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Designing a multiepitope mRNA-based vaccine against enterotoxigenic *Escherichia coli* infection in calves: Immuno-informatics and molecular modeling approach

Zainab Isam Alawadi[1], Amjed Alsultan[1], Dhama Alsallami[2], Sultan F. Alnomasy[3], Mohammed Alqasmi[4], Fawaz M. Almufarriji[3], Bader S. Alotaibi[3], Bi Bi Zainab Mazhari[5] and Rawaf Alenazy[4*]

[1]*Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, Al- Diwaniyah, Iraq*
[2]*Department of Physiology, Pharmacology and Biochemistry, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq*
[3]*Medical Laboratories Department, College of Applied Medical Sciences in Al- Quwayiyah, Shaqra University, Shaqra, Saudi Arabia*
[4]*Department of Medical Laboratory, College of Applied Medical Sciences-Shaqra, Shaqra University, Shaqra, Saudi Arabia*
[5]*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakakah, Saudi Arabia*

Abstract

Background: *Escherichia coli* is one of the serious pathogens causing various infections in the animal field, such as neonatal calf diarrhea, which is responsible for mortality associated with diarrhea during the first days of life.

Aim: Current work is aimed at designing an effective and safe multiepitope vaccine candidate against *E. coli* infection in calves based on the fimbrial protein K99 of Enterotoxigenic *E. coli* (ETEC) and Immuno-informatics.

Methods: A conserved sequence of K99 protein was generated, and then highly antigenic, nonallergic, and overlapped epitopes were used to construct a multiepitope vaccine. Five THL, six MHC II, and four beta cell epitopes were targeted to create the candidate. The candidate vaccine was produced utilizing 15 epitopes and three types of linkers, two types of untranslated region (UTR) human hemoglobin subunit beta (HBB), UTR beta-globin (Rabb), and RpfE protein as an immunomodulation adjuvant.

Results: Immuno-informatics analysis of the constructed protein showed that the protein was antigenic (antigenic score of 0.8841), stable, nonallergen, and soluble. Furthermore, the Immuno-informatics and physiochemical analysis of the constructed protein showed a stable, nonallergic, soluble, hydrophilic, and acidic PI (isoelectric point). of 9.34. Docking of the candidate vaccine with the toll-like receptor TLR3 was performed, and results showed a strong interaction between the immune receptor and the vaccine. Finally, the expression efficiency of the construct in *E. coli* was estimated via computational cloning of the vaccine sequence into Pet28a.

Conclusion: Results of immunoinformatics and *in silico* approaches reveal that the designed vaccine is antigenic, stable, and able to bind to the immune cell receptors. Our results interpret the proposed multiepitope mRNA vaccine as a good preventive option against *E. coli* infection in calves.

Keywords: Neonatal calf diarrhea, Fimbrial protein K99, Enterotoxigenic *E. coli*, Multiepitope vaccine, mRNA vaccine.

Introduction

Calf diarrhea is one of the important diseases that cause major losses to farm animals' producers (Coşkun *et al*., 2023). Several enteric pathogens are involved in the development of the disease, including viruses, bacteria, parasites, and fungi (Coşkun *et al*., 2023; Feuerstein *et al*., 2022). Pathogenic *Escherichia. coli* causes diarrhea and septicemia in newborn calves. The enteric form of the disease occurs during the first days of calves' life associated with a high prevalence and mortality rate. The disease causes severe economic losses and affects public health because the infected calves represent a reservoir of the bacterium to the contacting human (Feuerstein *et al*., 2022). Pathogenic *E. coli* has a set of virulence factors that are used to colonize the intestine and initiate the infection.

