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Original Research Article

Construction of a chimeric FliC including epitopes of OmpA and OmpK36 as a multi-epitope vaccine against *Klebsiella pneumonia*

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ABSTRACT

OmpA and OmpK36 from *K. pneumoniae* as the antigens and FliC from *Salmonella typhimurium* as the adjuvant were applied to construct a multi-epitope vaccine. B-cell, T-cell, and IL-17-epitopes were identified and a construct was modeled. Molecular docking was performed to assess the interaction between chimeric FliC and TLR-5. Downstream analysis including antigenicity, allergenicity, IFN-γ and IL-4 epitopes prediction was done. This construction covers both B-cell and T-cell epitopes as well as IFN-γ and IL-4 epitopes, but no IL-17 epitope was detected. We used two other known epitopes (IEDB epitope ID 43662 and epitope ID 57417) that induce the IL-17 release. According to the result, the multi-epitope protein is probable antigen and not an allergen. This construction was stable, hydrophilic, and has no transmembrane helixes. The computer-aided analysis imply that this protein is an acceptable candidate for immunization against *K. pneumoniae*.

***Keywords*:** *Klebsiella pneumoniae*, multi-epitope vaccine, chimeric protein, *in silico* analysis

INTRODUCTION

Klebsiella pneumoniae is a causative agent of a wide range of nosocomial and community-acquired infections mainly in immunocompromised and hospitalized patients [1]. The most important challenge associated with *K. pneumoniae* is the increasing prevalence of resistant strains, which reduces the chances of successful treatment [2]. In 1983, the emergence of isolates producing Extended-Spectrum β-Lactamases (ESBLs) capable of hydrolyzing all extended-spectrum

cephalosporins except carbapenems was reported [3]. Plasmids encoding ESBLs may also carry other resistance genes including aminoglycosides [4]. The first report of Carbapenemase-Producing *K. pneumonia* (CPKP) was presented in 2001 and resistance to carbapenems spread worldwide [5]. Infections due to CPKP are often associated with significant morbidity and mortality. The most clinically important carbapenemase, including KPC, NDM, VIM, and OXA-48- like are the major resistance mechanism among the CPKP isolates [6]. On the other hand, hypervirulent *K. pneumoniae* (hvKP) as a circulating pathogen, is capable of causing severe community-acquired infections even in healthy individuals [7]. Therefore, treatment and control of these strains especially in the hospital setting are troublous and research on other strategies such as prophylactic methods is needed.

One of the alternative solutions to control *K. pneumoniae* infections is vaccination [8]. In recent years, several studies have been performed on the identification of protected antigens and host immunization against *K. pneumoniae*. Most vaccinology studies have investigated LPS and Capsular Polysaccharide (CPS) antigens as vaccine candidates, but these antigens exhibit high antigenic variation. Moreover, immunity generated against LPS and CPS antigens is type-specific, and each type provides immunity only against itself [9]. Today, several types of research showed the high immunogenicity of Outer Membrane Proteins (OMPs) among pathogenic microorganisms, which made them appropriate targets for designing antimicrobial drugs and vaccines [10]. Some studies related to subunit vaccines have developed targeting OMPs of *K. pneumoniae* such as OmpK17, OmpK36 [11], and OmpA [12]. However, there is no FDA-approved vaccine against this pathogen yet.

OmpA and OmpK36 are the major OMPs on the *K. pneumoniae* outer membrane involved in resistance to phagocytosis processes and host immune evasion [13,14]. OmpA is a conserved protein in the *Enterobacteriaceae* family that can induce a specific humoral response [15]. You *et al*. observed that OmpA induces reactive oxygen species production and apoptosis of host cells. Moreover, OmpA induces IL-1β and IL-18 production and caspase-1 activation, stimulating pyroptosis and the death of the host cells [16]. In addition, OmpK36 as the major outer membrane porin of *K. pneumoniae* plays an essential role in both antimicrobial resistance and virulence. Therefore, OmpA and OmpK36

could be ideal targets for the design of the anti-*Klebsiella* therapeutic options especially in combination with efficient adjuvants [17].

