



Detecting the Dominant T and B Epitopes of *Klebsiella pneumoniae* Ferric Enterobactin Protein (FepA) and Introducing a Single Epitopic Peptide as Vaccine Candidate

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Abstract

Klebsiella pneumoniae causes various human infections. Ferric enterobactin protein (FepA) is a conserved protein of *K. pneumoniae* with high immunogenicity. In the present study, using comprehensive in silico approaches the T and B cell-specific epitopes of *K. pneumoniae* FepA were identified. The T (both class I and class II) and B (both linear and conformational) epitopes of FepA were predicted using prediction tools. The predicted epitopes were screened for human similarity, immunogenicity, antigenicity, allergenicity, toxicity, conservancy, structural and physicochemical suitability, and in case of T epitopes binding to HLA alleles, using numerous immune-informatics, homology modeling, and molecular docking approaches. These analyses led to introduce the most dominant FepA epitopes that are appropriate for vaccine development. Furthermore, we introduced an antigenic peptide containing both T and B epitopes which comprises suitable structural and physicochemical properties needed for vaccine development and it is conserved in many bacteria. Altogether, here the highly immunogenic T and B epitopes of FepA as well as a final epitopic peptide containing both T and B epitopes were found and introduced for future vaccine development studies. It is suggested that the actual efficiency and efficacy of our final epitopic peptide be investigated by in vitro/in vivo testing.

Keywords Epitope · Ferric enterobactin protein · Immune-informatics · *Klebsiella pneumoniae* · Molecular docking · Structure

Introduction

Klebsiella pneumoniae is a hospital-acquired pathogen of the Enterobacteriaceae family. This bacterium leads to various infections particularly in immune-deficient patients (Piperaki et al. 2017). The emergence of the antibiotics-resistant and hyper-virulent strains of *K. pneumoniae* greatly narrow down its therapeutic options, demanding other controlling strategies such as the infection prevention using vaccines (Piperaki et al. 2017; Malachowa et al. 2019). In spite of many researches to develop an effective vaccine for *K.*

pneumoniae, no vaccine has been yet approved for human use, and attempts to find an effective antigen as a vaccine, are ongoing (Pletz et al. 2016).

Ferric enterobactin protein (FepA) is a conserved protein of outer membrane proteins (OMPs) of gram-negative bacteria (Baghal et al. 2010). FepA from *K. pneumoniae* is an integral OMP that transports ferric enterobactin and it comprises 742 amino acid residues. Previous studies have shown that immune responses against FepA can inhibit *K. pneumoniae* infections. Baghal et al. produced a recombinant form of *E. coli* FepA and assayed its immunogenicity in BALB/c mice and rabbits. The results showed that FepA provoked significant antibody responses which protected mice and rabbits against *E. coli*, *Shigella flexneri*, *Salmonella typhi*, and *K. pneumoniae* (Baghal et al. 2010; Larrie-Bagha et al. 2013). Using an immunoproteome-based approach, Kurupati et al. identified many vaccine candidates for *K. pneumoniae*. Sera from *K. pneumoniae*-infected patients and healthy controls were analyzed for reactivity against *K. pneumoniae* OMPs separated by two-dimensional gel electrophoresis. FepA was one of the

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20 found highly immunogenic proteins (Kurupati et al. 2006). More previously, Lin et al. immunized cows with *E. coli* FepA and showed that it induced high titers of immunoglobulin-G in the cows serum and milk (Lin et al. 1998a), that were able to inhibit the growth of coliform bacteria such as *E. coli* and *K. pneumoniae* (Lin et al. 1999). They also showed that a monoclonal antibody against FepA could inhibit in vitro growth of coliform bacteria including *K. pneumoniae* (Lin et al. 1998b). All of these studies introduced FepA as an immunogen that was able to provoke robust immune responses. Furthermore, since FepA is highly conserved among bacteria of the Enterobacteriaceae family (Chakraborty et al. 2003), it can be an ideal vaccine candidate with the capability to cover a wider spectrum of pathogens.

In humans, immunity against *K. pneumoniae* is largely mediated by humoral responses (Campbell et al. 1996). However, there are some evidence supporting the protecting role of CD4+ T cells against *K. pneumoniae* (ten Hagen et al. 1998; Pletz et al. 2016). Therefore, an effective immunotherapeutic strategy against this pathogen should include both humoral and cell-dependent responses which are mediated by B and T cell-specific epitopes, respectively (Lee et al. 2015).

Epitope-based vaccines can lead to robust stimulation of both humoral and cell-dependent immune responses (Bijker et al. 2007). Additionally, epitope-based vaccines have some other advantages, such as cost-effective production, increased safety, and the ability to choose the immunity type (Sbai et al. 2001). These vaccines comprise highly immunogenic B and/or T cell-specific epitopes, which provoke B, helper T, and/or cytotoxic T cells. Since these cells play a significant role in the induction of protective responses against many pathogens, finding peptides that induce B and T cell responses is an essential step in epitope-based vaccine designing (Sidney et al. 2020). Computational biology and immune-informatics have intensely assisted to design epitope-based vaccines. In silico identification of potential epitopes to design such vaccines reduces the lengthy process of epitope discovery (He et al. 2010).

In the present study, the dominant T and B epitopes of *K. pneumoniae* FepA were identified using accurate immune-informatics, homology modeling, and docking approaches. Furthermore, we introduced and characterized a high immunogen and antigenic peptide of FepA which contains both T and B epitopes, and comprises suitable physicochemical properties needed for vaccine development.

Methods

Protein Sequence Retrieving

The amino acid sequence of *K. pneumoniae* FepA protein with the accession number of CDO13414.1 was retrieved from the

NCBI protein database (<https://www.ncbi.nlm.nih.gov/protein>).

