Turk. J. Fish.& Aquat. Sci. 24(9), TRJFAS25546
https://doi.org/10.4194/TRJFAS25546

PROOF
RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

A Novel Multi-epitope Vaccine Based on Virulence Stimulating Two-component Protein EsrA Against Fish Pathogenic *Edwardsiella tarda* – An Immunoinformatic Approach

Chathura Wikumpriya Gunasekara[1,*], LGTG Rajapaksha[2], WSP Madhuranga[1]

[1]Division of Fisheries Life Science, Pukyong National University, Busan 48513, Korea.
[2]Korean language program, Pukyong National University, Busan 48513, Korea.

How to Cite

Gunasekara, C. W., Rajapaksha, L.G.T.G., Madhuranga, W.S.P. (2024). A Novel Multi-epitope Vaccine Based on Virulence Stimulating Two-component Protein EsrA Against Fish Pathogenic *Edwardsiella tarda* –An Immunoinformatic Approach. *Turkish Journal of Fisheries and Aquatic Sciences*, *24(9)*, *TRJFAS25546.* https://doi.org/10.4194/TRJFAS25546

Article History
Received 02 February 2024
Accepted 11 June 2024
First Online 28 June 2024

Corresponding Author
E-mail: cwikumpr@jbnu.ac.kr

Keywords
E tarda
Edwardsielosis
Immunoinformatics
Multi-epitope vaccine
Immune responses

Abstract

Edwardsiellosis, caused by the gram-negative pathogen *Edwardsiella tarda*, is a major global aquaculture threat. Despite vaccination being a potential strategy, development of effective vaccines remains a challenge. The study aimed to develop a multi-epitope vaccine (EsrAmev) against virulent EsrA antigen in fish pathogenic *E. tarda* using an immunoinformatic approach. The study identified antigenic, non-allergenic, and non-toxic epitopes for cytotoxic T lymphocytes, helper T-lymphocytes, and linear B-lymphocytes. They were combined to create the EsrAmev construct, with TLR4 agonist added for the immunogenicity. The physiochemical analysis of EsrAmev by ProtParam server showed a molecular weight of 29 kDa, an antigenicity score of 0.5966, and a solubility of 0.823563 with immunogenic, non-allergenic and non-toxic properties. Robetta, Mod, and Galaxy refiner servers refined the EsrAmev 3D structure, achieving 97.4% RAMA-favoured region, stable interactions, binding stability, and structural stiffness with TLR4 receptor. The EsrAmev construct, optimized for high immune responses in *Escherichia coli* K12 model, was successfully cloned into the pET28a (+) vector *in silico* for future wet lab applications. These findings suggests that the EsrAmev multi-epitope vaccine might have potential for the prophylaxis of *E.tarda*-causing Fish Edwardsiellosis.

Introduction

Fish "edwardsiellosis" is caused by *Edwardsiella* spp belongs to the Gram-negative Enterobacteriaceae family (Miniero Davies et al., 2018). This systemic disease affects a variety of farmed and marine fishes including channel catfish, striped bass, tilapia, and flounders, and is considered one of the most devastating diseases around the globe (Kerie et al., 2019; Park et al., 2012). Fish mortalities caused by *Edwardsiella* infections have led to severe losses in the aquaculture industry over the last few years. *E. tarda* is the major pathogen among *Edwardsiella* spp capable of causing this disease (T. Xu & Zhang, 2014). Upon infection, fish exhibits clinical signs such as pale gills, skin lesions, eye tumefaction, excessive mucus secretion, scale erosion, and ulcers (Mohanty & Sahoo, 2007; Yu et al., 2009). The pathogenicity of *E. tarda* is thought to be associated with many different factors. For example, fish pathogenic *E. tarda* shows high survival capabilities in harsh environments (Li & Sun, 2018). Recent research suggested that under unfavorable conditions, this pathogenic bacteria can become nonculturable, but can remain viable, gradually decrease in size, and change shape to coccoid forms (Krzyżek et al., 2019). Once favorable conditions occur, they will become infective again, which shows their ability to thrive in the environment (Du et al., 2007; Oliver, 2010).

