.gov/protein) and the UniProt (accession number: A0A0H3YGJ3) (https://www.uniprot.org) databases in FASTA format. To determine its subcellular localization, the protein sequence was submitted to the PSORTb v.3.0.2 online server (www.psort.org/psortb/)[11]. Additionally, the TMHMM Server v. 2.0 web tool (http://www.cbs.dtu.dk/s.ervices/TMHMM/) was employed to identify any transmembrane helices present in the protein[12].

Antigenicity and allergenicity determination

The antigenic properties of OmpA were determined using the VaxiJen online server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a threshold of ≥ 0.4[13]. In addition, its allergenicity was evaluated using the AlgPred 2.0 web tool (https://webs.iiitd.edu.in/raghava/algpred2/batch.html) with a threshold of ≥0.5[14].

Homology of OmpA with the human proteome

To avoid cross-reactivity due to the similarity between OmpA and human proteins (Homo sapiens taxid: 9606), the online PSI-BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) from the NCBI database was utilized (identity >15%, max score >100, and E-value $<10^{-3}$)[15].

Physicochemical properties of OmpA

The Expasy ProtParam server (https://web.expasy.org/protparam/) was used to determine the physicochemical properties of OmpA, including the molecular weight, theoretical pI, estimated half-life, aliphatic index, and instability index[16]. The VICMpred (https://webs.iiitd.edu.in/raghava/vicmpred/) and Vaxign platforms (http://www.violinet.org/vaxign2) were utilized to predict the functional class of the proteins and the probability of adhesion, respectively[17]. Secondary structure of OmpA was illustrated using PDBsum generate algorithm (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/)[18].

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Identification of linear and conformational B-cell and T-cell epitopes

Linear B-cell epitopes of OmpA were predicted employing BepiPred, with a cut-off of 0.6[19]. The identified epitopes were then assessed for allergenic and antigenic properties. MHC-I and MHC-II binding sites were distinguished using the TepiTool web server (http://tools.iedb.org/tepitool/), a prediction tool from the Immune Epitope Database[20]. For MHC-I binding sites, a set of 27 common A and B alleles, representing 97% of the global population, with a peptide length of 9 was used. Similarly, MHC-II binding sites were predicted using a panel of 26 frequent alleles, with a fixed number of 10 overlapping residues. To determine toxic epitopes, the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/design.php) using SVM (support vector machine) method and default parameters was employed. The tertiary structure of OmpA was predicted using the I-TASSER tool (https://zhanggroup.org/I-TASSER/)[21]. The quality of the predicted 3D structure was validated using the ProSA-Web tool https://prosa.services.came.sbg.ac.at/prosa.php) and the Ramachandran plot (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/)[22]. Conformational B-cell epitopes were determined using the ElliPro server (http://tools.iedb. org/ellipro/), with a threshold of ≥0.8[23]. These predicted epitopes were visualized on the surface of the protein using Jmol software (https://jmol.sourceforge. net/). The Protein (FASTA) sequences of 50 *K. pneumoniae* strains with complete annotation were downloaded from the GenBank (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=573). The protein sequences of OmpA protein were blasted with all 50 *K. pneumoniae* strains and the conservancy of linear and conformational B-cell epitopes was reported using Epitope Conservancy Analysis web server (http://tools.iedb.org/conservancy/)[24].

Molecular dockings

The HDOCK web server (http://hdock.phys.hust.edu.cn/) was employed to analyze the binding of OmpA with TLR2 (PDB:2Z7X) and TLR4 (PDB:3FXI)[25], and the results were visualized by LigPlot (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/)[26].

Immune simulation

The C-ImmSim software (https://kraken.iac.rm.cnr.it/C-IMMSIM/index.php), employing default parametric values, was utilized as a predictive tool for assessing the molecular binding within the immune system simulation[27]. On the 7th day, IFN-γ, Th1, IgM, IgG, IgG1, and IgG2 levels were evaluated to gain insights into the immune response.

In vivo phase

Cloning, expression, and purification of rOmpA

The full length of *omp*A gene was amplified by the designed primers (Primer F *Nco*I: CATGCCATGGCA ATGAAAAAGACAGCTA and Primer R *Xho*I: AGT CTCTCGAGAGCCGCCGGCTGAGTTAC), utilizing the genomic DNA of *K. pneumoniae* ATCC 13883 strain. The methods described in the previous study were followed for cloning, expressing, and purifying the rOmpA[8].

Immunization of mice with the vaccine candidate

In the present study, two groups of mice, each comprising six female BALB/c mice aged 6-8 weeks, were examined. The study involved administrating rOmpA protein (50 µg) to one group, while the control group received PBS. Intranasal inoculations were given to the mice on days 0, 7, 14, and 21. Serum samples were obtained one week after the last inoculation.

Serological assessments

After administration of the last vaccine dose, the sera were collected from the mice in each group in order to assess the levels of specific total IgG against rOmpA and whole cell bacteria. To perform the ELISA assay, microtiter plates were first coated with 1 µg/well of purified rOmpA protein and also whole cells of *K. pneumoniae* ATCC 13883 (1.5×10^8 colony-forming unit/well). Following incubation at 4 °C overnight, the plates were washed with PBS 1× and blocked with a solution of 3% BSA in PBS. Subsequently, various dilutions of serum samples (ranging from 1:100 to 1:6400) in 1% BSA were added to the wells. As control, PBS was added to some of the wells coated with rOmpA and *K. pneumoniae* (ATCC 13883). Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) was added and incubated for 2 h. Finally, the reactions were carried out using tetramethyl-benzidine as the substrate. Also, the optical density at 450 nm was measured using an ELISA plate reader (Epoch2, Biotek Instruments, USA).

Determination of sensitivity and specificity

To assess the sensitivity of srum antibodies in detecting OmpA, the total IgG antibody levels in different serum dilutions (1:100-1:6400) aginst rOmpA and *K. pneumoniae* (ATCC 13883) were compared with the control groups. Experiments were performed in triplicate for each dilution. Given that rOmpA shares significant similarity with other Enterobacteriaceae species, particularly *E. coli*, it is crucial to examine the specificity of this protein as an immunogenic target. In order to ascertain the specificity, 1:100 serum dilution was used. A total of five bacteria, including *E. coli*,

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hvKp, *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* (n = 5 from each strain) were evaluated. In addition, for each isolate, three independent replicates were conducted.