Class I T Epitope Prediction

Class I T epitopes (CD8+ T cell-specific epitopes) of antigens are presented to T cells by human leukocyte antigen (HLA)-I alleles. Therefore, defining the HLA alleles is required for the epitope prediction. Here, 10 HLA-I alleles with high frequency in the world that have been determined in our previous study (Rostamian et al. 2020) were applied.

The epitope prediction was performed as previously report (Akya et al. 2019; Rostamian et al. 2020). Briefly, based on the highly frequent HLA-I alleles, FepA sequence was screened by three epitope prediction tools, namely IEDB (<http://tools.iedb.org>), (Fleri et al. 2017), ProPred-I (<http://crdd.osdd.net/raghava/propred1/>), (Singh and Raghava 2003) and SYFPEITHI (<http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm>), (Schuler et al. 2007)). The percentile rank of ≤ 1 for IEDB and the score of ≥ 10 for ProPred-I and SYFPEITHI were set as the thresholds. Only epitopes with 9-mer length were chosen to be predicted.

The ≥ 70 sequence similarity of the predicted epitopes was found and clustered by the IEDB Epitope Cluster Analysis tool (<http://tools.iedb.org/cluster/>). Of each cluster, only the epitope with the highest prediction score was selected for further analyses. Using IEDB Class I immunogenicity tool (<http://tools.iedb.org/immunogenicity/>), the immunogenicity of selected epitopes was evaluated (Calis et al. 2013).

Class II T Epitope Prediction

Class II T epitopes (CD4+ T cell-specific epitopes) of antigens, are presented to T cells by HLA-II alleles. These HLA-II alleles should be defined for epitope prediction tools. Here, eight previously determined highly frequent HLA-II alleles (Rostamian et al. 2020) were used as entries to three epitope prediction tools, IEDB, ProPred-II (<http://crdd.osdd.net/raghava/propred/>), and SYFPEITHI. The percentile rank ≤ 10 for IEDB and the score of ≥ 10 for ProPred-II and SYFPEITHI were used as the thresholds and 15-mer epitopes were chosen to be predicted.

Similar to what was mentioned about the class I T epitope prediction, the predicted class II T epitopes were also clustered, and for each cluster, the epitope with the highest score was selected. The ability of the epitopes in the production of interferon-gamma (IFN- γ) was also evaluated by the IFNepitope server (<http://crdd.osdd.net/raghava/ifnepitope/>).

Detecting Class II/Class I Windows and Finding Final Top 10 T Epitopes

Knowing that class I epitopes with nine residues may be windows of class II epitopes with 15 residues, the predicted class I and II epitopes were aligned to detect these windows using the IEDB Epitope Cluster Analysis tool. The similarity of $\geq 70\%$ was set as the tool cut off and the class II epitopes comprising at least one class I epitope were chosen.

Finally, based on the following criteria 10 best class II T epitopes were selected: 1—The class II epitope which comprises more class I epitopes, 2—The class II epitope which was predicted by more prediction tools, 3—The class II epitope which had higher prediction scores, and 4—The class II epitope which was predicted positive in the production of IFN- γ .

Similarly, 10 best class I epitopes was selected according to the following criteria: 1—The class I epitope which was a window of a high prediction score class II epitope, 2—The class I epitope which was predicted by more prediction tools, 3—The class I epitope which had a higher score, and 4—The epitope which had higher score in the immunogenicity prediction.

Tertiary Structures and Molecular Docking Studies

The final top 10 class II and class I epitopes were restricted to HLA-DRB1*01:01 and HLA-A*02:01, respectively. The 3D structures of HLA-DRB1*01:01 (PDB ID: 1AQD) and HLA-A*02:01 (PDB ID: 5HHP) were obtained from PDB databank (<https://www.rcsb.org/>).

The tertiary structures of the top 10 epitopes were modelled using the PEP-FOLD server (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/>; Thevenet et al. 2012; Shen et al. 2014)) and the models were validated by Ramachandran plot generation using MolProbity (<http://molprobity.biochem.duke.edu/>, (Chen et al. 2010)).

As the first step of docking studies, consensus prediction of interface residues in transient complexes (CPort) (<https://milou.science.uu.nl/services/CPort/>) was used to exactly predict which residues would be incorporated in the interactions (de Vries and Bonvin 2011). After predicting these residues, docking simulation was performed by HADDOCK 2.2 (<http://haddock.science.uu.nl/services/HADDOCK2.2>) applying the 3D structures of the epitopes and the HLA alleles.

Prediction of Linear B Epitope

The ABCpred (<http://crdd.osdd.net/raghava/abcpred/>) (Saha and Raghava 2006)) and IEDB (<http://tools.iedb.org/main/bcell/>), web servers were used to predict linear B epitopes. The IEDB B epitope prediction package contains five

tools that work based on different scales as follows: beta turn, hydrophilicity, surface accessibility, antigenicity, and flexibility. The prediction was performed as our previous study (Zargaran et al. 2020). Briefly, for each IEDB tool, the window size remained on seven residues and the average provided by the tool was used as the threshold. The charts provided by the IEDB tools were compared and the sequences that were predicted positively by at least three IEDB tools were chosen for further analysis. Also, IEDB supplies BepiPred 1.0 and BepiPred 2.0, two other prediction tools for linear B epitopes (Larsen et al. 2006; Jespersen et al. 2017). The sequence of FepA protein was entered into the tools and the predicted linear B epitopes were obtained.

Furthermore, the ABCpred server was also used for linear B epitope prediction. The default parameters of the tool were remained unchanged and more probable epitopes ranking from high to low scores were obtained.

Finally, using the Epitope Cluster Analysis tool, all the linear B epitopes predicted by IEDB tools (all seven tools) and ABCpred server were compared and the epitopes that were found in more tools or had higher scores were chosen as the final linear B epitopes.