***Corresponding Author:** Rawaf Alenazy. Department of Medical Laboratory, College of Applied Medical Sciences-Shaqra, Shaqra University, Shaqra, Saudi Arabia. Email: *ralenazy@su.edu.sa*

Depending on these factors, *E. coli* is classified into 6 groups including Enterotoxigenic (ETEC), Shiga toxin-producing, Enteroaggregative, Enteroinvasive, Enterohaemorrhagic, and Enteropathogenic. ETEC strains are the main cause of diarrhea in calves (Kolenda *et al*., 2015). To make colonization in the calf intestine, ETEC produces two fimbrial antigens (F5 and F41). Fimbrial antigen K99 also known as F5 is the main antigen that adheres to receptors in the gut epithelium and establishes the first step of colonization (Shams *et al*., 2012; Cho and Yoon, 2014). After being attached to the intestinal epithelium, ETEC produces an enterotoxin that acts on stimulating the mucosal cell to excrete sodium, chloride, and water which leads to disrupted fluid homeostasis and severe diarrhea in the infected calves (Kolenda *et al*., 2015).

According to recent reports and surveys, ETEC *E. coli* strains can resist a wide range of antibiotics including tetracycline, beta-lactam, flumequine, and sulfamethoxazole/trimethoprim. The increasing prevalence of multidrug resistance of ETEC in calves causes difficulties in the control of the disease (Kolenda *et al*., 2015; Cho *et al*., 2015). Several researchers suggested the vaccine policy to be a global strategy in combatting multidrug-resistant bacteria. However, hypersensitivity is one of the major limitations of traditional vaccines that are designed with large proteins (Khalid *et al*., 2022; Islam *et al*., 2022; Jalal *et al*., 2023). Compared to traditional vaccines, multiepitope using reverse vaccinology is a new vaccine approach with a lot of advantages including strong stimulation of cellular and humoral immune response, low-cost production, and high specificity and safety (Hashish *et al*., 2013; Feuerstein *et al*., 2022; Sanami *et al*., 2023). Fimbrial protein K99 was used as a target protein for epitope prediction. K99 was chosen to construct the candidate vaccine because it is responsible for the adherence of bacteria to the gut epithelial cell and starts the first step of colonization and infection. Previous studies suggest that the K99 fimbrial subunit provides good protection against ETEC (Feuerstein *et al*., 2022; Sanami *et al*., 2023). The antigenicity of the candidate mRNA vaccine was enhanced by incorporating the resuscitation-promoting factor E (RpfE) protein sequence in the main vaccine structure. The current study aims to design a multiepitope vaccine against ETEC using a reverse vaccinology approach.

Materials and Methods

A graphic depiction of the process employed for the construct of the candidate vaccine is represented in Figure 1.

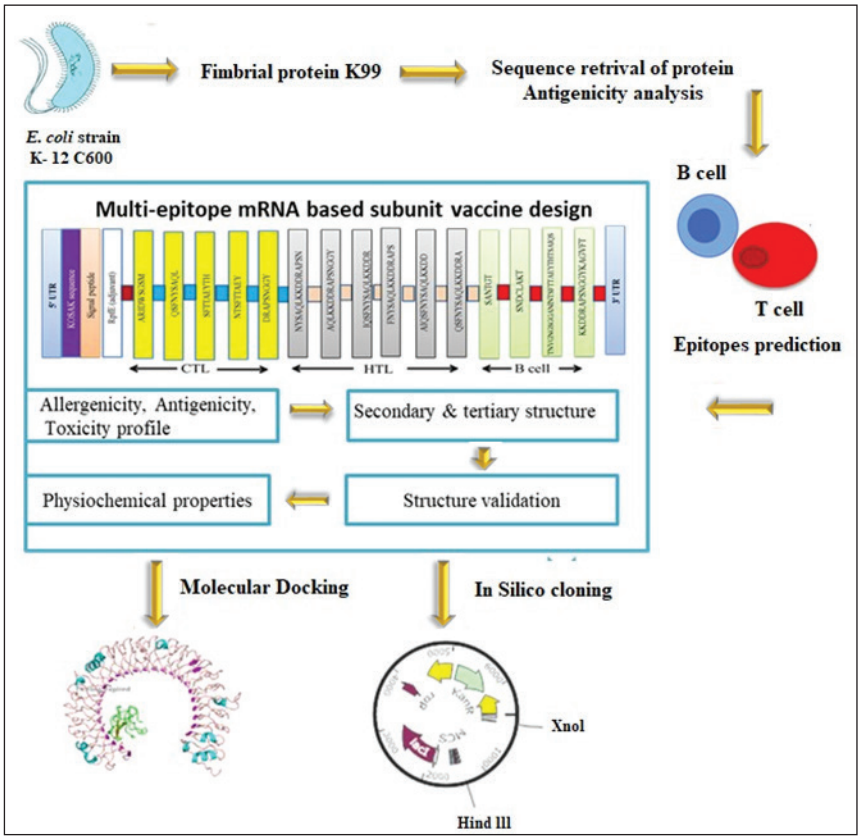

Fig. 1. Schematic flowchart of the study.

