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The Construction of A Multi-epitope Vaccine Against *Klebsiella pneumoniae* Using *in silico* Approach

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Background: *Klebsiella pneumoniae* is one of the bacteria that causes pneumonia infection. Even though the number of pneumonia cases is relatively high and has become a global problem, there is still no vaccine available to prevent this disease. This study was aimed to design a multi-epitope vaccine design through an *in silico* approach, against *K. pneumoniae.*
Materials and method: Vaccine candidate was constructed based on proteins of *K. pneumoniae*. These proteins were analyzed to identify the antigens sequence for multi-epitope vaccine design. The constructed vaccine was predicted for allergenicity, toxicity, population coverage, and its physicochemical properties. The vaccine structure was then docked with the toll like receptor 2 (TLR2) molecule to show the interaction. Expression analysis and cloning of the constructed vaccine was carried out in the pET-28a vector using SnapGene.
Results: The vaccine was 567 amino acids long, consisting of Cholera Toxin Subunit B as an adjuvant, 6 B-cell epitopes, 11 cytotoxic T-cell epitopes, and 10 helper T-cell epitopes connected with the appropriate linker. Epitopes analysis showed that the vaccine will be a non-toxic, has high antigenicity, but non-allergenic. The vaccine was predicted to be stable, hydrophilic, and had a low risk of triggering autoimmune response. The vaccine molecule was compatible to humans TLR2 molecule. Furthermore, visualization of the candidate vaccine protein on pET-28a showed that the vaccine protein might be expressed correctly.
Conclusion: The construction of multi-epitope vaccine has been developed, which might be a good vaccine candidate, containing 6 B-cell epitopes, 11 CTL epitopes, and 10 HTL epitopes. The construct may help scientists to experimentally formulate multi-epitope vaccine against *K. pneumoniae* in the future.

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

Introduction

Pneumonia is a disease that can be caused by bacteria, fungi, or viruses. One of the main causes is *Klebsiella pneumoniae* bacterial infection. *K. pneumoniae* is a gram-negative opportunistic pathogenic bacterium that belongs to the *Enterobacteriaceae* family which can infect the urinary tract, respiratory tract, blood circulation, and surgical infection.[1,2] The World Health Organization (WHO) designates the bacteria as hypervirulent and prone to

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antibiotic resistance.[3] With 21.7 million cases of pneumonia reported annually and a high mortality rate, this disease is a public health problem that needs special attention.[3] In Indonesia, pneumonia ranked as top ten for hospitalization cases in Indonesia.[4]

Pneumonia vaccine development have been carried out using various approaches by using outer membrane vesicles, capsular polysaccharides, or inactivated whole cell vaccines.[5] Since there is no live component, multi-epitope vaccine method is safer than using live attenuated vaccine/traditional approach. Moreover, the simple peptide sequences required in this work also can be produced at the laboratory level and doesn't require any pathogen. Although clinical trials and studies on experimental animals have been carried out for a long time, there is still no pneumonia vaccine that has been proven to be effective and safe in preventing *K. pneumoniae* infection available on the market. So that further exploration is needed regarding antigens that have the potential to be used as effective vaccines in preventing this disease.[6] Production of vaccines using the reverse vaccinology approach can shorten the time in the vaccine manufacturing process and has been widely used in several vaccine candidates for COVID-19[7], monkeypox[8], and tuberculosis[9].

As pneumonia places a significant burden on the economy and patient life expectancy, particularly in poorer nations, the development of vaccines to prevent diseases brought on by *K. pneumoniae* infection has become an essential public health concern.[10] Even though it is a global health problem, no vaccine has yet been approved for commercialization. Treatment of pneumonia infection by *K. pneumoniae* bacteria is generally difficult to do because this bacterium was easily developing resistance to various antibiotics commonly used in its treatment, as well as comorbidities of the patient.[3] Thus, preventive methods, like vaccines, are the first attempt to prevent infection.[11] Currently, manufacturing vaccine using modern methods, such as multi-epitope vaccine design, is preferable over traditional methods using attenuated bacteria. *In silico* prediction allows the production of vaccines that are more powerful, safer, low allergy risk vaccine that are both efficient in time and money.[12]

Given the relevance of the problem, this study aims to design multi-epitope vaccine candidate through an *in silico* approach. The candidate would consist of combinations of epitope for B cell and T cell, and adjuvant. By using *in silico* approach, we could predict humoral and cell-mediated immunity, terminate the chances of virulence reversal, and strengthen robust innate immune system.