FepA Modelling and Conformational B Epitope Prediction

To predict conformational B epitopes, the 3D structure of FepA was required. Since the 3D structure of *K. pneumoniae* FepA was not found in the PDB bank, it was modeled by the SWISS-MODEL server (<https://swissmodel.expasy.org/>, (Waterhouse et al. 2018)). The *E. coli* FepA (PDB ID: 1FEP) structure was used as a template for modeling. The model was validated by qualitative model energy analysis (QMEAN) scoring and generating a Ramachandran plot using MolProbity.

The conformational B epitope prediction was performed using DiscoTope 2.0 (Kringelum et al. 2012) and ElliPro (Ponomarenko et al. 2008) tools supplied by IEDB. The FepA model was submitted to the tools and the default parameters remained unchanged. The regions predicted as epitopes were saved and those predicted by both tools were considered as more probable conformational B epitopes.

Antigenicity, Allergenicity, Toxicity, Human Similarity, and Experimental Records

All top 10 class II and I T epitopes, linear B epitopes, and segment sequences of conformational B epitopes were checked for antigenicity, allergenicity, toxicity, human similarity, and experimental records.

The antigenicity was predicted using the tool provided by the European Molecular Biology Open Software Suite (EMBOSS) (<https://www.bioinformatics.nl/cgi-bin/emboss/>

antigenic) that employs Kolaskar and Tongaonkar algorithm (Kolaskar and Tongaonkar 1990) to predict antigenicity. The FepA sequence was submitted to the tool and other parameters were left as defaults. The tool predicted more probable antigenic regions. These regions were compared to the previously predicted epitopes and those with antigenic regions were determined.

To check the possible allergenicity of the epitopes, AllerCatPro (Maurer-Stroh et al. 2019) and Structural Database of Allergenic Proteins (SDAP) (Ivanciuc 2003) servers were used. The amino acid sequence of FepA was entered to the tools and other parameters were remained as default. The tools predicted regions with probable allergenicity. These regions were obtained, compared to the previously predicted epitopes, and those with allergenicity were excluded from further studies.

The potential toxicity of the predicted epitopes was evaluated by the ToxinPred server (Gupta et al. 2013) in which FepA sequence and the support-vector machine (SVM) algorithm were used.

For checking the human similarity of the predicted epitopes, BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) was used, in which human proteome (taxid 9606) was selected as the organism. The epitopes with $\geq 90\%$ similarity with human proteome (at both identity and coverage values) were excluded from further studies.

Since experimental records support the predictions, the predicted epitopes were checked for any experimental record using the IEDB home page (<https://www.iedb.org/>). Each epitope was entered separately, human was used as the host, and any found experimental record was obtained.

Finding the Epitope Regions Containing Both T and Linear B Epitopes

The sequences containing both T (class I and II) and linear B epitopes are much more favored as vaccines. Therefore, the top 10 classes I and II T epitopes were clustered with linear B epitopes by the Epitope Cluster Analysis tool, and the regions which had both T and linear B epitopes were selected.

Physicochemical Properties, Tertiary Structure, and Conservancy of the Final Epitopic Peptide

The 3D structure of the final epitopic peptide (the peptide that contained both T and B epitopes) was predicted by the PEP-FOLD server and their physicochemical properties were estimated using ProtParam from ExPASy (<https://web.expasy.org/protparam/>) (Adhikari et al. 2018).

Finally, the conservancy of the final epitopic peptide was checked using BLASTP server and NCBI conserved domain

search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

Results

Class I T Epitope Prediction

Based on 10 highly frequent HLA-I alleles, potential class I T epitopes were predicted using IEDB, ProPred-I, and SYFPEITHI. Each tool predicted different numbers of epitopes (Table S1).

If similar epitopes were predicted for different HLAs, only the best of them, which was that with the highest score, was selected. The selected epitopes of all tools were clustered and one epitope per cluster was chosen, which was the epitope with the highest prediction score or the epitope that had been predicted by more prediction tools.

The epitopes immunogenicity prediction indicated different scores for each epitope. These scores were recorded and higher ones were considered as positive points for selecting final epitopes. The immunogenicity scores of the top 10 class I T epitopes are shown in Table 1.

Class II T Epitope Prediction

Based on the highly frequent HLA-II alleles, potential class II T epitopes were predicted using IEDB, ProPred-II, and SYFPEITHI. No class II T epitopes with ≥ 10 scores (the threshold) were found in ProPred-II, while in IEDB and SYFPEITHI different numbers of epitope were predicted (Table S1). Similar to what was mentioned about the class I T epitope prediction, similar epitopes were found and clustered, and the best epitopes (the epitopes with the highest prediction scores or the epitopes that had been predicted by more prediction tools) were selected.

The selected epitopes were predicted positive or negative in IFN- γ production, however, no epitope was removed in this stage. The states (positive or negative) of each epitope were recorded for the final selections, where being positive in IFN- γ production was considered as a positive selecting point. The states of IFN- γ production of the top 10 class II T epitopes are shown in Table 1.

Class II/Class I Windows and Final Top 10 T Epitopes

All selected class I and class II T epitopes were compared by the cluster analysis tool to find class II/class I windows. Twenty-eight clusters, each containing at least one class II and one class I T epitope, were found. Based on the criteria mentioned about selecting best class II and class I T epitopes (see “Methods” section), the final top 10 T epitopes were selected (Table 1). It is noteworthy that each of the final