This study aimed to propose an immunoinformatics-based multi-epitope subunit vaccine by design a chimeric construction of FliC (flagellin) as an effective adjuvant including surface-exposed epitopes of the extracellular loops of OmpA and OmpK36 against *K. pneumoniae* infections. However, wet lab validation is necessary to verify the effectiveness of this proposed vaccine candidate.

MATERIALS AND METHODS

Sequence Collection

Given that, ST23 is the most circulating *K. pneumoniae* strain globally, and is highly associated with virulent strains [7,18,19]; it was selected as the reference strain. The amino acid FASTA sequences of FliC (accession number: EAM4110074) from *Salmonella typhimurium*, OmpA (accession number: BAH62679.1), and OmpK36 (accession number: BAH64431.1) from *K. pneumoniae* NTUH-K2044 (ST23) strain were retrieved from the NCBI protein database.

Prediction of B-cell & T-cell epitopes

Linear B-cell epitope prediction was performed using BepiPred v2.0 with the threshold ≥0.65 [20]. Major histocompatibility complex (MHC) II allele binding sites were predicted using CD4 T-cell immunogenicity prediction tool with threshold=50 % [21]. In addition, surface-exposed regions of OMPs were predicted by the PRED-TMBB database [22]. Finally, the conservation of whole proteins (OmpK36 and OmpA) and the location of all B-cell and T-cell epitopes were determined using the ConSurf web tool [23].

Construction of multi-epitope vaccine and 3D structure modeling

IL-17 is an essential chemokine for the control of extracellular bacteria [24]. To determine the most effective chimeric protein, the IL-17 inducing of epitopes was selected using IL17eScan web tool and the IEBD database. According to the immunoinformatics analyses, conserved B-cell, T-cell epitopes, IL-17 inducing epitopes, rigid (EAAAK), and flexible (GPGPG) linkers were used to design the chimeric FliC. The tertiary structure (3D) of intact FliC and chimeric FliC was modeled by the Robetta tool as a protein structure prediction server [25].

Molecular docking

Molecular docking was performed to assess the interaction between chimeric FliC (intact and chimeric) and Toll-like receptor-5 (TLR-5) using HADDOCK2.2 server [26].

Antigenicity and allergenicity prediction of chimeric FliC

The VaxiJen database was used to predict the antigenicity of chimeric FliC, with a cut-o*ff* ≥0.4 [27]. The allergenicity of chimeric FliC was predicted using the Algpred 2.0 tool with a cut-off ≥0.5 [28].

The physicochemical properties of chimeric FliC were evaluated by various databases. The number of amino acids, the estimated half-life, molecular weight, theoretical pI, aliphatic index, and instability index were examined using Expasy ProtParam Server [29]. Alpha helix, beta strand, disordered, and TM helices were determined using the Phyre2 webtool [30]. The functional class of the proteins was predicted using the VICMpred database. The number of disulfide bonds was predicted using the PROSITE database.

Prediction of conformational B-cell epitopes

Conformational B-cell epitopes of Chimeric FliC were identified using the ElliPro database with a threshold of ≥0.8 [31].

Prediction of IFN-gamma and IL-4 epitopes

Chimeric FliC was investigated for interferon-gamma (IFN-γ) and IL-4 epitopes potentiality. These capabilities of the cassette were predicted by the IFNepitope [32] and IL4pred servers [33], respectively. The SCAN module is designed to predict INF-γ inducing regions in a protein or antigen (window size= 15, SVM-based method).

Back translation and codon usage optimization

The chimeric FliC protein was reverse-translated into DNA and codon usage was optimized based on the *Escherichia coli* protein translation machinery using the Optimizer web server [34].

RESULTS

Topology analysis of OmpA and OmpK36

Topology of OmpA and OmpK36 to identify surface-exposed regions of OMPs were predicted by the PRED-TMBB database. Prediction of trans-membrane Beta-barrel of OMPs revealed 4 extracellular loops for OmpA and 8 extracellular loops for OmpK36. See Figure 1.