Statistical analysis

For statistical analyses and visualization of the data, Prism version 9 (GraphPad Software, Inc.) was employed. The experimental groups were compared using a two-way analysis of variance (ANOVA). A confidence level of 95% ($p \leq 0.05$) was considered statistically significance.

RESULTS

Bioinformatic analyses

Protein retrieval and subcellular localization

The complete sequence of the OmpA comprised 356 amino acids. Our analysis using web tools, PSORTb v.3.0.2 online server and TMHMM Server v.2.0, showed that the OmpA belongs to the Omps superfamily, with localization score of 10. No cytoplasmic signal peptide and no transmembrane helices were detected.

Assessment of antigenicity, allergenicity, and human proteome similarity

The OmpA protein sequence was imported to VaxiJen, and the server confirmed the potential antigenicity of OmpA with a high value (0.67). AlgPred analyses reported OmpA as a non-allergen variant. Additionally, the comparison of the OmpA sequence with the human proteome showed no significant similarity.

Physicochemical characteristics of OmpA

Physicochemical characteristics of OmpA were evaluated by the Expasy ProtParam server. Based on the results, the OmpA molecular weight and isoelectric point parameters were calculated as 37.9 kDa and 6.34, respectively. The molecular formula of OmpA was determined to be C1683H2604N468O519S9 with the net charge of -2.932 at pH 7.4. In *E. coli*, the estimated half-life of the protein was greater than 10 hours in vivo. In yeast, the half-life was greater than 20 hours in vivo, while in mammalian reticulocytes, it was 30 hours in vitro. The protein was classified as stable, with an instability index of 23.94. Additionally, the aliphatic index was evaluated to be 72.95, and the grand average of hydropathicity was -0.326. The functional class analyses suggested the possible involvement of OmpA in cellular processes. The secondary structural content of OmpA comprised 17% alpha-helix, 34% beta strands, 4% disordered regions, and the remaining 45% represented other secondary structural elements (Fig. 1).

Measurement of epitope density

In this study, five linear B-cell epitopes were identified in the OmpA protein (Table 1). The analysis also revealed MHC-I and MHC-II binding sites, with 166 and 94 peptides, respectively (Table S1). Based on the in silico analysis, none of the epitopes exhibited any toxicity. The 3D structure of OmpA was successfully predicted (Fig. 2A) and confirmed to be similar to other proteins determined by X-ray crystallography or NMR spectroscopy (Fig. 2B). The Ramachandran plot analysis indicated that the OmpA tertiary structure had a desirable conformation with more than 84% of residues located in the favored regions and 13% in the allowed regions (Fig. 2C). Detailed information about the predicted conformational B-cell epitopes is presented in Table 2 and Fig. 2D. In addition, the conservancy of theses epitopes was determined using IEDB Analysis Resource, indicating the relative stability of OmpA epitopes among *K. pneumoniae* strains (Tables 1 and 2).

Molecular docking results

The molecular docking analysis revealed favorable interactions between OmpA and TLRs. The docking scores of OmpA-TLR2 and OmpA-TLR4 docked complexes were -352.71and -412.52, respectively (Fig. 3).

Immune simulation

Based on C-ImmSim analysis, the high level of IFN-γ was detected for rOmpA with 390,000 ng/mL. Additionally, the levels of Th1, IgG1, IgM, and IgG2 were detected at 52,000, 13, 12, 1.5 cells/mm^3, respectively (Fig. 4).

In vivo phase results

Expression and purification of rOmpA protein

The *omp*A gene was amplified using PCR and then inserted into the pET28a vector through the cloning process (Fig. 5A). The resulting recombinant plasmid, pET28a-*omp*A, was confirmed through digestion with *Nco*I and *Xho*I restriction enzymes followed by sequencing. The digestion of pET28a-*omp*A produced the pET28a vector backbone and the *omp*A gene fragment (Fig. 5B). Successful transformation of the recombinant plasmid into *E. coli* BL21 (DE3) host was achieved, and induction with 0.5 mM IPTG led to the expression of a 40 kDa protein, as confirmed using SDS-PAGE and western blotting analysis (Fig. 5C and 5D). The histidine-tagged (6× his) recombinant protein was extracted from inclusion bodies obtained from an

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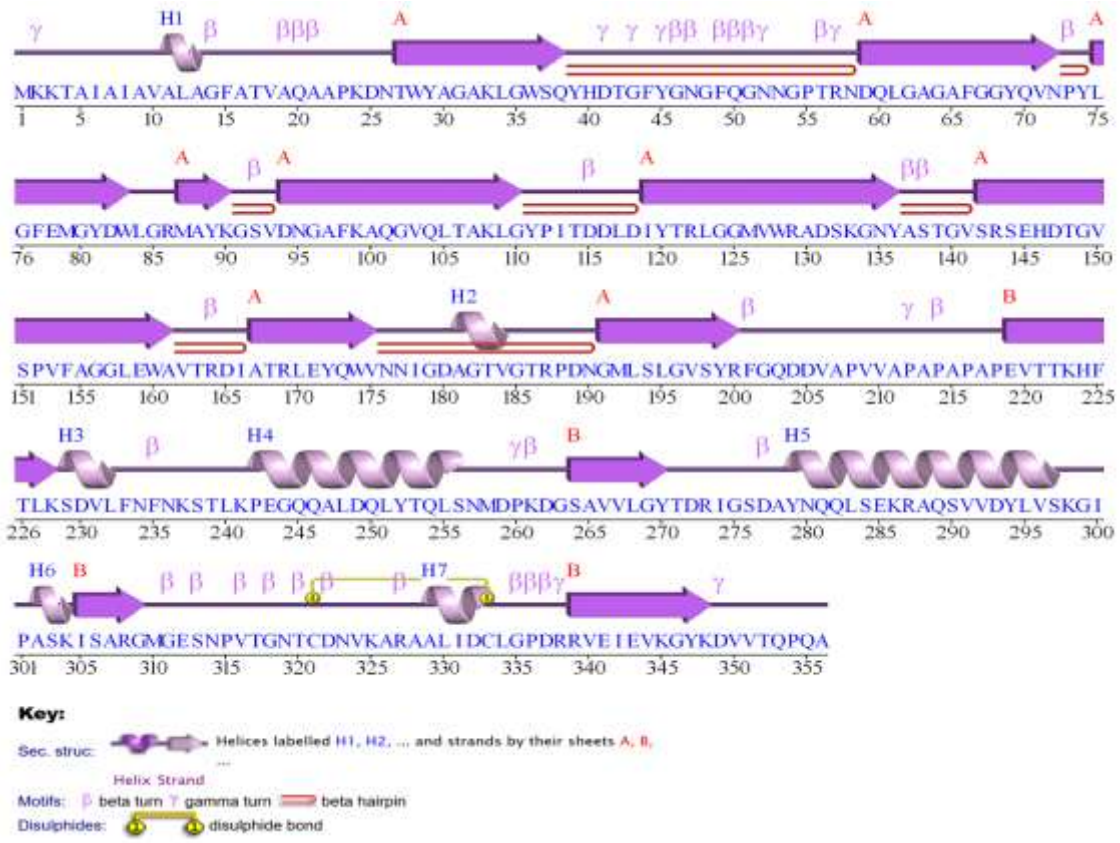