Windows of class II and I epitopes&	Class-II epitopes				Class-I epitopes							
	Epitope	HLA allele	Score*		Epitope	HLA allele	Score*					
			SYFPEITHI	IEDB			IFN γ	Docking [#]	SYFPEITHI	IEDB	Immunogenicity	Docking [#]
IFSLFAENN-MELTDT	DRB1*01:01	24	8	-	-129.3 \pm 3.5	LFAENNMEL	A*02:01	18	26.4	N/A	0.01	-87.4 \pm 4.5
ELTDTTMLT-PALRFD		21	6.2	+	-106.8 \pm 2.1	TMLTPALRF		13	N/A	0.75	0.08	-80.0 \pm 4.1
DVTKYVS-LTGGVDNV		22	6.4	+	-107.7 \pm 2.8	SLTGGVDNV		29	78.4	N/A	0.13	-76.8 \pm 3.9
HSIVGN-NWSP-SLNL		24	2.1	-	-101.3 \pm 4.3	VGNNWSPSL		15	26	N/A	-0.05	-77.2 \pm 3.7
GPENTLIL-IDGKPV		10	0.91	-	-100.7 \pm 3.4	GPENTLILI		23	484	N/A	0.16	-71.5 \pm 5.6
NNRIKSLA-LLVNLGI		17	4.7	-	-125.0 \pm 1.3	NNRIKSLAL		20	14.4	N/A	-0.2	-85.3 \pm 5.2
SQGLWDD-FTLKMGA		24	7	+	-116.1 \pm 6.9	GLWDDFTLK		27	80	0.2	0.25	-93.2 \pm 2.6
LVRWEFAP-MQSLEFE		24	8.8	+	-112.0 \pm 2.0	LVRWEFAPM		24	900	0.47	0.47	-83.8 \pm 2.0
SETVN-WTNNI-TYMLQ		24	1.7	-	-111.9 \pm 5.4	WTNNITYML		12	N/A	0.5	0.05	-85.6 \pm 1.9
GDRLSIPE-YTLNST		28	6.5	-	-113.0 \pm 1.9	RLSIPEYT		18	N/A	0.5	0.28	-106.9 \pm 12.2

#Docking scores are presented as affinity (kcal/mol) \pm standard deviation (SD)

⁵The ability and inability of the epitopes in IFN γ production is shown by “+” and “—”, respectively

epitopes was restricted to more HLA alleles, however; only one HLA allele was selected to make the issue simple and to make docking studies more feasible. These top 10 class II/class I T epitopes were all sent for docking studies.

Molecular Docking Studies

The 3D structures of the epitopes, which were modelled by the PEP-FOLD server, were evaluated by Ramachandran plots. The results showed the high quality of the models because high portions of the amino acid residues were located in the Ramachandran plot favored region (Ramachandran plots are not shown). Docking was performed using the HADDOCK server, which presents the outputs as docking scores and some more features (Dominguez et al. 2003; van Zundert et al. 2016). The docking scores of the top 10 epitopes are shown in Table 1.

Linear B Epitope Prediction

Using the linear B epitope prediction tools of IEDB and ABCpred, epitope prediction was performed. Each IEDB prediction tool provided a chart showing positive (with

scores higher than the threshold) and negative (with scores lower than the threshold) epitopic regions (Fig. S1). The positive epitopic regions in the charts were compared and those predicted in more tools were selected as more probable epitopes. The selected epitopes were also compared and clustered to the outputs of the ABCpred tool and only one epitope per cluster, which had the highest score or had been predicted by more tools, was selected. The number of initial linear B epitopes predicted by each tool is shown in Table S1. The top 10 linear B epitopes are presented in Table 2.

Conformational B Epitope Prediction

The quality of the FepA tertiary structure, modeled by the SWISS-MODEL server, was evaluated by the assessment tools provided by the server as well as generating Ramachandran plots. The scoring functions of SWISS-MODEL showed the high quality and accuracy of the model (Fig. 1A–C). Also, Ramachandran plotting showed that a large portion of amino acid residues was in the plot favored region, emphasizing the high quality of the model (Fig. 1D).

Table 2 The top 10 list of FepA predicted B epitopes

Linear epitopes	Antigenicity	Beta turn	Flexibility	Hydrophilicity	Surface accessibility	BepiPred 1.0	BepiPred 2.0	ABCpred
TYSVTWNGAWDNGVTT	–	+	+	+	+	–	+	+
MLHSEVSIPFDYLVNQ	+	–	–	–	–	–	+	+
MGIARAYKAPSLYQTN	+	+	+	+	+	+	–	+
YSKGQGCYASKDGCYL	+	+	–	+	–	+	+	+
NSTLSWQVRDDVSLQS	+	+	–	+	–	–	+	+
NTLILIDGKPVTSRNS	+	+	+	+	+	+	–	+
DSTGRSPYSQAEIFSL	–	+	+	+	+	–	–	+
RGERDTRGDTSWVP	–	+	+	+	+	–	+	+
QGNLYAGDTQNTNSND	–	+	+	+	+	+	+	+
KKTGDEWHGS	+	–	+	+	+	–	+	+
Conformational epitopes segments [‡]						DiscoTope 2.0		ElliPro
GNLDKTQADAWDINQGHQSERTGIYADTL PAGREGVKNKNI						+		+
WNGAWDNGVTT						+		+
QQRMKDNASNTQALSGGEIPGYDSTGRSPY						+		+
TYMNAPEHKDEGSTKRTNF						+		+
AGYSRQGNLYAGDTQNTNSNDLVKENYGKETNRLYRNTY						+		+
VDNVFDKRHWRAQAQTGGATGTMYGAGAETYNESGRTW						+		+
GCYASKDGCYLQGNDDLKAET						+		+
WYGKQEPKKYNYKGQPVGTGSEKNEVSPYSIL						+		+
DYRNKIEAGYAPVYQNNKG						+		–
DLYQWENVPKAVVE						+		–

The predicted epitopes by each tool are shown by “+”

[‡]Conformational epitopes are composed of many amino acid segments that may be near or far from each other. Each tool predicted only one large conformational epitope with many segments. Here the most dominant segments of the conformational epitopes are presented

Applying the FepA 3D model, conformational B epitopes were predicted by DiscoTope 2.0 and ElliPro tools. Each tool predicted one large conformational epitope which was composed of many amino acid segments

(Table S1). The segments were compared and the final conformational epitopic segments, which were those with the highest prediction score or those which were predicted in both tools, were selected (Table 2).