Sequence retrieval of protein

We used NCBI (the National Centre for Biotechnology Information) for the retrieval of the fimbrial protein K99 sequence of ETEC *E. coli* strain K-12 C600. K99 is a crucial protein that allows bacteria to adhere to receptors in the gut epithelium and establishes the first step of colonization (Kalita *et al*., 2014). Therefore, K99 was recruited as a target antigen for ETEC vaccine development.

Epitope mapping

MHC classes I, MHC class II, and B cell linear epitopes were mapped using the IEDB web server (https://www.iedb.org/) (Zhao and Li, 2009). The default parameter was used to predict epitopes. High-ranged and overlapped epitopes were chosen for further analysis.

Analysis of the conserved epitope

The conservation of B and T cell epitopes was analyzed using the IEDB conservancy analysis tool (Bui *et al*., 2007). Epitopes that show 100% conservation were selected for further analysis.

Prediction of antigenicity and allergenicity

Antigenicity of the candidate epitopes from IEDB analysis was filtered using VaxiJen v2.0 web server (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Saha and Raghava, 2006; Doytchinova and Flower, 2007). Bacteria were chosen as a target organism and the prediction of antigenicity was based on a 0.5 threshold. Highly antigenic epitopes were further analyzed for Allergy predictions using AllerTOP (https://www.ddg-pharmfac.net/AllerTOP/method.html) (Saha and Raghava, 2006). Only nonallergenic and antigenic epitopes were used to construct the multiepitope vaccine.

Assembly of the final multiepitope vaccine

To construct the final vaccine candidate, highly conserved, antigenic, and overlap epitopes were joined using GPGPG, AAY, and KK linkers. These Linkers act to preserve the independent immunogenic activity of epitopes via prevent epitopes from interacting with each other.

(Chen *et al*., 2013). RpfE of Mycobacterium tuberculosis (Uniprot ID: O53177) was linked to the N terminal of the construct via EAAAK linker as an adjuvant to improve the antigenicity of the construct. In several studies, RpfE was used as an adjuvant to induce the immune system where RpfE enhanced differentiation of CD4 and maturation of dentric cell (DC).

To increase the stability of the mRNA multiepitope vaccine two UTR sequences were added. Hemoglobin subunit beta (HBB) at N and rabbit beta-globin at C end (Chen *et al*., 2013).

Profile of physiochemical and immunological properties of the construct

A chemical and physical characteristic of the construct was predicted using the ProtParam web server https://web. expasy. org/ protp aram/ (Gasteiger *et al*., 2005). Molecular weight, number of AA, PI, aliphatic index, *in vitro*, and *in vivo* estimated half-life using the N-end rule, and hydropathicity index of the designed vaccine were tested. The antigenicity of the vaccine was checked with the VaxiJen v2.0 web server. While the Al-lerTOP server was used for the estimation of the Allergenicity of the construct.

Prediction of secondary structure

PSIPRED v3.3 web servers were used to estimate the secondary structure such as beta-turn, random coils, and alpha-helix of the construct vaccine (McGuffin *et al*., 2000).

Prediction of tertiary structure (3D)

The I-TASSER web server (https://zhanggroup.org/I-TASSER/) was used to build the three-deamination structure of the constructed vaccine. The good quality three-deamination structure of the model vaccine was chosen according to the confidence score which ranged from −5 to 2 (Roy *et al*., 2010).