Fig. 1. Secondary structure of OmpA using PDBsum.

induced culture using metal affinity chromatography under denaturing conditions, followed by purification. The purified protein underwent evaluation through SDS-PAGE (Fig. 5E) and western blotting analysis. Next, step dialysis was performed to remove urea from the purified protein in a reducing environment. Using the BCA method, the protein concentration was measured, which found to be 1.3 mg/mL. Additionally, the eluted protein was found to have minimal amounts of lipopolysaccharide (<0.1 EU/mL) according to the LAL test.

Total antibody response analysis and sensitivity determination

The findings indicated that the immunized group generated specific total IgG antibodies against rOmpA and *K. pneumoniae* ATCC 13883, showing a significant increase compared to the control group ($p < 0.0001$). Furthermore, there was no significant difference between the titers of antibodies produced in response to the bacterial strain compared to anti-rOmpA antibodies ($p > 0.05$; Fig. 6). For both recombinant protein and bacteria, there was no significant difference between dilutions of 1:100 to 1:6400 ($p > 0.05$). However, for other investigated dilutions, the antibody level significantly decreased with serum dilution ($p < 0.0001$). In the current study, for both rOmpA and *K. pneumoniae* ATCC 13883, the maximum dilution at which the antibody was significantly detected as compared to controls was 1:3200 (Fig. 6). This dilution value, indicative of the diagnostic sensitivity of the ELISA test, was the same for both rOmpA ($p < 0.0001$) and *K. pneumoniae* ATCC 13883 ($p < 0.001$).

Table 1. Linear and conformational B-cell epitopes of OmpA protein of *K. pneumoniae*

Linear B-cell epitopes	Start-end	Conservancy (%)
GFYGNGFQGNNGPTRNDQ	43-61	94.44
RMAYKGS	86-92	100.00
KGNYASTGVSRSEHDTG	133-149	100.00
QWVNNIGDAGT	173-183	100.00
LGVSYRFGQDDVAP	195-208	85.7

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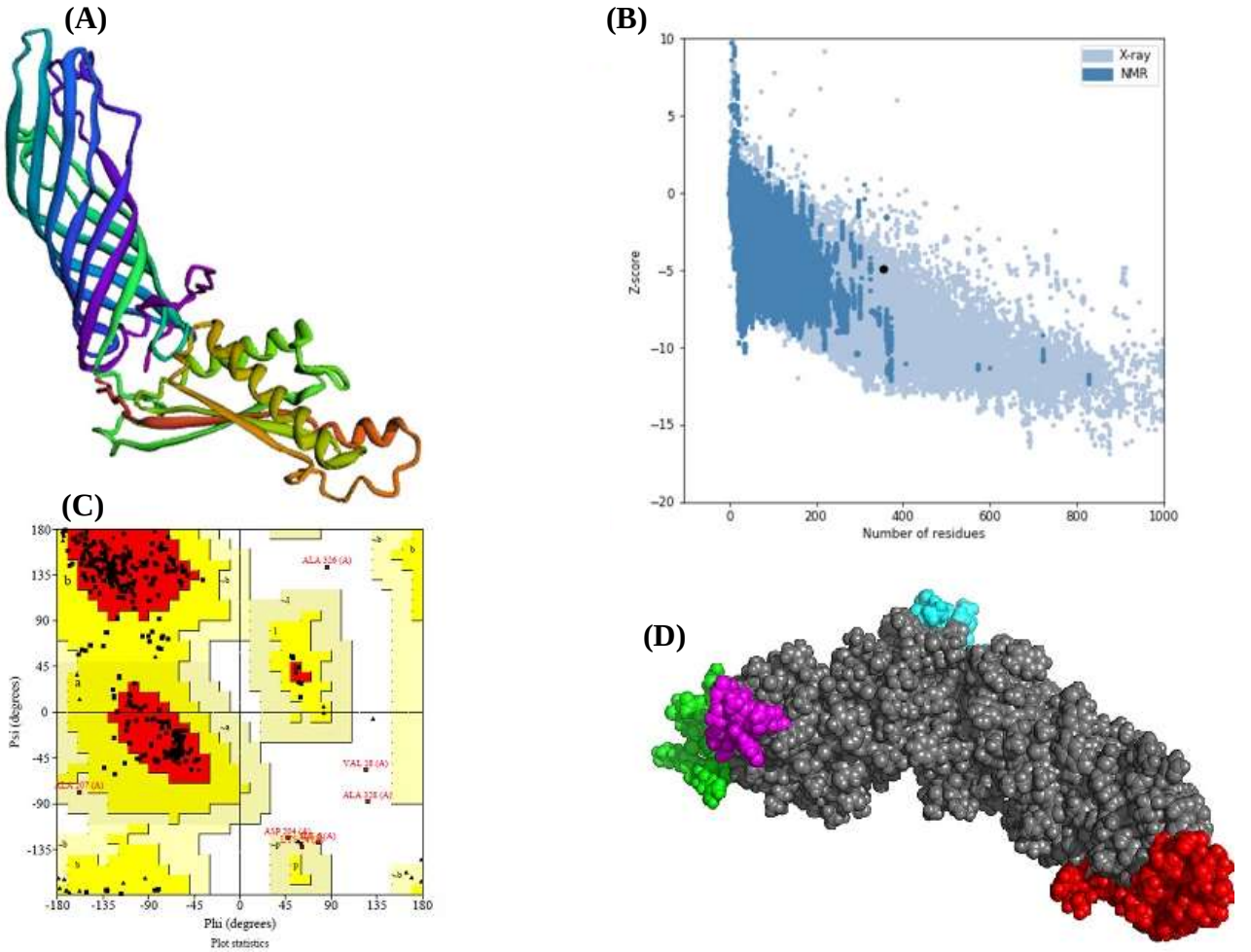