Among the identified virulence factors for *E. tarda,* hemolysin (HlyA) is one major protein associated with fish edwardsiellosis (Park et al., 2012; Wang et al., 2010). This protein is capable of stimulating bacterial invasion and replication inside host cells. In particular, *E. tarda* produces two types of hemolysins namely cell-

associated, iron-regulated hemolysin, encoded by *eth* A and *eth* B, that is secreted as an extracellular protein (ECP) and the other is an extracellular hole-forming hemolysin different from *eth* A and *eth* B (Gao et al., 2014; Park et al., 2012). A recent study demonstrated that *eth* A is a key determinant that facilitates the invasion and is regulated by a novel two-component system (TCS) called EsrA-EsrB complex. This novel two-component system also actively participates in the regulation of type III (T3SS) and type VI (T6SS) secretion systems (Guan et al., 2018; Rogge & Thune, 2011; Wang et al., 2010). Given that, the Esr proteins are considered as potential target antigens for vaccine development against fish disease-causing *E. tarda* spp. (Yang et al., 2015).

Immunization of host organisms against potential pathogens is intended to elicit an immune response and to prepare the body to infiltrate, destroy and/or control the toxicity of those particles. Based on prior research, introducing vaccination to hosts can prevent future outbreaks against those microorganisms (Islam, Mou, Sanjida, et al., 2022; Oliver, 2010). A few decades ago, the production of vaccines and the evaluation of their potential required very laborious protocols and a massive amount of time (Plotkin et al., 2017). However, with the recent technological advancement in immunoinformatic tools, it became easier to develop efficient, uncomplicated, economically feasible, and safe vaccines (Fatoba et al., 2022; Pyasi et al., 2021). In recent times, epitope-based vaccines have been successfully created *in silico* against human pathogens such as influenza, chikungunya, zika, Ebola, and, Middle East respiratory syndrome coronavirus (Antonelli et al., 2022; Dash et al., 2017; Jaan et al., 2022; Pyasi et al., 2021; Shi et al., 2015). However, so far, due to the lack of information on the differences in MHC class I, II, and HLAs, *in silico* techniques have not been utilized to develop epitope vaccines against fish diseases (Dijkstra et al., 2013; Grimholt & Dixon, 2016; Islam, Mou, Sanjida, et al., 2022). In most recent research conducted with cod and tilapia both MHC class I and II have been identified for the fish and as a result, the peptides, which have strong binding potential with previously reported HLAs (Such as HLA-A*0201, and HLA-B*3501) can be utilized to develop effective vaccines against specific diseases in Fish (Bolnick et al., 2014; Yamaguchi & Dijkstra, 2019). So far, *in silico* methods have been applied by researchers for fish pathogenic *Streptococcus agalactiae* and *Flavobacterium columnare*, to study the antigenic and immunogenic peptides whereas multi-epitope vaccines have been developed against pathogenic *V. harveyi* and seven-banded grouper necrosis viruses (Islam, Mou, Sanjida, et al., 2022; Joshi et al., 2021; Mahendran et al., 2016; Pereira et al., 2013).

The present study focuses on designing a cheap and alternative approach to craft a vaccine against *E. tarda* responsible for fish "edwardsiellosis". We designed a complete vaccine model by associating adjuvants and finalizing epitopes with the latest *in silico* peptide screening tools, molecular docking servers, molecular dynamic and immune simulation strategies. Moreover, the present study introduced immune stimulation and *in silico* cloning for the final vaccine construct which holds prospects for further testing in animal models and its easy wet lab synthesis.

Materials and Methods

Retrieval of Proteome and Antigen Selection

The National Center for Biological Information database (NCBI) was employed to detect the accessible *E. tarda* proteome sequences for antigen selection. Upon analysis, the EsrA protein, which is one of the most important virulenlence stimulating factors associated with fish infections, was extracted as FASTA files to design the multi-epitope vaccine (Wang et al., 2010; Yang et al., 2015). The VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/) and ANTIGENpro (https://scratch.proteomics.ics.uci.edu/) servers were employed to assess the antigenicity of EsrA protein with a server default threshold value of 0.4 (Doytchinova & Flower, 2007; Magnan et al., 2010). Subsequently, the EsrA protein with the highest antigenic score was chosen for further research by using various immunoinformatic tools (Figure 1).