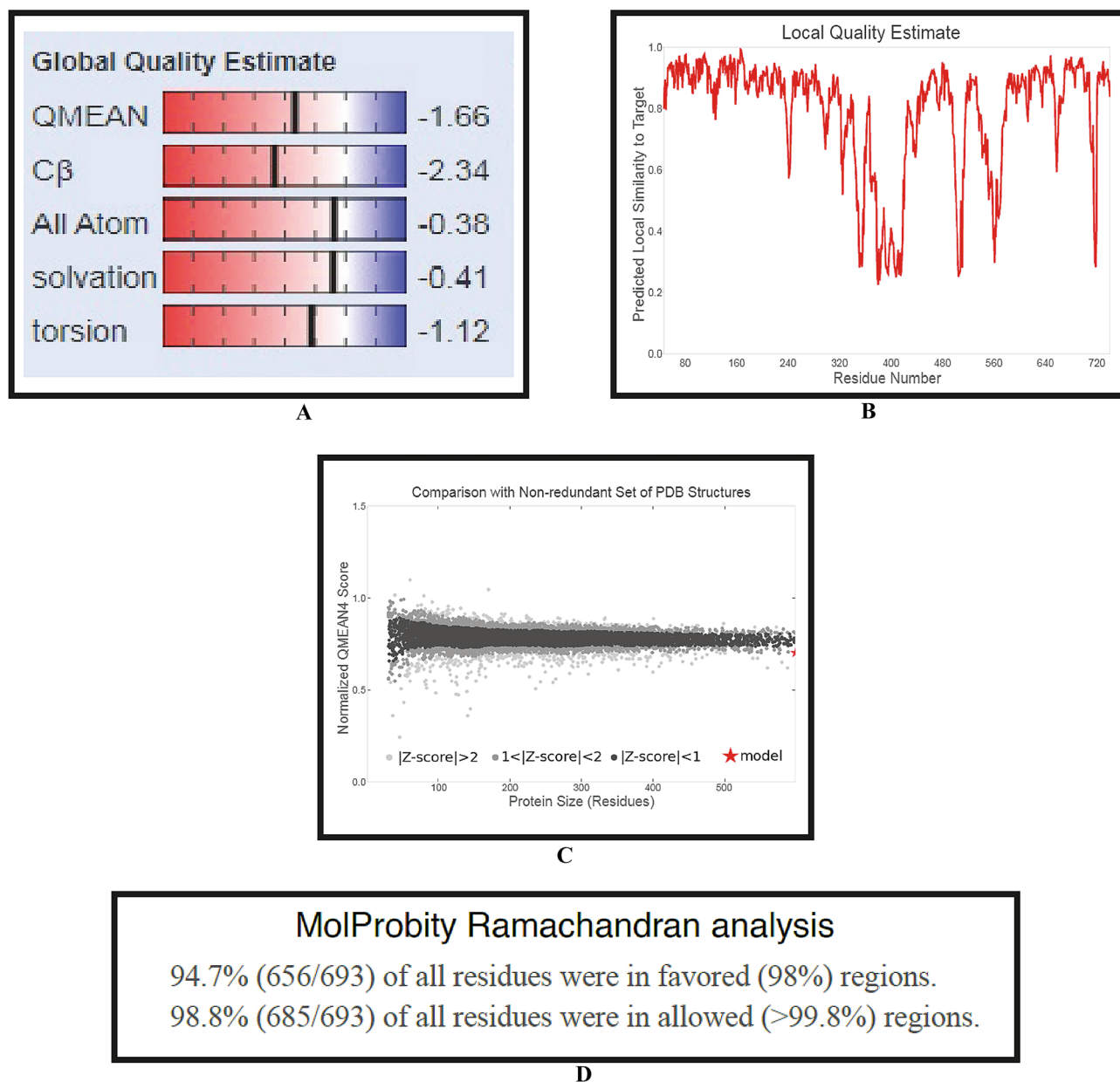


Fig. 1 Evaluation of the quality of FepA modeling. Following homology modeling of FepA, the quality of the model was evaluated by generating the Ramachandran plot and three scoring functions prepared by SWISS-MODEL server namely Global Quality Estimate (GQE), Local Quality Estimate (LQE), and Comparison with PDB structures. **A** GQE plot: The Z-score for each term around zero indicates the better agreement between the experimental structures and

the model. **B** LQE plot: The residues with a score >0.6 are assumed as acceptable quality. **C** Comparison plot: If the generated model (shown by the red star) is close to the grey regions (as it is for the FepA model), it has a high quality. **D** Ramachandran plot results: The main results of Ramachandran plot analysis are summarized in a table that shows the percentage of residues in favored and allowed regions

Antigenicity, Allergenicity, Toxicity, Human Similarity, and Experimental Records

Prediction of FepA antigenic regions led to many regions with different lengths and scores. These regions were compared and clustered with our final predicted epitopes. The common amino acids between antigenicity outputs and the previously predicted top 10 epitopes are shown in Table 3.

The results of allergenicity prediction by both AllerCat-Pro and SDAP indicated that no region of FepA was similar to previously determined allergens (Table 3). It means that none of our predicted epitopes were allergens.

The toxicity prediction results showed that no region of FepA was similar to previously toxins, meaning that none of the selected epitopes was toxic for humans (Table 3).

The human similarity of the selected epitopes was searched by BLASTP. None of the epitopes had $\geq 90\%$ similarity with the human proteome. The highest human similarities that were found for our top 10 epitopes are presented in Table 3.

Searching in IEDB to find experimental records showed that two of the linear and one of the conformational B epitopes had similar sequences with previously published experimental records (Table 3). All of these experimental records belonged to the *E. coli* ferrienterobactin receptor. No experimental record was detected for T epitopes (Table 3).