Fig. 2. (A) The tertiary structure of OmpA predicted using the I-TASSER web server. (B) ProSA-web analysis of OmpA 3D structure, showing a Z-score of -4.91. (C) The Ramachandran plot of the OmpA 3D structure, representing that over 84% of the residues were located in the favored and allowed regions. (D) The conformational B-cell epitopes of OmpA was identified using the ElliPro web server and demonstrated on the 3D structure of the protein using PyMOL software. Red: G43, G46, N47, G48, F49, Q50, G51, N52, N53, G54, P55, T56, R57, K90, G91, S92, V93, D94, N95, G134, N135, Y136, A137, S138, T139, G140, V141, S142. Cyan: P208, V209, V210, A211, P212, A213, P214, A215, P216, A217, P218, V350, V351, T352, Q353, P354, Q355, A356. Green: T317, G318, N319, T320, C321, D322, N323, V324, K325, A326, R327, A328, A329, L330, I331, D332, C333. Magenta: T271, D272, I274, G275, S276, D277, A278, Y279, N280, Q282, G311.

Table 2. Conformational B-cell epitopes of OmpA protein of *K. pneumoniae*

Conformational B-cell epitopes	Score (for conformational B-cell epitopes)	Conservancy (%)	Color
G43, G46, N47, G48, F49, Q50, G51, N52, N53, G54, P55, T56, R57, K90, G91, S92, V93, D94, N95, G134, N135, Y136, A137, S138, T139, G140, V141, S142	0.895	96.43	Red
P208, V209, V210, A211, P212, A213, P214, A215, P216, A217, P218, V350, V351, T352, Q353, P354, Q355, A356	0.884	94.44	Cyan
T317, G318, N319, T320, C321, D322, N323, V324, K325, A326, R327, A328, A329, L330, I331, D332, C333	0.877	100.00	Green
T271, D272, I274, G275, S276, D277, A278, Y279, N280, Q282, G311	0.832	90.91	Magenta

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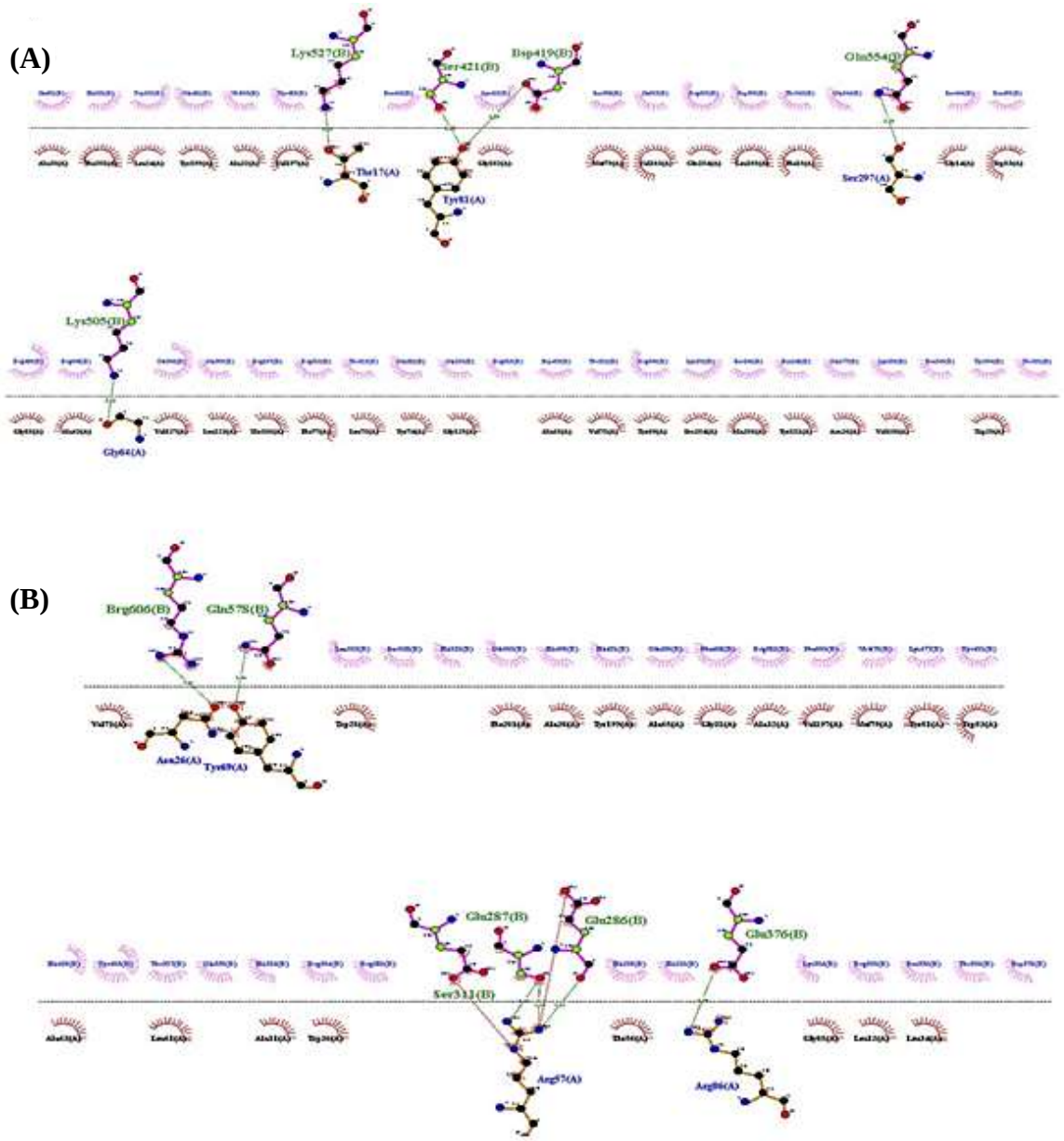

Docked complexes	Docking score	Confidence score	Ligand rmsd (Å)
OmpA-TLR2	-352.71	0.9828	73.68
OmpA-TLR4	-412.52	0.9948	59.24

Fig. 3. Molecular interactions of OmpA and human TLRs using the HDOCK web server and were visualized by LigPlot. Interacting residues of (A) OmpA-TLR2 and (B) OmpA-TLR4. The docking score, confidence score and ligand rmsd of each complex are represented in the figure.