Materials and methods

Protein Target Selection

Complete sequence of *K. pneumoniae* strain HS11286 was retrieved from the National Center for Bioinformatics Information (NCBI) database (*https://www.ncbi.nlm.nih.gov/nuccore/*) with the accession number of GCF_000240185.1. Selection for the presence of signal peptides was carried out with TargetP 2.0 webservers (*https://services.healthtech.dtu.dk/service.php?TargetP-2.0*), DeepSig (*https://deepsig.biocomp.unibo.it*), SignalP 6.0 (*https://services.healthtech.dtu.dk/service.php?SignalP-6.0*), Phobius (*https://phobius.sbc.su.se/*), and Signal BLAST (*http://sigpep.services.came.sbg.ac.at/signalblast.html*). Only proteins with signal peptides detected on the five webservers were continued for prediction of protein localization with the TMHMM 2.0 webserver (*https://services.healthtech.dtu.dk/service.php?TMHMM-2.0*) and PSORTb 3.0.3 (*https:// www.psort.org/psortb/*). The selected protein had outer and transmembrane sites.[13] To avoid cross-reactivity of the vaccine, the protein of the vaccine candidate was checked for homology with that protein in humans using the Basic Local Alignment Search Tool (BLAST) Protein from NCBI (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). The antigenicity prediction of vaccine candidate proteins was carried out using the VaxiJen 2.0 webserver (*http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html*). All settings on the webserver were default settings, except for Vaxijen where the threshold is increased to 0.6 so that only proteins with higher antigenicity were chosen in vaccine construction.

B Cell Epitope Prediction

Linear B cell epitopes were predicted using the ABCPred webserver (*https://webs.iiitd.edu.in/raghava/abcpred/*) with standard settings (16 amino acid length and 0.51 threshold).

Helper T Cell (HTL) Epitope Prediction

HTL epitope prediction was performed using the Immune Epitope Database (IEDB) MHC-II binding webserver (*http://tools.iedb.org/mhcii/*) using the entire selected Human Leukocyte Antigen (HLA) reference set, as well as standard settings (IEDB recommend 2.22 method and 15 peptide epitope result lengths). Furthermore, the HTL epitopes were checked for Interferon-Gamma (IFN-γ)

induction capabilities by the IFNepitope webserver (*http://crdd.osdd.net/raghava/ifnepitop/predict.php*).

Cytotoxic T Cell (CTL) Epitope Prediction

CTL epitopes were predicted using the NetMHCPan 4.1 webserver (*https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/*) by setting 12 HLA representative supertype alleles and the resulting length was set on 9 peptides.

Selected Epitope Characterization

All the candidate of B-cell, CTL, and HTL epitopes were re-checked for their antigenicity capabilities using the VaxiJen 2.0 webserver and confirmed that they did not have the potential to cause toxicity and allergies using the ToxinPred webserver (*https://webs.iiitd.edu.in/raghava/toxinpred/protein.php*) and AllergenFP (*https://ddg-pharmfac.net/AllergenFP/*). All settings on the web server used the default settings.

Population Coverage Prediction

The population coverage of the vaccine was predicted with IEDB Population Coverage webserver (*http://tools.iedb.org/population/*) because the frequency of HLA genotypes in each region will vary. All HTLs and CTLs along with their alleles were entered into the webserver and all settings on the webserver were used using the default settings.

Vaccine Construction

Multi-epitope vaccine construction was carried out by assembling adjuvants, B cell epitopes, HTL epitopes, and CTL epitopes with their appropriate linkers. Adjuvants and B cells were connected with the EAAAK linker, between B cells were connected with the KK linker, B cells with HTL were connected with the GPGPG linker, and finally HTL and CTL were connected with the AAY linker.