Prediction and Assessment of Cytotoxic T-Lymphocyte Epitopes

The cytotoxic T-lymphocytes (commonly referred to as CTLs) are primary type of immune response cells that has the capacity to infiltrate other infectious cells. These CTLs are capable of entering the infected cells and manipulating the host's defensive responses against pathogens (Andersen et al., 2006; Islam, Mou, Sanjida, et al., 2022). To predict the CTL epitopes, the selected EsrA protein was imported to NetCTL v1.2 (http://www.cbs.dtu.dk/services/NetCTL/) online platform (Larsen et al., 2007). This server delivers its output for a given protein by integrating information from 3 separate factors namely, TAP transport efficiency, proteasomal C terminal cleavage affinity (C-score), and MHC class I affinity. For the present protein, the prediction threshold parameter was set to 0.4 to obtain 0.940 and 0.89 specificity and sensitivity. Upon obtaining the 9-mer epitopes from NetCTL v1.2, they were exported to VaxiJen v2.0, ToxinPred (http://crdd.osdd.net/raghava/toxinpred/), AllerTop v2.0 (https://ddg-pharmfac.net/OP/) and IEDB (http://tools.iedb.org/immunogenicity/) servers to evaluate the antigenicity, toxicity, allergenicity and MHC class I immunogenicity (Calis et al., 2013; Dimitrov et al., 2013; Gupta et al., 2013). All of the forecasts were made using the default parameters of each server.