Validation and refinement of the model vaccine

Refining of the vaccine structure was performed using the Galaxy webserver (galaxy.seoklab.org/refine) (Heo *et al*., 2013). The RAMPAGE webserver was used to analysis of Ramachandran plot to validate the refined structure (Lovell *et al*., 2023).

Docking of the model vaccine with the immune receptor (TLR-3)

The ClusPro v2.0 webserver (cluspro.bu.edu/) was used to analyze the affinity between the model vaccine and receptor molecule (Abdellrazeq *et al*., 2020). The TLR3 receptor was selected and uploaded from the PDB webserver (PDB ID: IZIW). The educational version of the PyMOL(PyMOL-3.0-Windows-x86_64.exe2023-10-21 01:54433M) was used to draw figures and visualize the docked complex. HDOCK web server was used to further analyze of affinity between the modeled vaccine and the selected receptor (https://cluspro.bu.edu/).

In silico cloning and codon optimization

Codon optimization is essential for efficient gene expression in foreign hosts (Grote *et al*., 2005). The DNA sequence of the construct was uploaded to the JCat web server (www. jcat. de/) for codon optimization (Grote *et al*., 2005). Optimization of codon was based on guanine–cytosine (GC) concentration and codon adaptation index (CAI) which should be 0.1. Finally, *in silico* cloning of the optimized nucleotide sequence into the pET28a (+) expression vector was performed using the Snap Gene 4.2 tool (https:/snapgene.com/) (Pandey *et al*., 2018).

Ethical approval

Not needed for this study.

Results

Sequence of k99 protein

The selected K99 protein (accession no. P18103) had 181 amino acids. The k99 antigenicity was evaluated with the VaxiJen v2.0 webserver and results showed that of K99 protein is a probable antigen with an antigenic score of 1.003 double than threshold score.

Subsequent investigation was performed for the main sequence of the K99 protein.

Epitope mapping

One hundred% conserved, nonallergic, antigenic epitopes were screened in the K99 sequence. A shortlist of 15 epitopes that can induce B and T cells were chosen. As shown in Table 1, five immunogenic, nontoxic, and nonallergenic THL epitopes were chosen to create the candidate vaccine. the characterization of THL epitopes that are candidates for building the vaccine is summarized. Six CTL epitopes with lengths of 15 amino acids were chosen (Table 2). In the same manner, four linear B-lymphocyte (LBL) epitopes were inserted in the candidate vaccine (Table 3).

Construction of mRNA multiepitope vaccine

mRNA multiepitope vaccines were constructed utilizing T, and B cell epitopes that were previously nominated. AAY, GPGPG, and KK linker were used to fuse epitopes. To elevate the immunogenicity of the construct, RpfE (O53177) was used as an adjuvant. RpfE and Kozak's sequence were incorporated in the 5' region of the mRNA vaccine via the EAAAK linker. Human hemoglobin subunit beta (HBB) and rabbit beta-globin were added to the 5′ and 3′ regions, respectively (Fig. 1).

Analysis of physiochemical and immunological properties of the vaccine construct

The results of predication showed that the vaccine is nonallergic, antigenic, soluble, and nontoxic (Table 3). The physicochemical properties of the vaccine construct were predicated using the ProtParam tool. As mentioned in Table (4) protein has a molecular weight of 35 KD, the estimated half-life *in vitro* in mammalian cells is 1.9 hours, and the PI of 9.34. Other properties are summarized in Table 4. A protein analysis result reveals that the chimeric protein is stable and suitable as a vaccine candidate against *E. coli* infection.

Prediction of secondary structure

The secondary structure of the vaccine construct was predicated on utilizing PSI-PRED v3.3 and NPS@ server. The s structure consisted of 37% Helix, 48% Coil, and 13% stand. During infection antibodies detect the unfold region of protein. As shown in Figure 3, a

Table 1. Final selected CTL epitopes for the candidate vaccine.

Sr. no	Epitopes	Antigenicity score	Toxicity	allergencity	conservancy
1	ARIDWSGSM	0.7	No	None	100%
2	QSFNYSAQL	0.6	No	None	100%
3	SFTTAEYTH	0.7	No	None	100%
4	NTSFTTAEY	0.8	No	None	100%
5	DRAPSNGGY	0.7	No	None	100%

Table 2. Final selected HTL epitopes for the candidate vaccine.