Physicochemical Properties, Solvent Accessibility, and Secondary Structure Analysis

Secondary structure analysis was predicted using the NPS-SOPMA webserver (*https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl*), Proteus2 (*http://www.proteus2.ca/ proteus2/*), PSIRPRED 4.0 (*http://bioinf.cs.ucl.ac.uk/psipred/*), I-Tasser (*https://zhanggroup. org/I-TASSER/*), and NetSurfP 3.0 (*https://services.healthtech.dtu.dk/service.php?NetSurfP-3.0*). The ProtParam Expasy webserver (*https://web.expasy.org/protparam/*) was used to predict the physicochemical properties of vaccine constructs such as Grand Average Hydropathicity (GRAVY) values, isoelectric point (pI), molecular weight, stability index, aliphatic index, and half-life. Furthermore, for solubility prediction, SoluProt (*https://loschmidt.chemi.muni.cz/soluprot/*) Protein-Sol (*https://protein-sol.manchester.ac.uk*), and SoDoPe (*https://tisigner.com/sodope*) webserver were used. Solvent accessibility of vaccines could be predicted using the NetSurfP 3.0 and DeepREx-WS webservers (*https://deeprex.biocomp.unibo.it/*). All webserver settings used were default.

Modelling and 3-Dimensional Structure Validation

The I-Tasser webserver was used to create the 3-Dimensional structure model of multi-epitope vaccines. Furthermore, the vaccine model was validated using the Protein Structure Validation Software Suite (PSVS) webserver (*https://montelionelab.chem.rpi.edu/PSVS/PSVS/*) to verify the reliability of the method.[14] Further refinement of the vaccine structure was carried out with the help of the GalaxyRefine tool webserver (*https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE*) and validated again using the PSVS webserver. All settings on the webserver that are used use the default settings.

Docking with Toll Like Receptor-2 (TLR2) Molecule

HDOCK webserver (*http://hdock.phys.hust.edu.cn/*) was used to perform molecular docking between A chain of TLR2 (PDB ID: 6NIG) and the vaccine model. The docking results were then visualized with PyMol software (*https://pymol.org/2/*). All webserver settings used were default.

Codon Optimization and Molecular Cloning

The Java Codon Adaptation Tool/JCat webserver (*http://www.jcat.de/*) was used to convert the amino acid sequences in the vaccine into nucleotide base sequences using *Escherichia coli* as preference organism. Then the appropriate restriction enzymes in the pET-28a vector plasmid were added and the final results were visualized using the SnapGene software 3.2.1 (GSL Biotech LLC, Boston, MA, USA).

Results

Protein Selection

Out of a total of 5316 proteins found on the genome of *K. pneumoniae* strain HS11286, 6 proteins fulfilled

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all the specified criteria (proteins 1-6), namely zinc resistance protein (ID: AEW58919.1); putative porin (ID: AEW59317.1); hypothetical protein (ID: AEW59552.1); the Porin Lamb family (ID: AEW59875.1); putative fimbrial-like protein (ID: AEW61306.1); and a hypothetical protein (ID: AEW63631.1).

B-Cell, CTL, HTL Epitope Selection

The selected B-cell, CTL, and HTL epitopes had the highest test scores from each webserver used, great antigenicity properties, and did not have the potential to cause allergies and toxicity. There were 6 B-cell epitopes, 11 CTL epitopes, and 10 HTL epitopes representing the six selected proteins which could be seen in Table 1, Table 2, and Table 3.

Population Coverage

The allele combination of CTL and HTL found in the vaccine covered 96.97% of the alleles of the world's population. On the continents of Africa, America, Europe, and Australia, it covers 89.65%, 95.28%, 97.88% and 96.84% of the population in the area. Meanwhile, in the Asian region, the coverage reached 93%, specifically in Indonesia itself, the coverage was 86.48%.

Vaccine Construct Analysis

The final vaccine construct length was 567 amino acids and consist of a cholera toxin B (CTB) sequence (UniProt ID: P01556), 6 B-cell epitopes, 11 CTL epitopes, and 10 HTL epitopes linked by their appropriate linkers. The vaccine had a molecular weight of 58.39 kDa; a pI of 8.66; instability index of 28.09; an aliphatic index of 78.69; and a GRAVY score of -0.09. The vaccine also had a predictive half-life of 30 hours if injected *in vitro* in mammalian reticulocytes, >20 hours in *in vivo* trials in yeast, and >10 hours in *in vivo* trials in *Escherichia coli*. While the solubility prediction, the SoluProt, Protein-Sol, and SoDoPe webservers showed the number 0.839; 0.431; and 0.736 respectively. Solvent accessibility from vaccines with the DeepREx webserver showed 316 residues (55.73%) exposed to solvents while 251 residues (44.27%) were not accessible to solvents, on the I-TASSER webserver 353 residues (62.26%) were exposed, and on the NetSurfP 3.0 webserver, 391 residues (68.96%) were exposed. Table 4 showed a secondary structural analysis on all five webservers.