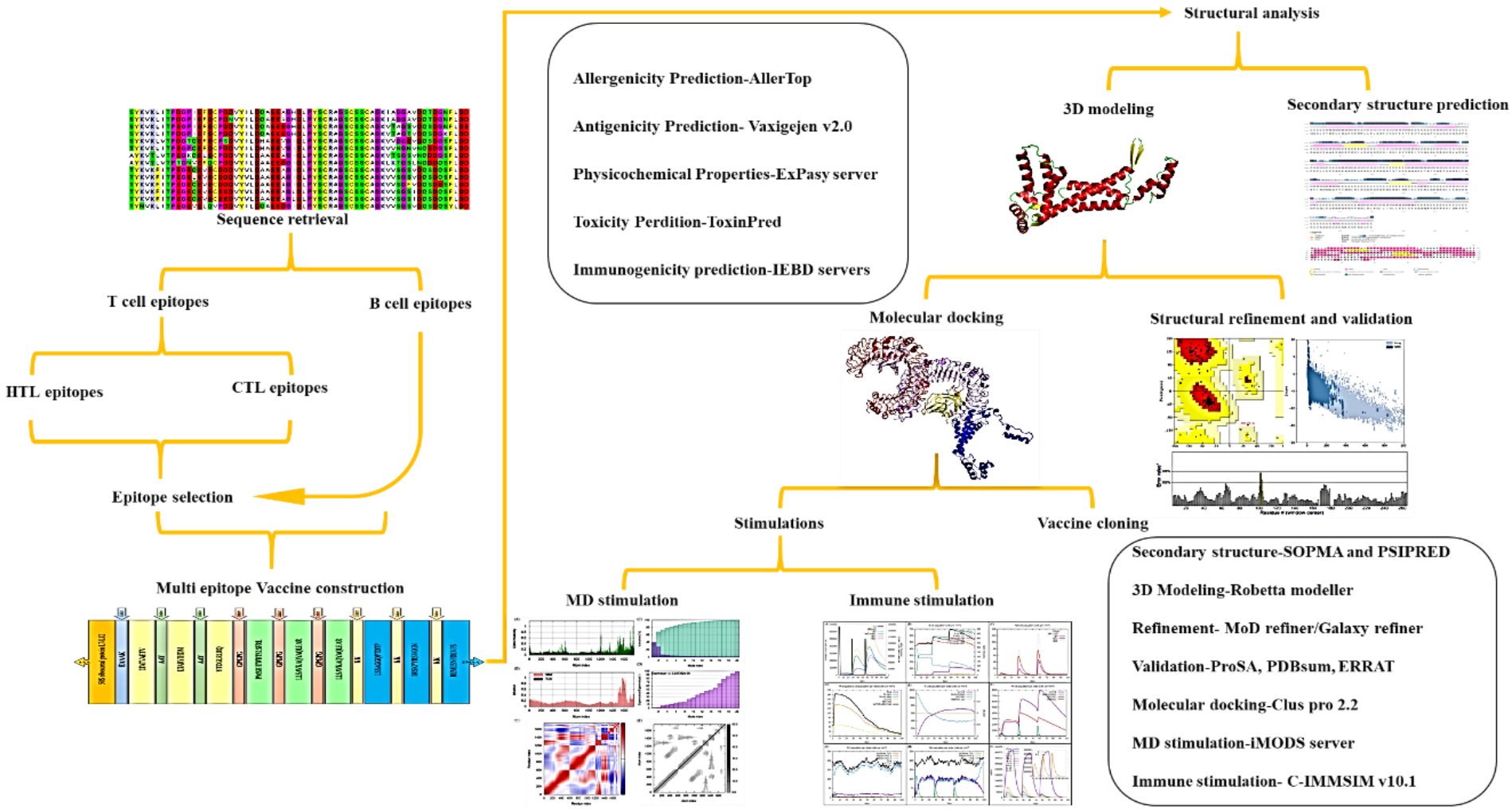

Figure.1. Overview of the study design

Prediction and Assessment of Helper T-Lymphocyte Epitopes

The helper T-lymphocytes (Referred to as HTLs) can activate B-lymphocytes and CTLs, as a response to outside antigens (Islam, Mou, Sanjida, et al., 2022; Z. Xu et al., 2020). Hence, the presence of HTL epitopes in the present protein was observed using IEBD's MHC class II binding allele tool (http://tools.iedb.org/mhcii/) with Consensus 2.22 prediction method (Nielsen et al., 2010). To obtain suitable HTLs, a set of seven HLAs namely, HLA-DRB3*02:02, HLA-DRB3*01:01, HLA-DRB1*15:01, HLA-DRB1*07:01, HLA-DRB1*03: 01, HLA-DRB4*01:01, and HLA-DRB5*01:01 were utilized. The HTLs were selected based on their binding capabilities towards each allele by considering their IC_{50} scores. The server recommends IC_{50} values < 50 nM, < 500 nM, and < 5000 nM as strong, moderate, and weak binding affinities of epitopes for MHC-II. Moreover, the percentile rank, which is inversely related to the binding affinities, was taken into consideration when selecting suitable epitopes (Islam, Mou, Sanjida, et al., 2022; Moutaftsi et al., 2006). The cytokine-inducing abilities, namely interferon-γ (IFN-γ), interleukin-10 (IL-10), and interleukin-4 (IL-4) were further assessed for the selected epitopes by employing IFN epitope (http://crdd.osdd.net/raghava/ifnepitope/), IL4pred (http://crdd.osdd.net/raghava/il4pred/), and IL10pred (http://crdd.osdd.net/raghava/IL-10pred/) online platforms with default parameters (Dhanda, Gupta, et al., 2013; Dhanda, Vir, et al., 2013; Nagpal et al., 2017). The antigenicity was predicted using the VaxiJen v2.0 server.

Prediction and Evaluation of Linear B Cell Lymphocytes Epitopes (LBL)

The B cell epitopes are required to stimulate/promote humoral or antibody-mediated immunity. Hence, the online platform iBCE-EL (http://thegleelab.org/iBCE-EL/) was employed to predict the linear B cell lymphocyte epitopes of the present protein. This server utilizes a combination of amino acid compositions and physiochemical properties of a given protein as input features (Manavalan et al., 2018). The anticipated 12-15 mer epitopes were exported and further analyzed by VaxiJen v2.0, ToxinPred, and AllerTop v2.0 servers as previously described.