Determination the specificity of anti-rOmpA

In terms of the specificity of polyclonal antibody developed against the rOmpA, we found that the produced antibodies could react with hvKp and *K. pneumoniae* ATCC 13883. The difference was found to be significant when compared to the control group ($p < 0.0001$). We observed similar results for *E. coli*, although the level of antibodies reacting with this bacterium was lower compared to *K. pneumoniae*. There was also no significant difference between the vaccinated and control groups with respect to the other investigated bacteria (Fig. S1).

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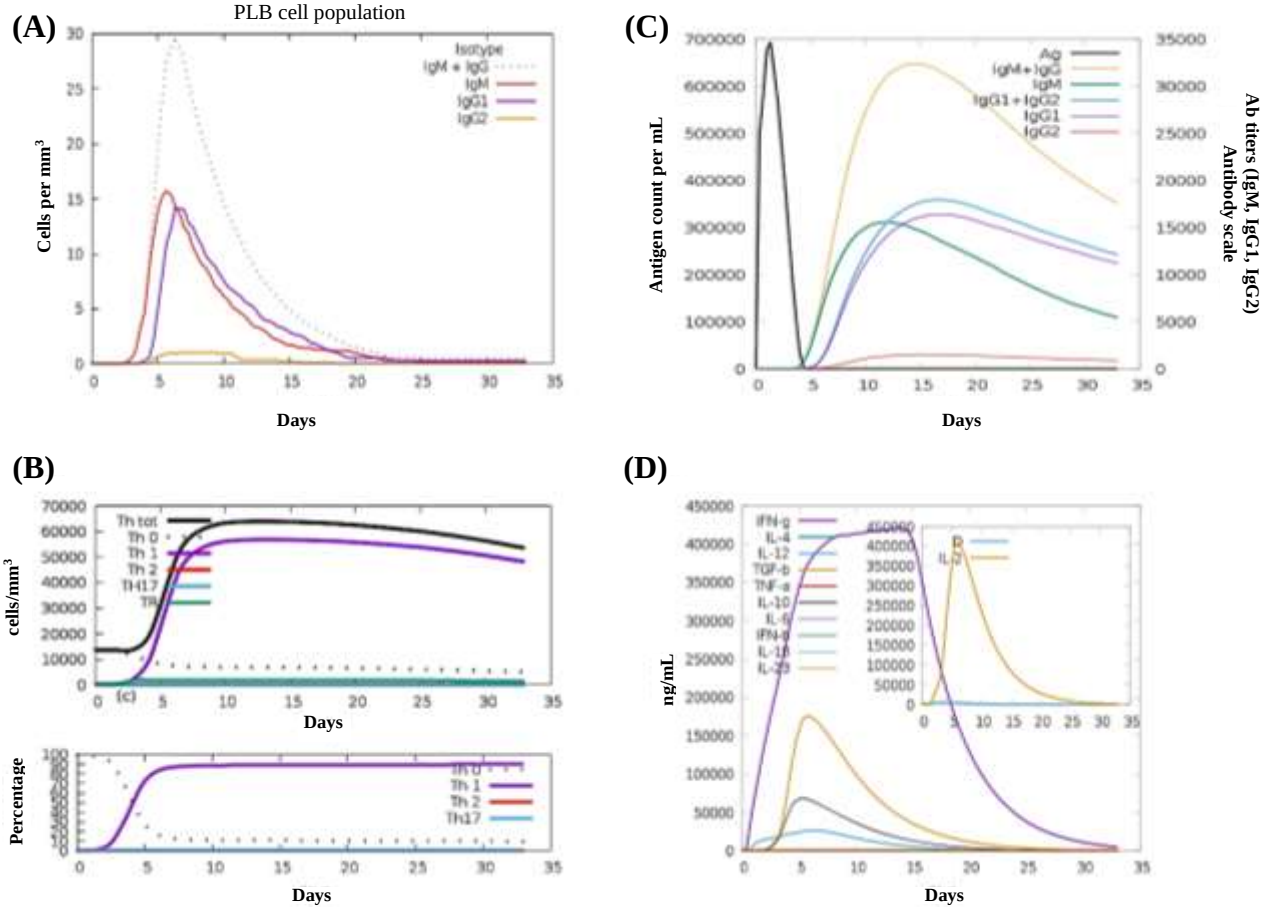

Fig. 4. C-ImmSim presentation of an in silico immune simulation with OmpA. (A and C) Immunoglobulin production in response to antigen injections; specific subclasses are showed as colored peaks. (B and D) Concentration of cytokines and interleukins.

DISCUSSION

In this study, we utilized bioinformatics tools to investigate the rOmpA. The efficacy of the vaccine candidate was also evaluated under in vivo conditions. In silico study of vaccine candidates is a valuable approach that enables the assessment of potential efficacy and immunological characteristics before proceeding into in vitro or in vivo experiments[28,29].

Overall, our study provides compelling evidence for the potential of rOmpA as a vaccine candidate against *K. pneumoniae* infection. Its desirable characteristics, including antigenicity, allergenic properties, adhesive prosperity, stability, interaction with TLRs, conservancy among strains, and ability to stimulate both humoral and cellular immune responses make it a promising candidate for further development. Challenges in vaccine development can be attributed, in part, to the scarcity of suitable candidates among various circulating strains[30]. As a result, the selected candidate was evaluated to determine its presence in different strains of *K. pneumoniae* in this study. Our results indicated the prevalence of ≥93% for rOmpA among different *K. pneumoniae* strains. The instability index of ompA was found to be <40, indicating stable nature of this candidate[31]. Adhesion probability is regarded as a criterion for vaccine candidates due to its important role in virulence and host-pathogen interaction[32]. In our study, investigating the subcellular localization and secondary structures of OmpA, a protein consisting of 356 amino acids, revealed the presence of alpha-helical structures and beta strands. The results indicated that OmpA belongs to the Omps superfamily, with no cytoplasmic signal peptide and without any transmembrane helices, which aligns with findings from previous studies[33,34].