Sr. no	Epitopes	Antigenicity score	Toxicity	allergencity	conservancy
1	NYSAQLKKDDRAPSN	0.8480	No	None	100%
2	AQLKKDDRAPSNGGY	0.7955	No	None	100%
3	IQSFNYSAQLKKDDR	0.7762	No	None	100%
4	FNYSAQLKKDDRAPS	0.8075	No	None	100%
5	AIQSFNYSAQLKKDD	0.7162	No	None	100%
6	QSFNYSAQLKKDDRA	0.8001	No	None	100%

Table 3. Final selected LBL epitopes for the candidate vaccine.

Sr. no	Epitopes	Antigenicity score	Toxicity	allergencity	conservancy
1	SANTGT	2.2	No	None	100%
2	SNDCLAKT	0.6364	No	None	100%
3	TNVGNGSGGANINTSFTTAEYTHTSAIQS	1.2	No	None	100%
4	KKDDRAPSNGGYKAGVFT	0.9902	No	None	100%

Table 4. Immunogenic and physicochemical characteristics of the vaccine construct.

Characteristic	Measurement	Remark
Total number of amino acids	333	Suitable
Molecular weight	35042.09	Appropriate
Theoretical pI	9.34	Basic
Formula	$C_{1521}H_{2320}N_{448}O_{502}S_4$	-------
Predicted half-life (*Escherichia coli*, *in vivo*)	10 hours	-----
Predicted half-life (mammalian reticulocytes, *in vitro*)	10 hours	------
Predicted half-life (yeast-cells, *in vivo*)	20 hours	-----
Grand average of hydropathicity (GRAVY)	-0.807	Hydrophilic
Instability index of vaccine	23.95	stable
Antigenicity	0.8841	antigenic
Allergenicity	Non-allergic	Non-allergic
Toxicity	Nontoxic	Nontoxic

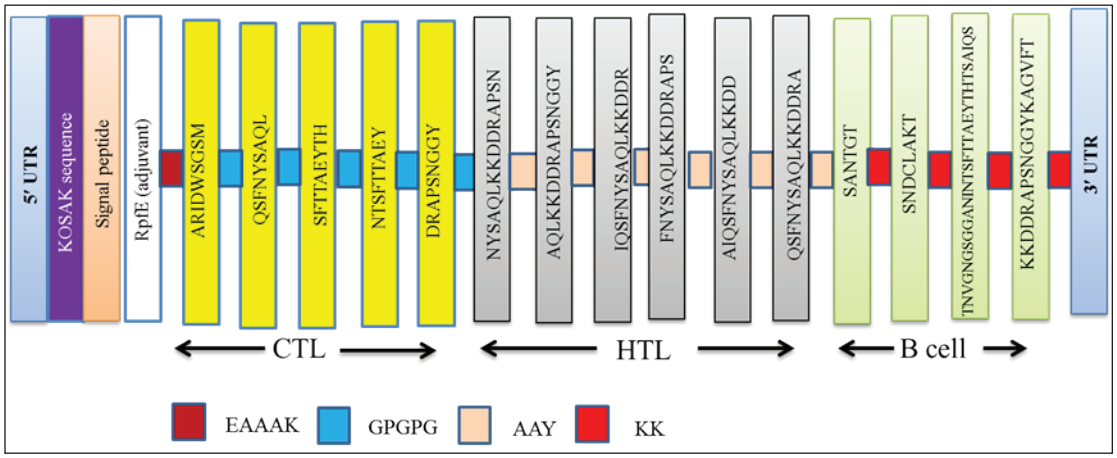

Fig. 2. Graphical map of the mRNA multiepitope vaccine construct: HBB (UTR), Kosak sequence, signal peptide, and adjuvant were linked at N terminal with help of EAAAK linker. CTL epitopes fused via GPGPG linker while HTL epitopes linker via AAY linker. B cell epitopes fused via KK linker. Rabbit beta-globin was linked to the C terminal as UTR.

random coil shows these unfold regions in the vaccine construct.