Modelling and 3-Dimensional Structure Validation

The first model from the results of modeling with the I-TASSER webserver was chosen because it has the best C value (-1.11) with a topological similarity (TM) value of 0.58±0.14 and a RMSD value of 10.2±4.6Å. After improvements were made with the Galaxy Refine webserver, the Ramachandran Plot value for the most favored regions increased from 66.7% to 89%. The mean value and Z-score of the Procheck G-factor were also increased from -1.3 to -0.35 and from -7.69 to -2.07.

Docking with TLR2 Molecule

The results of blind docking between the vaccine construct and the A chain of TLR2 molecule could be seen in Figure 1. The docking results showed a docking value of -311.74 with a confidence level of 96.21%. In addition, interactions of 103 residue pairs occurred between vaccine candidate and the TLR2 molecule below 5 Å.

Codon Optimization and Molecular Cloning

Codon optimization of the vaccine construction using the JCat webserver resulted in a sequence of 1701 nucleotides with a Codon Adaptation Index (CAI) value of 1 and a GC content of 53.14%. Additionally, NdeI and SacI restriction sites were added at the N-terminale and C-terminale ends of the DNA sequence to facilitate a sticky end ligation. The vaccine DNA sequence was then simulated for insertion

Table 1. Selected B-cell epitope.

Sequences	ABCPred Score	VaxiJen Score	Protein
AGMGYGGCRGSAGGHM	0.91	1.54	1
DGSQTGGVGAGYNLEV	0.86	2.50	2
KQLAATPQTDTAGANE	0.95	1.05	3
AWIRGDANDVDYRVDG	0.93	1.10	4
AKTAETPFTINLTGCP	0.94	0.74	5
TSPMDARRDLSKKADE	0.88	0.77	6

Vaxijen Cut-off score: 0.4.

Table 2. Selected CTL epitope.

Sequences	Allele	NetMHCPan Score	VaxiJen Score	Protein
ALRQQLQSK	HLA-A*03:01	0.92	0.96	1
DYYAQTSAL	HLA-B*39:01	0.28	0.48	1
AYVGGGVTF	HLA-A*24:02	0.94	0.52	2
TLTDSIHVF	HLA-B*15:01	0.77	0.42	2
GTSTSQLQK	HLA-A*03:01	0.89	1.35	3
ALNKRINEL	HLA-B*08:01	0.88	0.87	3
GELELGSEV	HLA-B*40:01	0.86	0.91	4
TEFGIDYAM	HLA-B*40:01	0.86	0.55	4
STEGTQLTF	HLA-A*01:01	0.67	1.80	5
KADELGGKY	HLA-A*01:01	0.91	2.02	6
KVGSITTSR	HLA-A*03:01	0.76	0.54	6

Vaxijen Cut-off score: 0.4.

into the pET-28a plasmid with the same restriction enzyme. The inserted DNA has a size of ~1.7 kb on a vector of ~5.3 kb (Figure 2).

Discussion

In this study, a multi-epitope vaccine based on a *in silico* approach uses six target proteins to select appropriate candidate epitopes. The selected proteins come from *K. pneumoniae* strain HS11286 which is the standard/reference strain of *K. pneumoniae*.[15] The B cell epitope is an important area of an antigen for recognition and binding to antibodies of a host, so that it may trigger an immune response.[16] Helper T cell lymphocytes/HTLs play an important role in the adaptive immune system. HTL is involved in the activation of cytotoxic B cells and T cells to produce antibodies and the removal of infective target cells.[17] Meanwhile, cytotoxic T cell lymphocytes/CTLs induce an MHC-I immune response by neutralizing infected and damaged cells by releasing cytotoxic proteins such as granzyme and perforin.[17]

The best-performing linear B and T cell epitopes in each analysis showed that the oligopeptide was not allergic, non-toxic, and had antigenicity above the threshold. After selecting the epitope, an adjuvant was added to the N-terminus of the vaccine. Adjuvants were used to assist and stimulate the active adaptive immune responses to vaccines.[18] CTB is the selected adjuvant in the vaccine candidate in this study which has the advantages of being stable and non-toxic. In addition, CTB as an adjuvant has been widely used in various *in vivo* vaccine studies and has

Table 3. Selected HTL epitope.