In the context of *K. pneumoniae* infection, B-cells play a critical role in generating a robust humoral immune response, while $CD4^+$ T-cells are essential for initiating and sustaining pathogen-specific humoral and cellular immune responses. Therefore, identifying B-cell epitopes and linear $CD4^+$ T-cell epitopes within bacterial antigenic Omps holds great potential for enhancing our understanding of the protective

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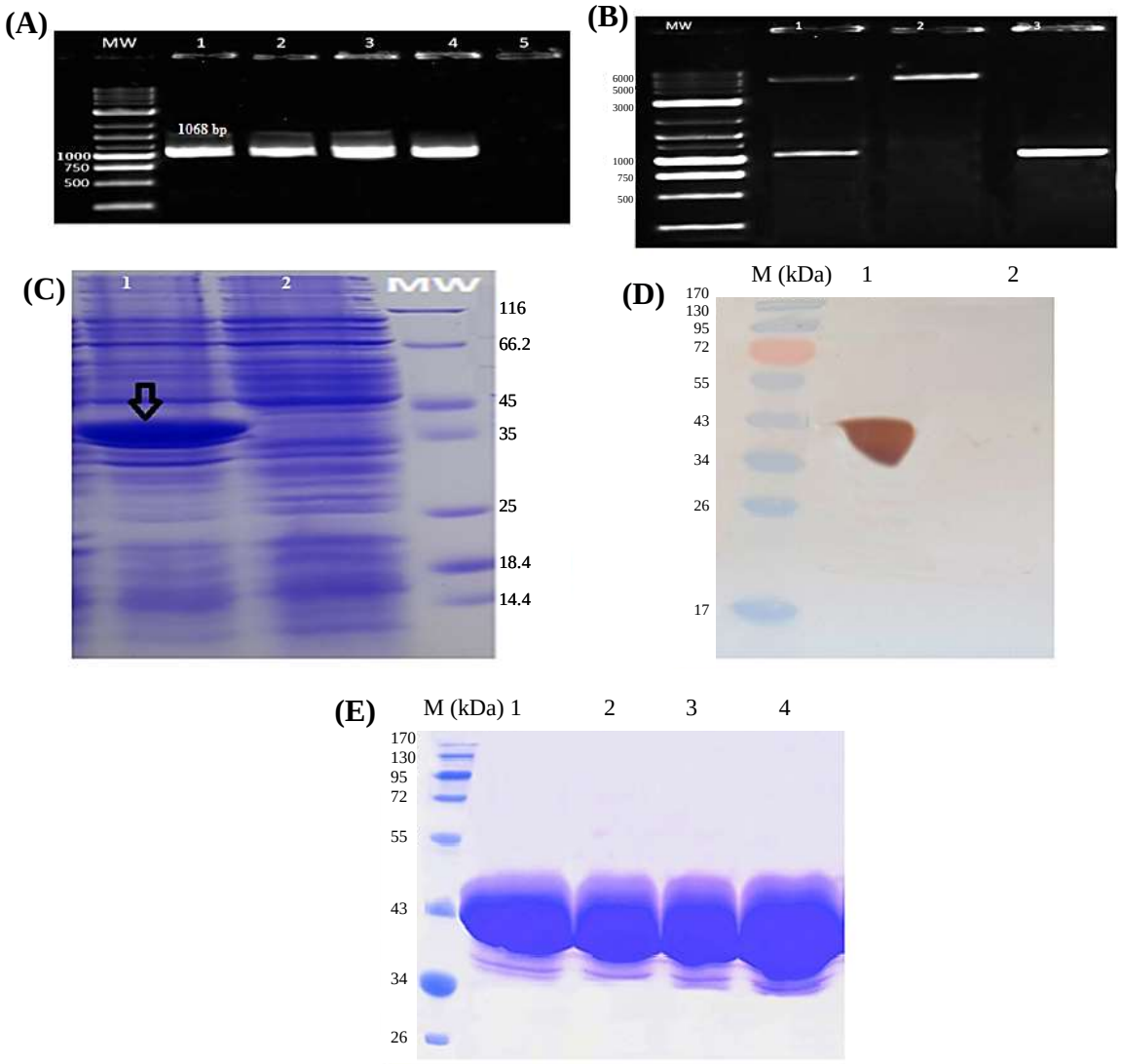

Fig. 5. (A) Amplification of the *omp*A gene in the recombinant pET28a vector by PCR reaction (line 1-4: *omp*A gene (1068 bp) and 5: negative control); (B) confirmation of cloning of *omp*A gene in pET28a vector by digestion using *Nco*I and *Xho*I restriction enzymes (line 1: pET28a-*omp*A digested clone; line 2: pET28a; line 3: *omp*A gene); (C): SDS-PAGE of the induced rOmpA in pET28a (lane 1: induced clone [0.5 mM IPTG]; lane 2: uninduced clone). Arrow shows expressed OmpA protein; (D) Western blotting of the induced protein (line 1: induced clone; line 2: uninduced *E. coli* BL21 [DE3]); (E): SDS-PAGE of the purified protein in different elutes (lines 1-4). MW: molecular weight; M: protein marker.

immunity against *K. pneumoniae*[35]. In our study, we specifically identified five linear B-cell epitopes in OmpA. Additionally, we found that there were 166 peptides capable of binding to MHC-I and 94 peptides to MHC-II, highlighting the potential for these epitopes to elicit a cellular immune response. Importantly, our in silico analysis revealed that none of these epitopes demonstrated signs of toxicity, further supporting the potential of OmpA for vaccine development against *K. pneumoniae*. This protein also demonstrated stable interactions with TLR2 and TLR4. The importance of these receptors in producing an effective immune response has been shown in various immunoinformatics studies[33,36]. The high levels of IgG1 (13 cells/mm^3), IFN-γ (390,000 ng/mL), Th1 (52,000 cells/mm^3), IgM (12 cells/mm^3), and IgG2 (1. 5 cells/mm^3) were detected for rOmpA. Hence, this particular protein has the potential to stimulate the immune response and can be considered as a viable candidate for vaccine development.