Modeling, validation, and refinement of the 3D model

The prediction is based on identifying similar structures in the PDB library. Five top models were predicated with confident scores for each model (−3.19, −2.09, −3.65, −4.15, and −3.91). C-score ranged from −5 to 2 and predicated structure with C-score below −1.5 is an atypically good model. Out of the five models, Model 1 with C-score −3.19 with an estimated TM-score 0.36 ± 0.12 and estimated RMSD = 14.2 ± 3.8Å was advised by the server; therefore, we used it as 3D of the vaccine construct (Fig. 4 A). The selected structure was refined with the Galaxy web server (Fig. 4B). According to the Ramachandran plot analysis of the refined structure with PROCHECK; 90.5% of the residues are in the preferred area while 8.4% and 0.7% of the residues reside in permitted and disallowed areas, respectively (Fig. 4 C). Furthermore, another analysis of the selected model reveals that MolProbity is 1.7., poor rotamers are 0.4%, clash score is 10.5, RMSD is 0.428, and GDT-HA is 0.9707. The quality factor of the structure which estimates with ERRAT was 91.98. ProSA-web server was used to calculate *Z*-score which indicates to overall quality structure of the model. The estimated *Z*-score was −0.77 (Fig. 4 D). Overall, the results of the structural analysis of the vaccine construct revealed that it's a highly flexible protein which makes it a good preventive option against *E. coli* infection in calves.

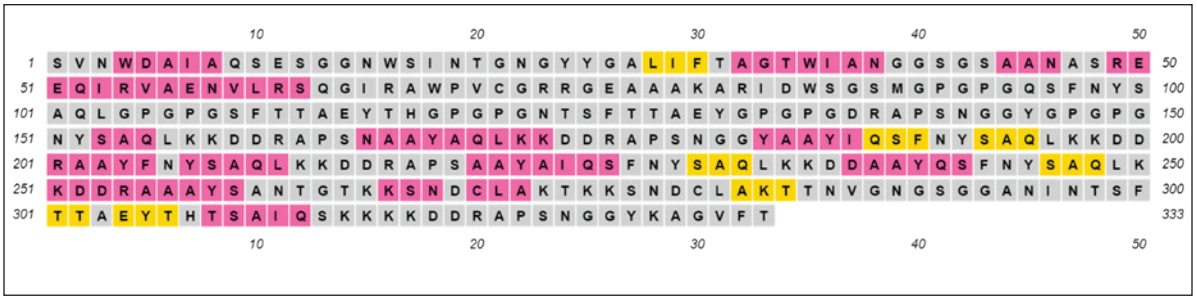

Fig. 3. Secondary structure elements predication of designed mRNA multiepitope-based vaccine using PSIPRED 4.0, Gray: coil, Pink: Helix, and yellow strands.