B-cell and T-cell epitopes selection

In this section, our goal was to determine the exposed linear B-cell epitopes and to characterize T-cell epitopes. Our analysis showed that there were three exposed linear B-cell epitopes and three T-cell epitopes in OmpA, while six B-cell and three T-cell epitopes were predicted for OmpK36. A total of 15 epitopes of OmpA and OmpK36 were detected. The data of Pred-TMBB and 3D structure modeling showed that all B-cell epitopes were surface-exposed, while all T-cell epitopes were buried. The conservation of epitopes was visualized using the ConSurf web tool. B-cell epitopes are shown in black boxes and T-cell epitopes are shown in blue boxes. All results are shown in Figure 2A and Figure 2B.

IL-17 epitopes selection

IL -17 plays an essential role in controlling *K. pneumoniae* infection by recruiting neutrophils [35]. Among the identified B-cell and T-cell epitopes of OmpA and OmpK36 proteins, no IL-17 inducing epitope was detected using IL17eScan. Because of the importance of the IL-17 response against *K. pneumoniae* infections, we used two other known epitopes that activate the IL-17 response when eukaryotic cells are exposed to these epitopes. One of these two epitopes was NEKYAQAYPNVS, a linear peptide epitope (epitope ID 43662) that has been studied as part of Listeriolysin O from *Listeria monocytogenes*. This epitope was studied for immuno-reactivity in 22 publications and tested in 64 T-cell assays. The other was SEFAYGSFVRTVSLPVGADE, a linear peptide epitope (epitope ID 57417) that was studied as part of alpha-crystallin from *Mycobacterium tuberculosis*. This epitope was screened for immuno-reactivity in 21 publications and tested in 77 T-cell assays, 6 B-cell assays, and 17 MHC ligand assays.

Construction of multi-epitope vaccine

For the construction of a multi-epitope vaccine protein, two IL-17 inducing epitopes, eleven B-cell and T-cell epitopes were brought together using flexible linkers. FliC is an L-shaped protein, the middle part of the protein (amino acid 178-374) was removed, and the epitopes construct was coupled to the N- and C-terminal of FliC protein using rigid linkers (see Figure 3). The protein sequence of chimeric FliC was deposited in the supplemental data.

Tertiary structure modeling and docking

The 3D structure of intact FliC and chimeric FliC proteins was determined

using Robetta server (Figure 3). Protein-protein interaction with TLR-5 using HADDOCK server showed that chimeric FliC could interact with TLR-5 as if the intact FliC protein. The HADDOCK scores of chimeric and intact FliC are −237.3 ± 4.7 and −226.3 ± 1.6, respectively. See Table 1.

Table 1. Physicochemical characteristics of chimeric FliC protein

Protein / **Characteristics**	**Intact FliC**	**Chimeric FliC**
Number of amino acids	495	530
Predicted functional class	Virulence factors (score: 1.61)	Virulence factors (score: 2.18)
Molecular weight (kDa)	51.59	54.3
Theoretical pI	4.79	5.02
The estimated half-time	>10 hours (*E. coli, in vivo*)	>10 hours (*E. coli, in vivo*)
Predicted solubility upon overexpression in *E. coli*	0.50	0.82
Aliphatic index	83.66	74.08
Instability index	24.67 Stable	30.44 Stable
Grand average of hydropathy (GRAVY)	-0.39	-0.53
Ag overall prediction	0.82 (probable antigen)	0.83 (probable antigen)
Allergenicity	0.32 (non-Allergen)	0.31 (non-Allergen)
Alpha helix	59 %	42 %
Beta strand	2 %	4 %
Disordered	45 %	44 %
HADDOCK score (FliC-TLR5)	−226.3 ±1.6	−237.3 ±4.7
Van der Waals forces	−114.2 ±2.5	−111.2 ±2.5
Electrostatic energy	−310.3 ±1.3	−317.3 ±8.3
De-solvation energy	−11.0 ±4.1	−12.0 ±2.4
Restraints violation energy	0.0 ±0.00	0.0 ±0.00
IL-4 motif	20 motifs	17 motifs
IFN-gamma epitopes (SVM, Score ≥0.5)	14 motifs	22 motifs