The Epitopic Region Containing Both T and Linear B Epitopes

The comparison of final T epitopes with linear B epitopes showed only one short epitopic region containing both T (class I and II) and linear B epitopes. This epitopic region (the final epitopic peptide) is shown in Table 4.

Physicochemical Properties, Tertiary Structure, and Conservancy of the Final Epitopic Peptide

The estimated physicochemical properties of the final epitopic peptide including molecular weight, hydrophobicity, net charge, grand average of hydropathicity index (GRAVY), isoelectric pH (pI), and instability index are presented in Table 4.

The peptide comprises 19 amino acid residues with a molecular weight of 2011.26 Dalton and pI equal to 6.07 (Table 4). The estimated half-life of the peptide was 30 h in mammalian reticulocytes, in vitro, > 20 h in yeast, in vivo, and > 10 h in *E. coli*, in vivo (Table 4). The peptide was stable and hydrophilic (Table 4).

The 3D structure prediction revealed that the final epitopic peptide is a mixture of four turns and two strands (Fig. 2). The peptide is buried in the FepA tertiary structure (Fig. 2).

Checking conservancy of the final epitopic peptide showed that it is a highly conserved region in bacteria, especially the Enterobacteriaceae (Fig. 3).

Discussion

Due to the challenging issue of *K. pneumoniae* control via common treatments (Kim et al. 2002; Piperaki et al. 2017), attempts to find prevention strategies such as vaccines are of great interest (Ahmad et al. 2012; Pletz et al. 2016). Here we focused on *K. pneumoniae* FepA as a promising vaccine candidate and comprehensively studied it through immunoinformatics methods. Previous experimental and animal researches have shown the immunogenicity of FepA (of *K. pneumoniae* or other members of Enterobacteriaceae) (Lin et al. 1998a, b, 1999; Baghal et al. 2010; Larrie-Bagha et al. 2013). However, there was no accurate data on FepA T or B epitopes in the literature except one in silico study in which they identified potential epitopes of many OMPs including FepA, and designed two vaccine constructs from these epitopes (Farhadi et al. 2015). However, they mainly focused on the final two constructs and did not robustly predict and analyze the FepA epitopes specially class-I T and linear B epitopes which were completely ignored (Farhadi et al. 2015). It is noteworthy that one of their predicted class-II T epitope (WEFAPMQAL) was among our final top 10 predicted class-II T epitope as well, emphasizing the importance of this epitope. Due to the importance of FepA and lack of accurate data on its epitopes, here we deeply investigated all probable T (class I and II) and B (linear and conformational) epitopes of this highly immunogenic protein to find and introduce the most significant epitopes as vaccine candidates.

Since initial predictions led to a large number of epitopes, they should have been reduced in number to be more manageable. Therefore, in the present study, many analyses were applied to reduce the epitope numbers and to find the best ones. These analyses include the selection of the epitopes with higher scores, the epitopes predicted by several tools, and the epitopes that were not similar to human peptides. Furthermore, other criteria were applied to select the final epitopes as mentioned in the Methods. These analyses led to introduce 10 class I T epitopes, 10 class II T epitopes, 10 linear B epitopes, and 10 conformational B epitope segments for FepA which were highly immunogenic and antigenic, not allergen, not toxic, not similar to human peptides, and in case of T epitopes bind with high affinity to the HLA alleles.

Due to the low number of experimental studies on *K. pneumoniae* antigens and epitopes, only three of our final epitopes were similar to the previous experimental records. These three experimental records which were all found in *E. coli* FepA (Murphy et al. 1990), were very similar to

Table 3 Antigenicity, allergenicity, toxicity, human similarity, and experimental records of the top 10 list of FepA predicted epitopes

	Epitope	Antigenic regions*	Allergenicity [‡]	Toxicity [‡]	Human similarity [‡]		Experimental records [€]
					Coverage (%)	Identity (%)	
Class II T epitopes	IFSLFAENNMELTDT	–	–	–	40	100	–
	ELTDTTMLTPALRFD	–	–	–	40	100	–
	DVTKYVSLTGVDNV	DVTKYVSLTG	–	–	46	100	–
	HSIVGNNWSPSLNLS	SLNLS	–	–	40	100	–
	GPENTLILIDGKPV	TLILIDGKPV	–	–	40	100	–
	NNRIKSLALLVNLGI	KSLAL	–	–	46	100	–
	SQGLWDDFTLKMGA	–	–	–	40	100	–
	LVRWEFAPMQSLEFE	LVRW	–	–	40	100	–
	SETVNWTNNITYMLQ	–	–	–	40	100	–
	GDRLSIPEYTLNST	LSIPEYT	–	–	100	87.5	–
Class I T epitopes	LFAENNMEL	–	–	–	100	77.8	–
	TMLTPALRF	–	–	–	88	100	–
	SLTGVDNV	SLTG	–	–	100	77.8	–
	VGNNWSPSL	SL	–	–	100	77.8	–
	GPENTLILI	NTLILI	–	–	77	100	–
	NNRIKSLAL	KSLAL	–	–	100	71	–
	GLWDDFTLK	–	–	–	66	100	–
	LVRWEFAPM	LVRWE	–	–	100	77.8	–
	WTNNITYML	–	–	–	55	100	–
	RLSIPEYT	LSIPEYT	–	–	66	100	–
Linear B epitopes	TYSVTWNGAWDNG-VTT	TYSVT	–	–	100	39.3	ALTWNGGWDNG-VTTSNWWQYE-HTRNSRIP-EGLAGGTEGK-FNEKATQDFVD
	MLHSEVSIPFDYLVNQ	MLHSEVSIPFDYLVNQ	–	–	68	72.8	–
	MGIARAYKAPSLYQTN	ARAYKAPSLYQTN	–	–	81	61.5	–
	YSKGQGCYASKDGCYL	YSKGQGCYASKDGCYL	–	–	93	58.3	–
	NSTLSWQVRDDVSLQS	RDDVSLQS	–	–	100	66.7	–
	NTLILIDGKPVTSRNS	TLILIDGKPV	–	–	93	66.7	–
	DSTGRSPYSQAEIFSL	PYSQAEIFSL	–	–	37	100	–
	RGERDTRGDTSWVP	–	–	–	42	100	–
	QGNLYAGDTQNTNSND	–	–	–	87	75	NLYAGDTQNTNSDSYTRSKYGDENRLYRQNYA
	KKTGDEWHGS	–	–	–	90	71.4	–
Conformational B epitope segments	GNLDKTQADAWDIN-QGHQSERTGIYADTL-PAGREGVKKNKI	–	–	–	–	–	–
	WNGAWDNGVTT	–	–	–	–	–	ALTWNGGWDNG-VTTSNWWQYE-HTRNSRIP-EGLAGGTEGK-FNEKATQDFVD
	QQRMKD-NASNTQALSGGEIP-GYDSTGRSPY	–	–	–	–	–	–