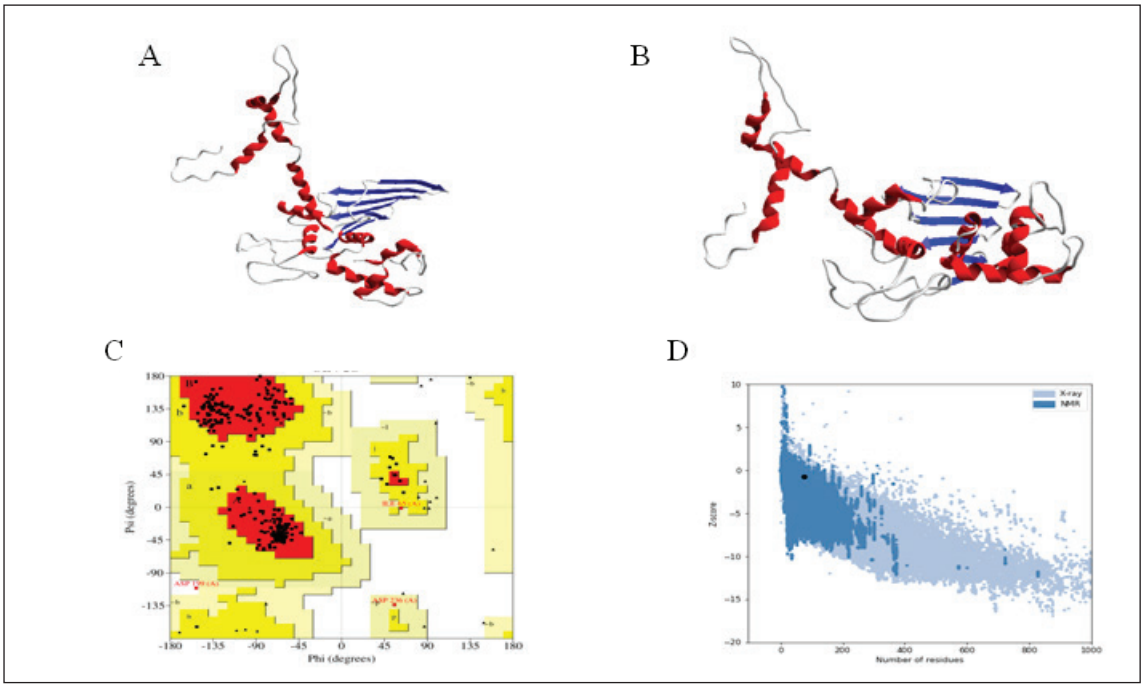

Fig. 4. Modeling refinement, and validation of 3D model. (A): Creation of vaccine construct model via I-TASSER. (B): Refinement of the model using Galaxy-web. (C): Ramachandran plot of the model. (D): Validation of the model using Z-score and Pro-SA server.

Molecular docking of the modeled structure with TLR-3 receptor

The results of the server showed 10 possible models between the modeled vaccine and the TLR-3 receptor. The selected model had 91 members and the lowest binding energy of -694. HDOCK web servers were used to further analysis of TLR-3 and the modeled vaccine. The result of the analysis showed that the lowest binding energy was −299 (Fig. 5A).

Computerized cloning

The vaccine construct sequence was reversed transcribed then *in silico* cloning analysis. The results show that the GC content of the construct was 69.4 and the CAI-Value 0.95 (Fig. 5B, C). According to the results, modeled mRNA is predicated on optimal expression within the target host. As shown in Figure 5D, the XhoI and HindIII sites were flanked at positions 158 and 173 to clone the DNA sequence.

Discussion

Escherichia coli K99 is one of the important causes of diarrhea in neonatal calves and it is responsible for mortality associated with diarrhea during the first week of life. In general, the bacterium *E. coli* K99 can resist a wide range of antibiotics. In the screening test of antibiotic resistance genes in neonatal calves, Astorga and his colleagues found that 92% of *E. coli* K99 isolates were resistant to at least one antibiotic and 2.3% of isolates were resistant to more than one antibiotic (Poirel *et al.*, 2018; Astorga *et al.*, 2019). Also, several