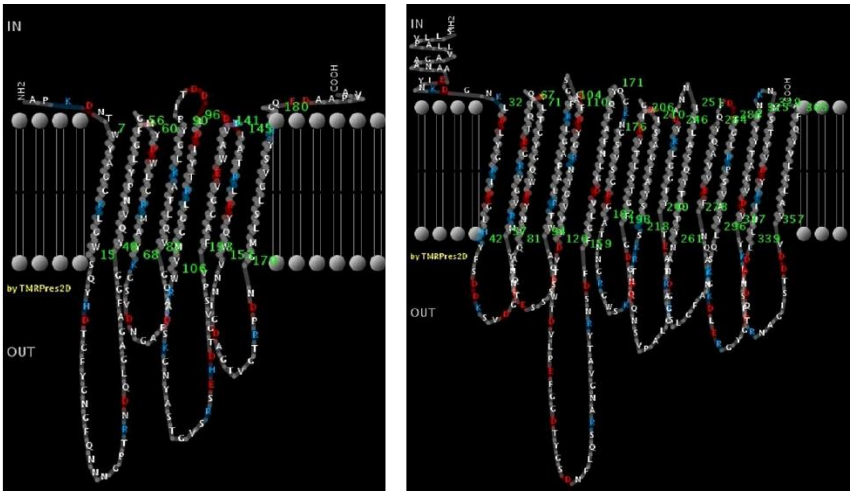

Figure 1. Topology analysis of OmpA (A) and OmpK36 (B) to identify surface-exposed regions of OMPs by the PRED-TMBB database. Prediction of trans-membrane Beta-barrel of OmpA and OmpK36 revealed 4 extracellular loops for OmpA and 8 extracellular loops for OmpK36.

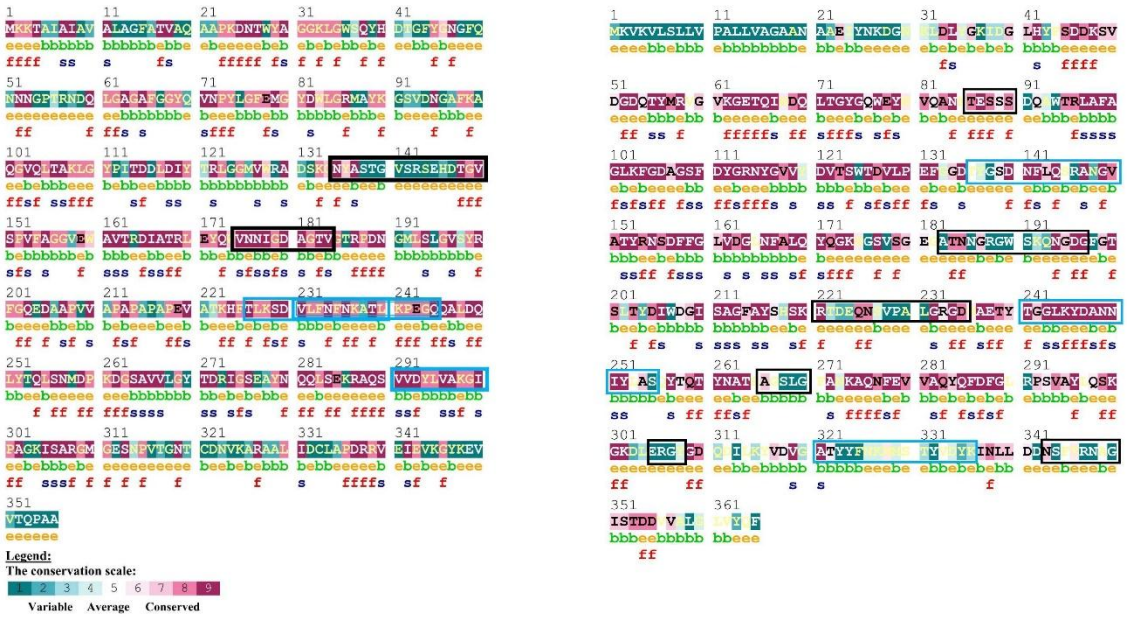

Figure 2. The conservation analysis of full length OmpA (2A) and OmpK36 (2B) and the location on linear B-cell (black boxes) and T-cell (blue boxes) epitopes. There are 3 exposed linear B-cell epitopes and 3 T-cell epitopes of OmpA, while 6 B-cell and 3 T-cell epitopes were predicted for OmpK36.