The ELISA findings revealed that immunized mice generated robust immune responses with significant levels of IgG antibodies reacting specifically to rOmpA and *K. pneumoniae*. The developed anti-OmpA antibody exhibited high sensitivity, showing reactivity at a dilution of 1:3200. This observation suggests the

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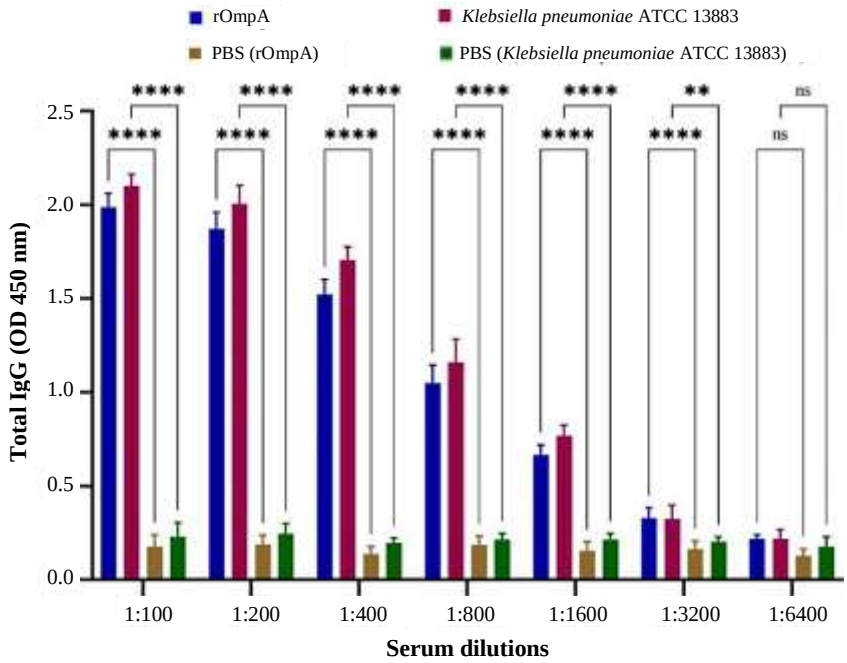

Fig. 6. Specific systemic IgG antibody responding to rOmpA and *K. pneumoniae*. BALB/c mice (six mice per group) were intranasally inoculated with rOmpA. The control groups were administered with PBS. The level of total IgG after the final vaccine was measured by ELISA. The ELISA plates were coated with rOmpA or *K. pneumoniae* ATCC 13883 antigens, and the serum collected from mice immunized with rOmpA or mice that received PBS were applied to the plates for analysis. As control, PBS was added to some of the wells coated with rOmpA and bacteria: PBS (rOmpA) and PBS (*K. pneumoniae* ATCC 13883). The results represent the mean values (±SD) of three-repeated experiments at serum dilutions 1:100-1:6400 ($^{****}p < 0.0001$, $^{**}p < 0.001$). ns: not significant.

potential efficacy of the immunization strategy in generating a specific and strong immune response against *K. pneumoniae*. Cross-reactivity with other Enterobacteriaceae bacteria, such as *E. coli*, *P. aeruginosa*, and *A. baumannii*, was investigated due to the sequence similarity of rOmpA. Concerns about potential disruptions to host microbiota were raised, but bioinformatics tools can be utilized to identify unique immunogenic epitopes for targeting pathogens, assisting in evaluating the effectiveness of the constructed entities in combating various bacterial strains[35]. In terms of the specificity, we found that the produced antibody could significantly react with hvKp and *K. pneumoniae* (ATCC 13883) compared to the control group ($p < 0.0001$). Similar results were observed for *E. coli*, although the level of antibodies reacting with this bacterium was lower compared to *K. pneumoniae*. Nevertheless, *P. aeruginosa* and *A. baumannii* were not identified by the anti-rOmpA polyclonal antibodies. While cross-reactivity can be beneficial for therapeutic intentions[37], there is a significant apprehension regarding the possible adverse impacts on the host gut microbiota. However, additional research is required to investigate the potential influence of the rOmpA of *K. pneumoniae* as a vaccine candidate on the host microbiota in forthcoming studies. Given the potential hazard of cross-reaction with the host gut microbiota, the utilization of the vaccine must be judiciously employed, taking into account local epidemiological monitoring and precise diagnosis in the future.

CONCLUSION

The present study demonstrates the potential of *K. pneumoniae* OmpA as a promising vaccine candidate against *K. pneumoniae* through a comprehensive analysis combining bioinformatics and in vivo experiments. The in vivo study confirmed the generation of specific IgG antibodies in response to immunization with rOmpA, indicating a robust immune response. The ELISA results further supported the specificity of the generated antibodies towards both rOmpA and whole cell of *K. pneumoniae*, with no significant difference in antibody titers. Additionally, the determined sensitivity of the ELISA test highlights its potential for future diagnostic applications. This research represents a significant step forward in the development of a potential vaccine against *K. pneumoniae* utilizing OmpA. However, to further validate its efficacy and safety, additional experimental analyses are warranted.

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DECLARATIONS

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Ethical approval

All the experimental procedures in this study were conducted in accordance with the Institutional Animal Care guidelines and approved by Pasteur Institute of Iran (ethical code: IR.PII.REC.1400.068).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

ShSh: writing–original draft, methodology, investigation, and conceptualization; FB: review and editing and validation; MH: software, investigation, and data curation; SS: writing-original draft and methodology. NNG: methodology. MF: review and editing, project administration, methodology, investigation, and conceptualization. MRAK: review and editing, project administration, methodology, investigation, and conceptualization.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version contains supplementary material.