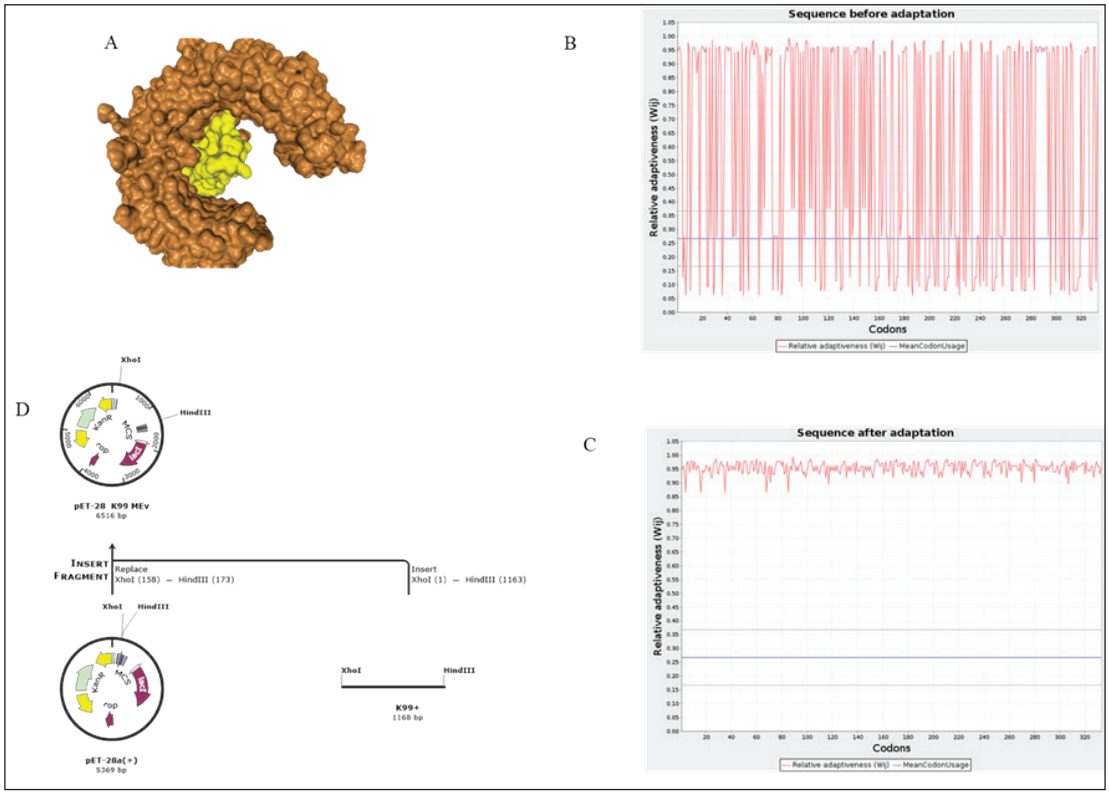

Fig. 5. (A) HDOCK docking complex, yellow part represents vaccine and brown part represents TLR receptor. (B) DNA sequence of the chimeric protein before optimized for expression in eukaryotic cell. (C) optimizing DNA sequence of the chimeric protein for expression in eukaryotic cell. (D) *in silico* cloning of vaccine construct in *E. coli* expression system.

studies suggested that the bacterium *E. coli* K99 which is isolated from calve represents a reservoir for antibiotic resistance genes for other bacteria. The modeled vaccine consists of 15 T, and B cell epitopes (Fig. 2). RpfE was linked to the N terminal of the modeled vaccine as an adjuvant to increase the adaptive immune response. It is well documented that RpfE acts to induce maturationDC by increasing the expression level of surface molecules and the production of interleukins (Choi *et al*., 2015). To increase the stability and transitional efficiency of the modeled mRNA multiepitope vaccine in the target host, HBB and Rabb as UTR were added at 5′ and 3′ to the N and C of the modeled vaccine, respectively (Fig. 2).

The physiochemical structure of the modeled vaccine was tested, as mentioned before the results show that the modeled protein is stable and soluble, and the estimated half-life in mammalian cells is 1.9 hours. Furthermore, the modeled protein shows good affinity with the immune receptor (TLR3) at low energy (Desta *et al*., 2020). Moreover, CAI-Value of modeled vaccine DNA refers to optimal expression within the target host. The results revealed that the modeled multiepitope mRNA vaccine is a good preventive option against *E. coli* infection in calves. Nevertheless, further evaluation is needed to confirm its efficacy and safety through *in vivo* and *in vitro* testing.

Conclusion

With the rapid increase of multidrug resistance phenomenon among pathogenic bacteria, identifying a new strategy to combat these bacteria is urgently needed. The current study employed several Immunoinformatics tools to design a safe, effective, quick, and low-cost multiepitope mRNA-based vaccine against *E. coli* infection in calves. The designed vaccine shows high antigenicity, stability, and good ability to bind to the immune cell receptors. The modeled vaccine that was suggested in the current study can be a potential candidate for immunizing cows against *E. coli* K99. However, confirmation of the efficiency and safety of the model vaccine needs further experimental research.

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Authors' contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Not applicable.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that there are no conflicts of interest.

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