Antigenicity and allergenicity prediction

The overall prediction for total antigenicity was 0.8363, which indicates that chimeric FliC is a probable antigen. The AlgPred score was 0.31. Thus, this protein is not an allergen. The antigenicity of intact FliC was 0.8286 and the allergenicity score was 0.32.

Physicochemical properties

The ProtParam analysis demonstrated that the estimated half-life of chimeric FliC was 30 h (mammalian reticulocytes, *in vitro*), >20 h (yeast, *in vivo*), and >10 hours (*E. coli*, *in vivo*). TMHMM result demonstrated that chimeric FliC protein has no transmembrane helices. All physicochemical properties are presented in Table 1.

Conformational B-cell epitope

The amino acid sequence is as follows: F190, R192, T193, V194, S195, L196, P197, V198, G199, A200, D201, E202, G203, P204, G205, P206, G207, N208, E209, K210, A212, Q213, Q233, L234, G235, G236, P237, G238, N241, G257, P258, G259, P260, G261, V262, N263, N264, V271, G272, P273, G274, P275, G276, T277, E278, S279, S280, S281, P283, G284, P285, G286, A287, T288, N289, N290, G291.

Gene optimization

The chimeric FliC sequence was back-translated and codon-optimized using the Optimizer web server according to the codon usage of *E. coli* with low G+C % (33.6 %). The DNA sequence of chimeric FliC was deposited in the supplemental data.

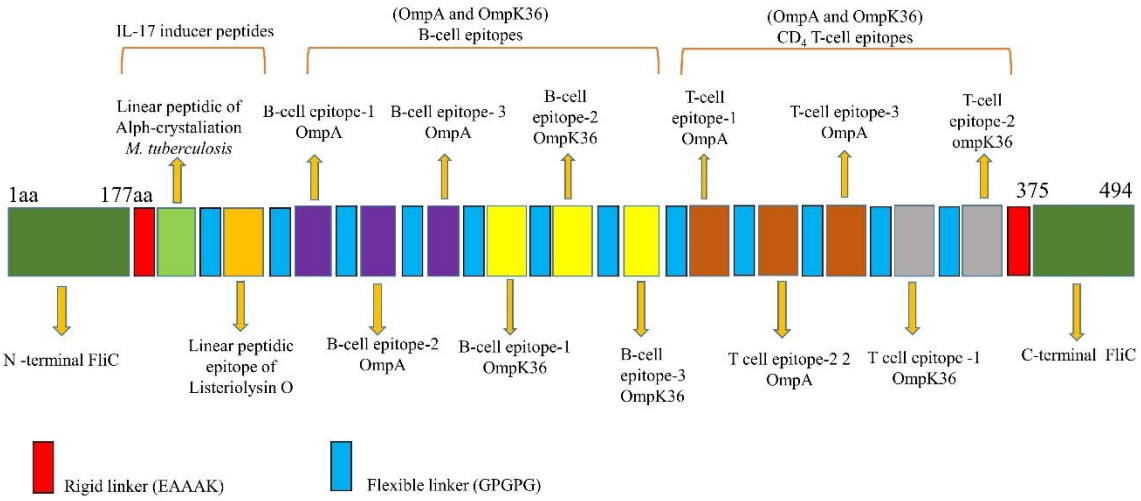

Figure 3. Schematic diagram of chimeric FliC multi-epitope vaccine construct. The middle part of FliC (amino acid 178-374) that don't have interaction with TLR-5 was removed, and the multi-epitope region (e.g. IL-17 inducer peptides, B-cell and T-cell epitopes of OmpA and OmpK36 as well as flexible linkers) was inserted to the N- and C-terminal of FliC protein using rigid linkers.