Peptide Modeling and Molecular Docking

The Peptide structure prediction server (PEP-FOLD 3) (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) was used to stimulate the 3D structure of the CTL and HTL epitopes (Lamiable et al., 2016). As the reference, crystal structures of HLA-B*3508 and DRB1*1101 were downloaded from the RCSB protein data bank (http://www.rcsb.org/pdb/home/home.do). However, the crystal structures were in the combined form of protein and ligand, and hence, they were separated by Maestro 3.3 software (https://www.schrodinger.com/). All heteroatoms and water molecules were also removed. Following, the docking simulation was performed through ClusPro v2.0 (https://clusp ro.bu.edu/home.php) online server (Alekseenko et al., 2020). The models were visualized through the PDBSum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) online server (Laskowski et al., 2018).

Development of a Multi-Epitope Vaccine

A combined vaccine was designed by using the chosen epitopes (CTLs, HTLs, and LBLs) with a selected adjuvant and linking them with the proper linkers in a sequence-wise manner. In the final vaccine construct, a total of 3 CTL epitopes which showed the highest combined score, 3 HTL epitopes with the lowest percentile rank, and 3 LBL epitopes were joined together by using Ala-Ala-Tyr (AAY) linkers, Gly-Pro-Gly-Pro-Gly (GPGPG) linkers and Lys-Lys (KK) linkers respectively. The AAY linker has the potential to stabilize the vaccine construct and improve the epitope presentation (Islam, Mou, Sanjida, et al., 2022). With the GPGPG linker, "Junctional epitope" can be avoided which in return simplifies the immune processing, while the KK linkers help to maintain the unique immunogenetic features of the vaccine construct (Hammed-Akanmu et al., 2022; Mahmud et al., 2021). Moreover, innate immunity can be activated by toll-like receptors (TLR). Because toll-like receptor 4 (TLR4) can be recognized by glycoproteins, and adjuvants are essential for overcoming the constraints of translation and synthesis of the target vaccine construct, the adjuvant used here was TLR4 agonist named 50S ribosomal protein L7/L12 (NCBI ID: P9WHE3) with an EAAAK linker (Islam, Mahfuj, et al., 2022).

Structural Properties of the Designed Vaccine

For a particular protein, understanding its physicochemical properties is a fundamental requirement. Hence, the physicochemical properties of the constructed vaccine (named as $EsrA_{mev}$) were assessed by the ProtParam online server (https://web.expasy.org/protparam/) to gain a comprehensive understanding of the nature of the developed vaccine (Gasteiger et al., 2005). Following this, the immunological properties were further evaluated by VaxiJen v2.0, MHC-I immunogenicity, AllerTop, and SOLpro (https://scratch.proteomics.ics.uci.edu/) servers (Magnan et al., 2009). The structural properties and quality of $EsrA_{mev}$ construct, 2D structural characteristics, such as the α-helix, β-turn, and random coils were assessed by SOPMA (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html) (Self-Optimized Prediction Method with Alignment) and PSIPRED v4.0

(http://bioinf.cs.ucl.ac.uk/psipred/) (PSI-blast based secondary structure prediction) servers with default parameters (Buchan & Jones, 2019; Geourjon & Deléage, 1995).

Prediction, Confirmation, and Refinement of the Tertiary Structure

The 3D structure of the designed $EsrA_{mev}$ construct was assessed to understand its biological properties. Among the 3D modeling software available online, the Robetta server (https://robetta.bakerlab.org/) which utilizes a cutting-edge algorithm was used to develop the most precise 3D structure of the current $EsrA_{mev}$ construct. This server is capable of producing protein models with a defined confident-score range from 0 to 1 in which the higher value indicates the most acceptable model (D. E. Kim et al., 2004). Hence the generated structure with a high confidence score was saved in PDB format for further analysis. The primary and secondary refinements of the constructed 3D model were carried out by ModRefiner (https://zhanggroup.org/ModRefiner/) and Galaxy refines server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) respectively (Heo et al., 2013; D. Xu & Zhang, 2011). The refined structures were downloaded and among them, a candidate 3D structure was chosen based on its energy scores of the lowest and highest RMSD values. The molecular representation was conducted by PyMOL v2.3.4 (https://www.schrodinger.com/) and the validations of the refined structure were carried out by using Ramachandran plots developed through PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) web server (Laskowski et al., 1993). The validation of the model quality was conducted by ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) and ERRAT (https://saves.mbi.ucla.edu/) online servers (Colovos & Yeates, 1993). Furthermore, the flexibility of the constructed model was evaluated by CABS-Flex 2.0 server (http://biocomp.chem.uw.edu.pl/CABSflex2) which is a well-developed tool to effectively perform simulations of a protein's structural flexibility (Kuriata et al., 2018).