Sequences	Allele	IEDB Score	IFN Score	VaxiJen Score	Protein
DSGKIEAVAQEMEGL	HLA-DQA1*05:01/DQB1*02:01	1.20	0.09	0.66	1
QQRVKFDIALAEAGV	HLA-DPA1*02:01/DPB1*14:01	1.20	0.23	1.06	1
LTAVSGSAMAVGFTV	HLA-DQA1*05:01/DQB1*03:01	0.85	0.70	0.91	2
RKLNILILAALTAVS	HLA-DRB1*01:01	0.24	0.72	0.5	2
AATLTLNAPLLMLVA	HLA-DRB3*02:02	0.33	0.33	1.00	3
ALAAALIAPSAFAAT	HLA-DQA1*05:01/DQB1*03:01	0.27	0.68	0.43	4
AFNFIADVKSDSSQD	HLA-DRB1*03:01	0.83	0.11	0.93	5
NGTSAAVLALSDSAD	HLA-DQA1*01:02/DQB1*06:02	0.56	0.19	0.76	5
LLALSLATVTTARAA	HLA-DRB1*08:02	0.52	0.21	0.61	5
KYFVVIAGQKNEKTV	HLA-DRB5*01:01	0.54	0.21	0.5	6

Vaxijen Cut-off score: 0.4.

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Table 4. Vaccine's secondary structure analysis.

Webserver	Coil	Helix	Sheets
NetSurf	332 residues	111 residues	124 residues
Proteus	388 residues	94 residues	85 residues
NPS-SOPMA	188 residues	208 residues	171 residues
PSIRPRED	248 residues	186 residues	133 residues
i-TASSER	417 residues	105 residues	45 residues

been proven safe, so it was approved for use as a vaccine adjuvant by European and Canadian authorities.[19]

Vaccine construction was made using adjuvants linked to the first B-cell epitope with the EAAAK linker, then between linear B-cell epitopes linked with the KK linker. The GPGPG linker connects the HTL epitope with the last B-cell epitope as well as between the HTL epitopes. Then the last HTL epitope and CTL epitope are connected to the AAY linker so that a total of 567 amino acids made up the vaccine. The EAAAK linker is a linker that is commonly used in the preparation of *in silico* vaccines and has been tested to increase its structural stability and immunogenicity. This linker also has a rigid helical structure.[20] Whereas the KK, GPGPG, and AAY linkers are hydrophilic amino acids and can prevent domain function disturbances and vaccine folding. The GPGPG linker can stimulate the HTL response and conformation of the bound immunogenicity of HTL, while the AAY linker can increase epitope presentation and eliminate junctional epitopes.[21,22]

In general, vaccine candidates cover up to 96.97% of the world's population alleles, specifically in Indonesia itself, the coverage is quite high, namely 86.48%. In addition, the vaccine candidate also meets good vaccine parameters, namely having a low molecular weight (below 110 kDa), as well as an instability index below 40 which proves that the vaccine molecule is stable.[23] A negative GRAVY value indicates that the vaccine is hydrophilic[24] and is supported by its solubility prediction with 3 different web servers with high results. Meanwhile, an aliphatic index of more than 50 in the vaccine indicates that the vaccine is thermostable.[25] The vaccine also had a predictive half-life of 30 hours if injected *in vitro* in mammalian reticulocytes, more than 20 hours in *in vivo* trials in yeast, and more than 10 hours in *in vivo* trials in *E. coli*. The prediction of solvent accessibility shows results above 55% which indicates that the high possibility of a vaccine being immunogenic.[26]

The results of modeling using the I-TASSER webserver are then refined to improve the most favored regions, which shows the stability of the structure. Furthermore, the perfected vaccine molecule is docked with the TLR2 molecule. The docking results showed many interactions between proteins, good docking values with a high level of confidence. Codon optimization is carried out to ensure high gene expression in the host cell, considering the inconsistency of mRNA codons is caused by variations in foreign gene expression in the host cell genome.[27] The vaccine candidate was successfully visualized on pET-28a vector that might be expressed in *E. coli* cells.