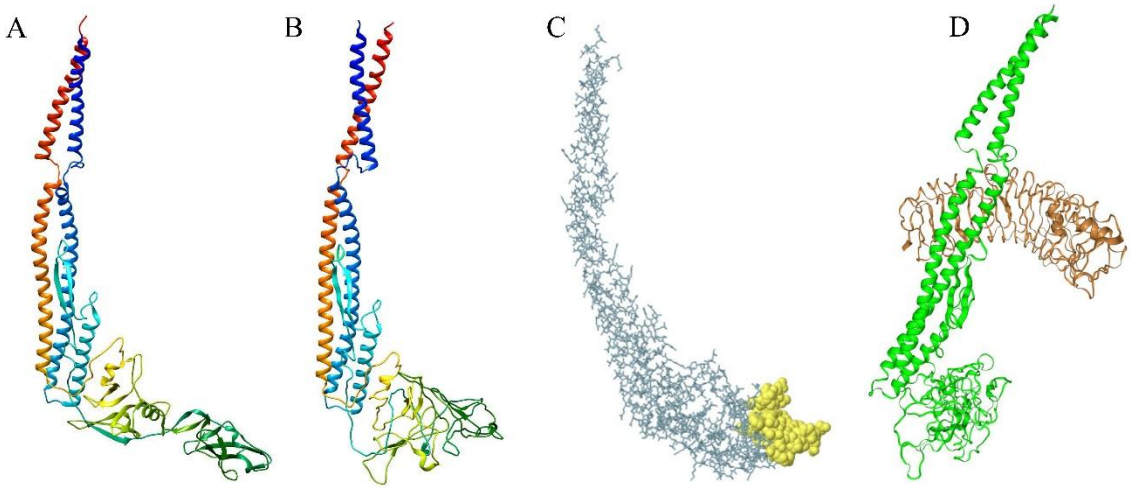

Figure 4. Tertiary structure of protein. A, 3D structure of intact FliC protein; B, 3D structure of multi-epitope FliC. C, conformational B-cell epitope of multi-epitope region. D, interaction of chimeric FliC with TLR-5.

DISCUSSION

K. pneumoniae is quickly becoming known for its resistance properties to most of the last-line antibiotics that are commonly used. It is particularly problematic in hospitals, where it causes very diverse infections [36,37]. Despite some advances in the development of treatments for *K. pneumoniae* infections until now, a safe and effective vaccine is highly desirable to prevent the spread of this opportunistic pathogen [38]. Therefore, there is a significant need to find a new generation of vaccines against *K. pneumoniae*. The development of vaccines is labor-intensive, however with advancements in immunoinformatics and molecular biology fields polytypic vaccines have received extend attention as innovative procedure [39].

Various approaches have been used to develop vaccines against against *K. pneumoniae* [40-42]. Members of the OMPs family are immunogenic surface antigens and are therefore ideal candidates for vaccines preparation [39]. Koropati *et al.*, identified OmpA as a potential antigen of *K. pneumoniae* using immune-proteome analysis. [43]. In another study, mice were vaccinated using plasmid DNA containing *ompA* or *ompK36* of *K. pneumoniae*, which generated humoral as well as Th1 cell-mediated immune responses [12].

In *K. pneumoniae* infection, B cells can induce strong humoral immunity and $CD4^+$ T cells have a basic role in priming and maintenance of pathogen specific humoral and cellular immune responses. Hence, identification of the B cell epitopes and linear $CD4^+$ T cell epitopes of bacterial antigenic OMPs could contribute to better understanding of the protective immunity to *K. pneumoniae* and, additionally facilitate the preparation of effective anti *Klebsiella* vaccines [39]. In this research, it has been attempted to *in silico* construction of a chimeric FliC including epitopes of OmpA and OmpK36 as a multi-epitope vaccine against *Klebsiella pneumonia*, using various bioinformatics approaches.

B cell epitope region of the bacterial membrane proteins, including OMPs, is surface-exposed that reacts with infected host antibodies [39]. For this reason, while predicting B cell epitopes from OMPs, the prediction of location of membrane spanning segments along the sequence is often important. In the current study, topology map of OmpA and OmpK36 was predicted to find extracellular regions. Selected T cell epitopes were found to be

protected among bacterial strains and were predicted to be naturally immunogenic, so they are perfect candidates for broad-spectrum vaccine development. In this study, IFN-γ inducing MHC II epitopes were also predicted. The production of IFN-γ is essential for the innate immune response against pulmonary *K. pneumoniae* infection and contributes to the clearance of *K. pneumoniae* infections, especially for hypervirulent strains [38]. Therefore, proper epitope can produce more specific, safer, more effective and permanent immune responses as well as prevent all undesired effects. Multi-epitope vaccines are considered a better alternative to monovalent vaccines because of their ability to activate humoral and cellular immune responses [38].