Prediction of Discontinuous and Linear B Cell Epitopes

For a vaccine construct to stimulate a humoral response, it needs to have B-cell lymphocyte- epitopes in its regions. However, in most cases, B cell epitopes can be discontinuous (conformational). For this reason, the Online server Ellipro (http://tools.iedb.org/ellipro/) was utilized to model the possible linear and conformational B cell epitopes. This server utilizes algorithms to determine three factors namely, the protrusion index (PI) of residues, (ii) estimation of the protein shape, and (iii) cluster formation of the neighboring residues of a given protein (Ponomarenko et al., 2008).

Molecular Docking

The binding structures of the receptors and modeled proteins can be assessed through molecular docking experiments. To archive this purpose, the refined $EsrA_{mev}$ construct was submitted to the ClusPro v2.0 server as the ligand whereas, the TLR4 protein (PDB ID: 4G8A) was considered as the potential receptor molecule for docking (Islam, Mou, Sanjida, et al., 2022). As the initial step, modifications of the receptor molecule and associated ligand (water and the other chemical residue removal) were carried out by using PyMOL v2.3.4 program. The binding interactions and residues in the interacting surface were detected by PBDSum online server.

Simulation of Molecular Dynamics (MDs)

Molecular dynamic (MD) simulations are necessary to stabilize the docked molecular structure. Hence, the iMODS server (https://imods.iqfr.csic.es/) was employed to perform MD simulations. This software is a well-developed tool composed of a fully integrated web-service-oriented platform to perform molecular dynamics simulations. The server evaluates protein stability by using normal mode analysis (NMA) to generate the internal coordinates of the protein. In the present study, the $EsrA_{mev}$-TLR4 complex was submitted to iMODS server with default settings. The parameters including deformability plot, eigenvalue value, B-factor value, covariance matrix, and elastic network model were used to describe the stability of the $EsrA_{mev}$ construct (Jos´ et al., 2014).

Immune Response Simulation

The entire $EsrA_{mev}$ construct was uploaded to the C-IMMSIM v10.1 server (http://kraken.iac.rm.cnr.it/C-IMMSIM/) to assess the vaccine potential for the immunological response. The minimum gap of the two-vaccine dosage was set to 28 days apart. A total of 3 injections were administrated as 1 time, 84 times, and 168 times respectively, considering 1 time equals 8h in real life. The maximum stimulation value was set to 100 whereas all other parameters were used at their default settings (Rapin et al., 2011).

Codon Adaptation and *in Silico* cloning

In order to express a foreign gene of interest inside a vector, it is a must to have conformity between the codons of the vector and the gene of interest so that high levels of activity can be archived at the time of purification (Islam, Mou, & Sanjida, 2022). For this purpose, the vaccine construct was uploaded to the Novapro vector server (https://www.novoprolabs.com/tools/codon-optimization) to optimize the codon adaptation. The strain *E. coli* K12 which is known for its quick reproduction rates and survival potential was used as the expression host for the entire procedure while

avoiding three criteria namely, sites of restriction enzyme cleavage, binding sites of prokaryotic ribosomes, and rho-independent transcription termination (Fadaka et al., 2021; Islam, Mahfuj, et al., 2022). The sequence quality was evaluated by using codon adaptation index (CAI) value and the guanine-cytosine (GC) concentration. The *in silico* cloning procedures were carried out with SnapGene tool by utilizing PET28a (+) expression vector and by adding *XhoI* / *NdeI* sites to the N and C-terminal ends of the DNA construct.

Results

Retrieval of Protein Sequence

The initial step of the designed workflow was to obtain the sequence of *E. Tarda* two-factor component system protein EsrA from the NCBI database (Supplementary Table 1). Upon analysis, the protein sequence AAX55230.1 which had 856 amino acids (AA) and a molecular weight of 92.22 kDa was selected as the potential candidate for multi-epitope vaccine design. The selected EsrA sequence had greater scores than the threshold value of 0.4 through VaxiJen (0.450) and ANTIGENpro (0.617) for antigenicity, suggesting the nature of the EsrA protein.