There is absence of experimental validation of the protective antigenicity of the identified proteins with vaccine potential in this study, therefore, further analysis via *in vivo* or *in vitro* experiments is needed to allow the vaccine candidate to be used in various immunological studies that could confirm the results of this study.

Figure 1. The docking between TLR2 molecules and vaccine candidate using the HDDOCK webserver and shown in cartoon form. The green color indicates the TLR2 molecule, while the blue color indicates the vaccine molecule.

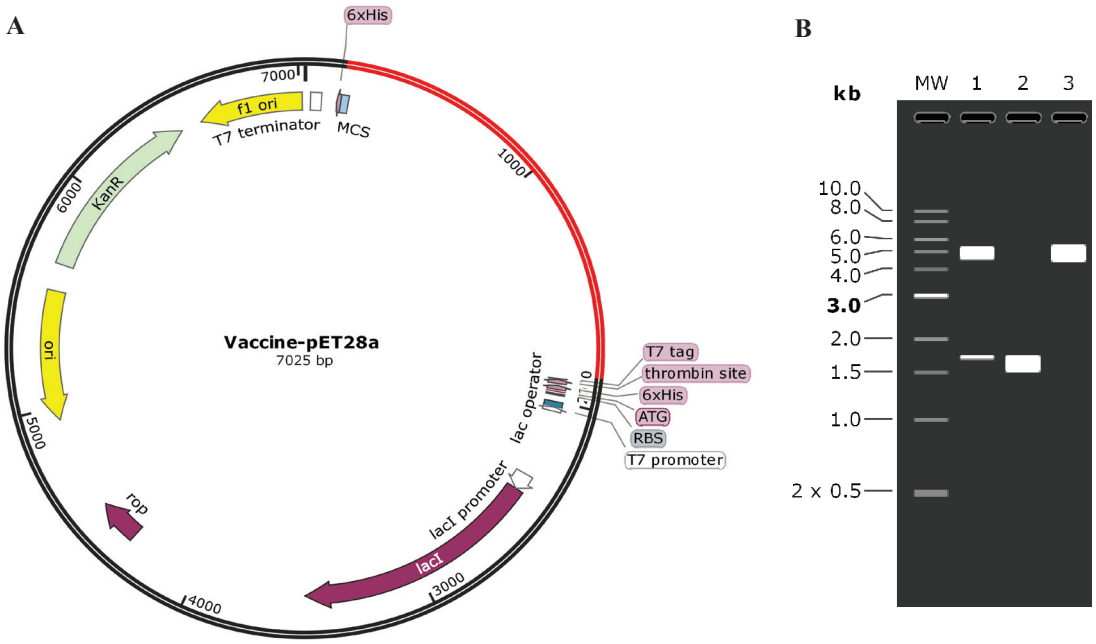

Figure 2. *In silico* cloning of multi-epitope vaccines using SnapGene software. A: The multi-epitope vaccine sequence was drawn in red colour, while the black line colour is pET-28a backbone vector. B: Virtual conformation of the vaccine digested with 2 restriction enzymes, NdeI and SacI using SnapGene software. MW row: the marker; row 1: the recombinant plasmid digested with NdeI and SacI; row 2: the vaccine insert; row 3: pET-28a plasmid.

Conclusion

The multi-epitope vaccine designed in this study is expected to be a vaccine candidate for pneumonia caused by *K. pneumoniae* infection, given the absence of a vaccine that specifically prevents infection by this bacterium. The vaccine construct consists of B-cell, CTL, and HTL epitopes with various HLA that can trigger immunity in the human body when injected. The vaccine also includes a high allele population, as well as no allergic reactions or toxicity, high antigenicity, and minimal risk of autoimmune reactions as there is no homology with human proteins. The vaccine fulfills all the parameters of a good vaccine candidate. In addition, the vaccine can also be expressed in *E. coli* bacteria for overproduction. With high-cost requirements and limitations for developing the traditional vaccine preparation, this multi-epitope vaccine can be valuable candidate for therapeutic, preventive, as well as diagnostic and are useful for further experimental validation to prevent pneumonia disease.

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Authors Contribution

DW and MW were involved in concepting and planning the research. DW performed the data acquisition/collection, calculated the experimental data and performed the analysis, drafted the manuscript, and aided in interpreting the results. DW and MW designed the figures. MW took parts in giving critical revision of the manuscript.

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