REFERENCES

1. Shahkolahi S, Shakibnia P, Shahbazi S, Sabzi S, Badmasti F, Asadi Karam MR, et al. Detection of ESBL and AmpC producing Klebsiella pneumoniae ST11 and ST147 from urinary tract infections in Iran. Acta Microbiol Immunol Hung. 2022; 69(4):303-13.
2. Lucena ACR, Ferrarini MG, de Oliveira WK, Marcon BH, Morello LG, Alves LR, et al. Modulation of Klebsiella pneumoniae outer membrane vesicle protein cargo under antibiotic treatment. Biomedicines. 2023; 11(6):1515.
3. Shivaee A, Shahbazi S, Soltani A, Ahadi E. Evaluation of the prevalence of broad-spectrum beta-lactamases (ESBLs) and carbapenemase genes in Klebsiella pneumoniae strains isolated from burn wounds in patients referred to shahid motahari hospital in Tehran. Med Sci J Islamic Azad Univ. 2019; 29(3): Autumn 2019.
4. Shahbazi S, Sabzi S, Goodarzi NN, Fereshteh S, Bolourchi N, Mirzaie B, et al. Identification of novel putative immunogenic targets and construction of a multi-epitope vaccine against multidrug-resistant Corynebacterium jeikeium using reverse vaccinology approach. Microb Pathog. 2022; 164:105425.
5. Choi M, Tennant SM, Simon R, Cross AS. Progress towards the development of Klebsiella vaccines. Expert Rev Vaccines. 2019; 18(7):681-91.
6. Dar HA, Zaheer T, Shehroz M, Ullah N, Naz K, Muhammad SA, et al. Immunoinformatics-aided design and evaluation of a potential multi-epitope vaccine against Klebsiella pneumoniae. Vaccines (Basel). 2019; 7(3):88.
7. Jeannin P, Bottazzi B, Sironi M, Doni A, Rusnati M, Presta M, et al. Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. Immunity. 2005; 22(5):551-60.
8. Shahbazi S, Habibi M, Badmasti F, Sabzi S, Farokhi M, Karam MRA. Design and fabrication of a vaccine candidate based on rOmpA from Klebsiella pneumoniae encapsulated in silk fibroin-sodium alginate nanoparticles against pneumonia infection. Int Immunopharmacol. 2023; 125(Pt B):111171.
9. Assoni L, Girardello R, Converso TR, Darrieux M. Current stage in the development of Klebsiella pneumoniae vaccines. Infect Dis Ther. 2021; 10(4):2157-75.
10. Kurupati P, Ramachandran N, Poh CL. Protective efficacy of DNA vaccines encoding outer membrane protein A and OmpK36 of Klebsiella pneumoniae in mice. Clin Vaccine Immunol. 2011; 18(1):82-8.
11. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010; 26(13):1608-15.
12. Emanuelsson O, Brunak S, Von Heijne G, Nielsen H. Locating proteins in the cell using targetp, signalp and related tools. Nat Protoc. 2007;2(4):953-71.
13. Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC bioinform. 2007; 8(1):1-7.
14. Sharma N, Patiyal S, Dhall A, Pande A, Arora C, Raghava GP. AlgPred 2.0: an improved method for predicting allergenic proteins and mapping of IgE epitopes. Brief Bioinform. 2021; 22(4):bbaa294.

[DOI: 10.61186/ibj.4023]

15. Bhagwat M, Aravind L. PSI-BLAST tutorial. Methods Mol Biol. 2008; 395:177-86.
16. Duvaud S, Gabella C, Lisacek F, Stockinger H, Ioannidis V, Durinx C. Expasy, the swiss bioinformatics resource portal, as designed by its users. Nucleic Acids Res. 2021; 49(W1):W216-W27.
17. He Y, Xiang Z, Mobley HL. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. J biotechnol biomed. 2010; 2010:297505.
18. Sachdeva G, Kumar K, Jain P, Ramachandran S, Ramachandran S. SPAAN: A software for prediction of adhesins and adhesin-like proteins using neural networks. Bioinformatics. 2005; 21(4):483-91.
19. Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. Nucleic Acids Res. 2017; 45(W1):W24-9.
20. Astle WJ, Elding H, Jiang T, Allen D, Ruklisa D, Mann AL, et al. The allelic landscape of human blood cell trait variation and links to common complex disease. Cell. 2016; 167(5):1415-29.
21. Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc. 2010; 5(4):725-38.
22. Badmasti F, Siadat S, Bouzari S, Nasiri O, Nemati H, Shahcheraghi F. Molecular analysis of AbOmpA type-1 as immunogenic target for therapeutic interventions against MDR Acinetobacter baumannii infection. Vac Res. 2015; 2(1):9-18.
23. Ponomarenko J, Bui H-H, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinform. 2008; 9:1-8.
24. Bui H-H, Sidney J, Li W, Fusseder N, Sette A. Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. BMC bioinform. 2007; 8:1-6.
25. De Vries SJ, Van Dijk M, Bonvin AM. The HADDOCK web server for data-driven biomolecular docking. Nat Protoc. 2010; 5(5):883-97.
26. Wallace AC, Laskowski RA, Thornton JM. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng Des Sel. 1995; 8(2):127-34.
27. Rapin N, Lund O, Bernaschi M, Castiglione F. Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. PloS one. 2010; 5(4):e9862.
28. He Y, Xiang Z. Databases and in silico tools for vaccine design. Methods Mol Biol. 2013; 993:115-27.
29. Sabzi S, Badmasti F, Noori Goodarzi N, Habibi M, Shahbazi S, Asadi Karam MR, et al. In silico evaluation, cloning, and expression of omp22 as a promising vaccine candidate against Acinetobacter baumannii. Vac Res. 2023; 10(1):11-7.
30. Sabzi S, Shahbazi S, Noori Goodarzi N, Haririzadeh Jouriani F, Habibi M, Bolourchi N, et al. genome-wide subtraction analysis and reverse vaccinology to detect novel drug targets and potential vaccine candidates against Ehrlichia chaffeensis. Appl Biochem Biotechnol. 2023; 195(1):107-24.
31. Kar T, Narsaria U, Basak S, Deb D, Castiglione F, Mueller DM, et al. A candidate multi-epitope vaccine against SARS-CoV-2. Sci Rep. 2020; 10(1):1-24.
32. Casadevall A, Pirofski La. Host-pathogen interactions: the attributes of virulence. J Infect Dis. 2001; 184(3):337-44.
33. Mehdinejadiani K, Bandehpour M, Hashemi A, Ranjbar MM, Taheri S, Jalali SA, et al. In silico design and evaluation of Acinetobacter baumannii outer membrane protein A (OmpA) antigenic peptides as vaccine candidate in immunized mice. Iran J Allergy Asthma Immunol. 2019; 18(6):655-63.
34. Smith SG, Mahon V, Lambert MA, Fagan RP. A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol Lett. 2007; 273(1):1-11.
35. Farhadi T, Nezafat N, Ghasemi Y, Karimi Z, Hemmati S, Erfani N. Designing of complex multi-epitope peptide vaccine based on omps of Klebsiella pneumoniae: an in silico approach. Int J Pept Res Ther. 2015; 21:325-41.
36. Goodarzi NN, Fereshteh S, Sabzi S, Shahbazi S, Badmasti F. Construction of a chimeric FliC including epitopes of OmpA and OmpK36 as a multi-epitope vaccine against Klebsiella pneumonia. Health Biotechnol Biopharma. 2021; 5:44-60.
37. Hu G, Chen X, Chu W, Ma Z, Miao Y, Luo X, et al. Immunogenic characteristics of the outer membrane phosphoporin as a vaccine candidate against Klebsiella pneumoniae. Vet Res. 2022; 53(1):1-13

[DOI: 10.61186/ibj.4023]