Given the role of IL-17 in controlling *K. pneumoniae* infection through neutrophil recruitment [35], we employed two IL-17 inducing peptides to construct this multi-epitope vaccine. Th17 cells recognize conserved antigens among *Enterobacteriaceae*, consequently confer serotype independent immunity. Chen *et al*., reported that intranasal administration of recombinant OmpX protein of *K. pneumoniae* induced mucosal Th17 responses strongly, which is necessary for protection against *K. pneumonia* [44].

One of the significant drawbacks of multi-tope vaccines is the low immunogenicity [45]. To overcome these problems, various approaches have been used to enhance the immunogenicity of polytopic vaccines [46]. In a study conducted by Farhadi *et al.*, some of these approaches were used to increase the immunogenicity of the constructs at different levels of designing. They increased the size of the constructs by multiplication of the epitopes and incorporation of immunogenic sequence tags (adjuvants) because of the fact that construct which has small size may be rapidly cleared from the body [47]. Previously, Arshad Dar *et al.*, developed a multi-epitope vaccine against *K. pneumoniae* using a total of four antigens, identified through reverse vaccinology approach, and Cholera Toxin B as adjuvant [38]. In the present study, FliC as an adjuvant added to the construct of multi-epitope vaccines to effectively stimulate the immune system [48]. Bacterial FliC is a direct TLR-5 agonist that induces the MyD88 signaling pathway and regulates the production of regulatory cytokines. Flagellin is a safe and potent immunomodulatory molecule that acts as an adjuvant, stimulates Antigen-Presenting Cells (APCs), and enhances the innate immune response via binding to TLR-5

[49]. The constant D0 and D1 domains, located in the N and C terminal sites of bacterial FliC, are involved in TLR-5 interaction [50]. Thus we selected the D0 and D1 domains of FliC of *K. pneumoniae* as an adjuvant to increase the immunogenicity of the multi-epitope vaccine. *K. pneumoniae* infection leads to the overexpression of TLRs in human airway epithelial cells [51]. The docking score showed significantly high interaction between vaccine and innate immune receptors which suggests that the vaccine can trigger TLR activation and increasing immune responses against *K. pneumoniae*.

The chimeric FliC was a probable antigen (0.8363) and is not an allergen (0.32). The physicochemical analyses of the vaccine projected it to be virulence factors, stable, hydrophilic, alkaline in nature, and has no transmembrane helixes. Furthermore, in the field of peptide-based vaccine designing, multi-epitope construct must mimics the structure of protein epitopes [34]. For this reason, 3D structure of the B-cell epitope was determined. According to the result epitope region of FliC including 57 amino acids with a 0.829 Ellipro score was considered as a potent conformational B-cell epitope**.** Finally, to attain a high-level expression of recombinant construct in the *E. coli* host, codon optimization was performed to improve the transcription efficiency and transcript stability. Solubility of overexpressed recombinant proteins in the *E. coli* host is one of the important requirements of many functional and biochemical assessments. The solubility chance of the Chimeric FliC construct was higher than Intact FliC (0.82 vs. 0.50), which manifests that Chimeric FliC construct shows an acceptable percentage of solubility in an overexpressed mode. As a result, the subunit protein along with proper adjuvant can be considered as a candidate immunogenic for the development of therapeutic option against *K. pneumoniae*.

CONCLUSION

This study presented a novel chimeric FliC multi-epitope protein by an *in silico* approach as an effective vaccine for protection against *K. pneumoniae* infectious diseases. This multi-epitope vaccine covers both B-cell and T-cell epitopes of OmpA and OmpK36 proteins, IL-17 inducing epitopes, and TLR5-interacting domains of FliC protein. All of these features make this protein as an acceptable target candidate for immunization against *K. pneumoniae*. However, wet lab validation is necessary to

verify the effectiveness of this proposed candidate.

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