Table 3 (continued)

Epitope	Antigenic regions*	Allergen- icity [‡]	Toxicity [‡]	Human similarity [‡]		Experimental records [‡]
				Coverage (%)	Identity (%)	
TYMNAPEHKDEGST-KRTNF	–	–	–	–	–	–
AGYSRQGNLYAG-DTQNTNSNDLVKE-NYGKETNRLYRNTY	–	–	–	–	–	–
VDNVFDKRHWAG-NAQTTGGATGTMY-GAGAETYNESGRTW	–	–	–	–	–	–
GCYASKDGCYLQGND-DLKAET	–	–	–	–	–	–
WYGKQEPKKYNYK-GQPVTGSEKNEVS-PYSIL	KGQPVTGSEK-NEVSPYSIL	–	–	–	–	–
DYRNKIEAGYAPVY-QNNKG	AGYAPVYQNNKG	–	–	–	–	–
DLYQWENVPKAVVE	VPKAVVE	–	–	–	–	–

*This column shows amino acid residues of the epitopes which were predicted by antigenicity prediction tools

[‡]None of our epitopes were allergen or toxic (shown by “–”)

[‡]The highest percentages of human similarity (at query coverage and identity values) to the epitopes are shown. No significant similarity found for the conformational epitope segments

[‡]This column shows the previously epitopes found experimentally

The common sequences with our predicted epitopes are shown with bold underlined fonts. All three experimental records belonged to the *E. coli* ferrienterobactin receptor

Table 4 The final epitopic peptide and its physicochemical properties

The final epitopic peptide	GPENTLILIDGKPVTSRNS
Class II T epitope	GPENTLILIDGKPV
Class I T epitope	GPENTLILI
Linear B epitope	NTLILIDGKPVTSRNS
Number of amino acid residues	19
Molecular weight	2011.26 Dalton
Hydrophobicity ratio	36.84%
Net charge at pH 7.0	0
Isoelectric pH (pI)	6.07
Grand average of hydropathicity index (GRAVY)	−0.453
Instability index	38.15-stable
Estimated half-life	30 h (mammalian reticulocytes, in vitro)
	> 20 h (yeast, in vivo)
	> 10 h (<i>Escherichia coli</i> , in vivo)

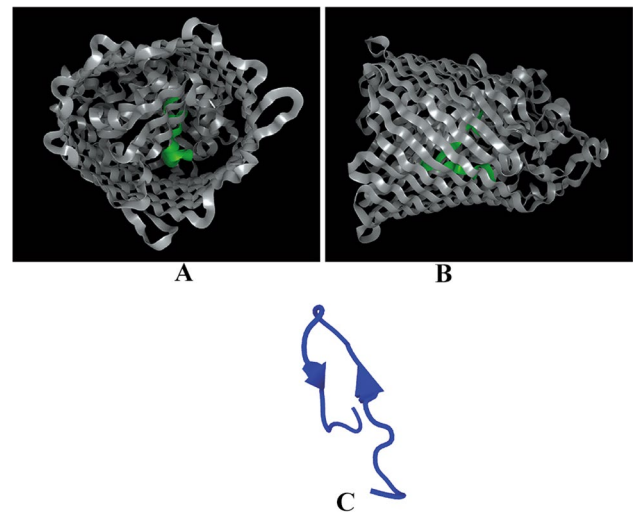


Fig. 2 Structure of the final epitopic peptide in and out of the FepA model. The position of the final epitopic peptide is shown by green color in the whole structure of FepA in top view (A) and lateral view (B). C The tertiary structure of the final epitopic peptide which was generated by the PEP-FOLD server (strands are depicted by arrows)

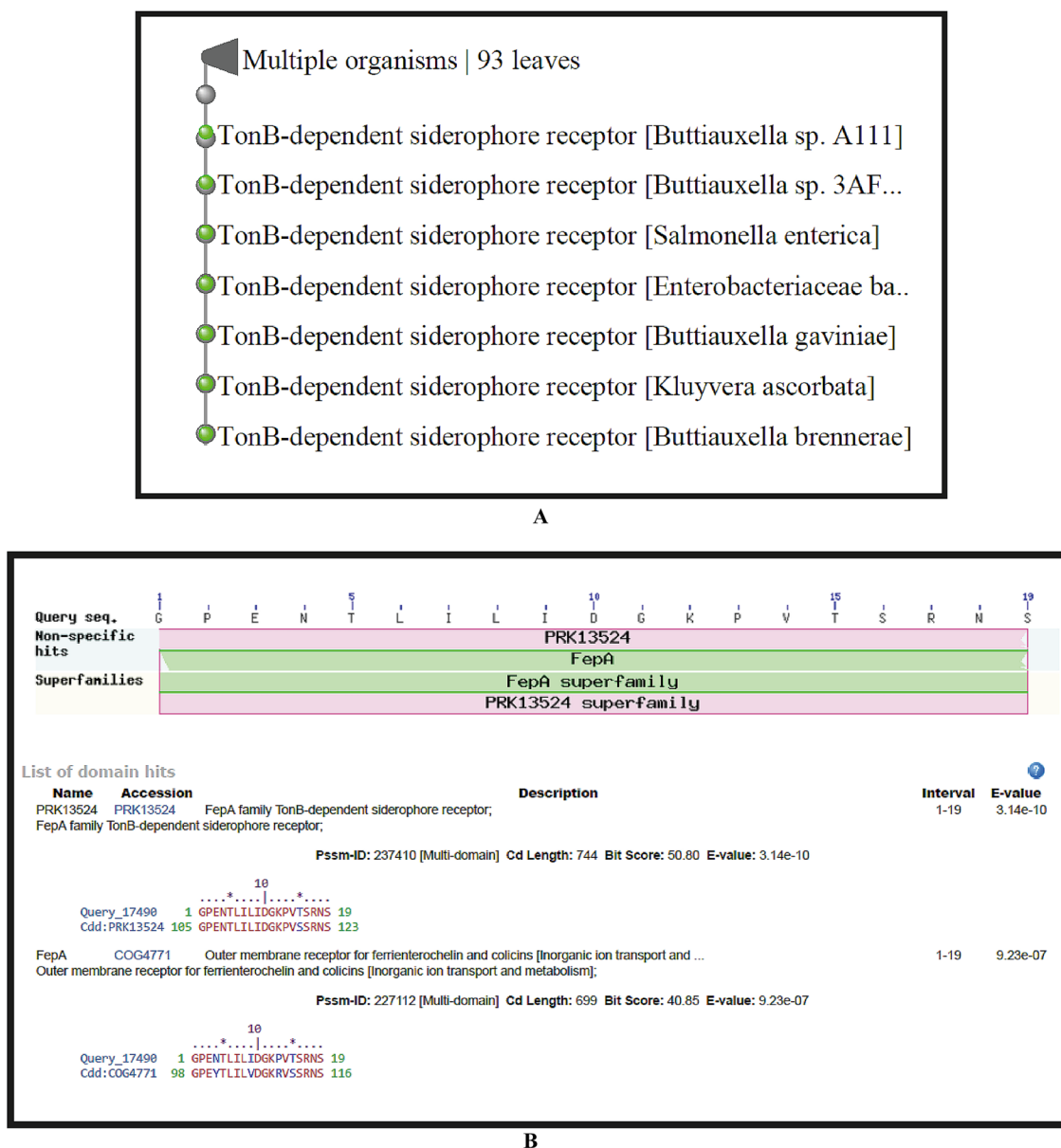


Fig. 3 The conservancy of the final epitopic peptide. **A** The summarized BLAST results are shown. The diagram shows the most significant protein families in which our final epitopic peptide is conserved.

B The results of NCBI conserved domain search which shows two superfamilies (FepA and PRK13524, both belong to siderophore receptors), for our final epitopic peptide

our predicted B epitopes indicating them as more probable epitopes. However, it does not mean that other predicted epitopes are not valid because more experiments are needed to evaluate them. The inadequately experimental studies cause a serious limitation for validation of in silico predicted epitopes.

Conformational B epitopes comprise atoms from distant amino acids joined on the antigen surface, therefore they only shaped when the whole antigen with correct folding exists (Chyau Liang 1998). Because of this issue, here only

the conformational B epitopes and 10 of their most significant segments were introduced, but none of them were applied for selecting the final epitopic peptide. The final epitopic peptide introduced here contained both T (class II and I) and linear B epitopes. If this peptide is used as a vaccine, it may result in both cell-dependent (CD4+ and CD8+ specific) and humoral responses.

If a peptide is used as a vaccine, elucidation of its structure and physicochemical properties will be essential. In the present study, many physicochemical properties

of the final epitopic peptide were investigated including half-life, molecular weight, instability index hydrophobicity, GRAVY, net charge, pI, and 3D structure, all valuable assessments to increase the accuracy of the epitope designs (Adhikari et al. 2018). One of the factors that should be considered for epitope selection is pI (Campos-Pinto et al. 2019) which should not be close to the pH of the body tissues (pH 7.2–7.6) because the immunogenicity of peptides is reduced in their pI (Wang et al. 2018). The pI of the final epitopic peptide of our study was 6.07 which was far from the body's physiological pH.

The hydrophilicity/hydrophobicity profile of a peptide could be indicated by the GRAVY index. Positive GRAVY shows that the peptide is more hydrophobic, while negative GRAVY is an indicator of being more hydrophilic (Kyte and Doolittle 1982; Adhikari et al. 2018). The GRAVY index was negative for our final epitopic peptide indicating it as a more hydrophilic peptide. Moreover, the prediction of stability via instability index revealed that our final epitopic peptide was stable. These factors also suggest the final epitopic peptide as a good vaccine candidate.

Checking conservancy of the final epitopic peptide showed that its sequence belongs to the FepA family siderophores and is highly conserved among bacteria, especially Enterobacteriaceae. Therefore, if this peptide is developed as a vaccine, it may protect humans against many bacteria, although its real efficiency and efficacy need much more specific studies.

Altogether, in the present study, the highly immunogenic T and B epitopes of *K. pneumoniae* FepA were found and introduced for future studies. Furthermore, we introduced a high immunogen and antigenic peptide which contains both T and B epitopes and comprises suitable physicochemical properties that are needed for vaccine development. It is suggested that its actual efficiency and efficacy be investigated by in vitro/in vivo testing.

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Data Availability All data are included in the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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