Prediction and Assessment of Cytotoxic T-Lymphocyte Epitopes

To design effective vaccine candidates, accurate predictions of CTL epitopes are crucial. Hence the NetCTL v1.2 server was utilized to predict CTL epitopes with a length of 9 mers for the chosen EsrA protein (Supplementary Table 2). There was a total of 18 epitopes and, among them, only 5 epitopes were shown to be antigenic, non-toxic, and non-allergenic. In the present study 3 suitable epitopes were selected based on the highest antigenic scores and the position of the epitopes to design the final vaccine ($EsrA_{mev}$) construct (Table 1)

Prediction and Assessment of Helper T-Lymphocyte Epitopes

Initially, the IEDB MHC-II tool was utilized to obtain a total of 803 HTL epitopes with a length of 15 mers in reference to 7 HTAs (Supplementary Table 3). Upon analysis, 3 potential candidate epitopes with low percentile ranks and high binding affinities were selected to develop the $EsrA_{mev}$ construct. These epitopes were found to be antigenic, non-allergenic, and non-toxic with IFN-γ, IL-4, and IL-10-inducing capabilities (Table 2).

Prediction and Assessment of Linear B Cell Lymphocyte Epitopes

The designed vaccine's effectivity can be stimulated by the presence of B cell epitopes which alternatively helps to prevent the disease. Hence, the B cell epitopes analysis conducted through iBCE-EL provided a total of 137 LBLs with a length of 12 mers (Supplementary Table 4). Among them, 3 epitopes with antigenic, non-allergenic, and non-toxic capabilities were selected for the multi-epitope vaccine construct (Table 3).

Construction of Multi-epitope Vaccine ($EsrA_{mev}$)

The selected epitopes for CTL, HTL, and LBL were arranged and coupled together in a sequence-wise manner with the help of specific adjuvants and linkers to develop the final $EsrA_{mev}$ construct (Figure 2). It was composed of 9 antigenic, non-allergenic, and non-toxic epitopes. Among them the 3 CTL epitopes were connected using AAY linkers, the 3 HTL epitopes were connected using GPGPG linkers and the 3 LBL epitopes were joined using KK linkers. To enhance the immunogenicity, adjuvant sequence 50S ribosomal protein L7/ L12, which is a TLR4 agonist, was connected to the initial CTL epitope of the vaccine with EAAAK linker (Figure 3). As a result, 270AA long antigenic (score of 0.5966) $EsrA_{mev}$ construct was designed with non-allergen, non-toxic properties.

Peptide Modeling and Molecular Docking

Molecular docking was conducted to confirm the binding interactions of the CTL and HTL epitopes to the selected HLA alleles. The CTL and HTL epitopes had binding energies that ranged from -681.0 to –779.7 kcal/mol. The PDBSum analysis showed best interacting models in between the CTL epitope YTDGLRLRQ with 14 hydrogen bonds associated with TYR7, TYR9, ARG62, TYR99, GLN155, ARG156, TYR159, and TRP167. On the other hand, the HTL epitope LLSAVKAQYA had 5 hydrogen bonds in between residues ARG65, LYS146, and GLN155 (Figure 4).

Structural Properties of the Designed Vaccine

The properties of vaccine construct were computed using various web-based servers. The $EsrA_{mev}$ construct had a chemical formula of $C_{1283}H_{2075}N_{345}O_{388}S_5$ with a 28.70197 kDa molecular weight. The other physicochemical and immunological properties including theoretical isoelectric point (pI), aliphatic index, instability index, and grand average of hydropathicity along with toxicity, allergenicity immunogenicity, solubility and antigenicity score immunological for the vaccine construct are shown in Table 4.

Table 1. The selected CTL epitopes for the final vaccine construction

Position	Epitopes	Prediction score	Antigenicity score	Immunogenicity score	Allergenicity	Toxicity
71	LTATVAETV	1.0969	0.4748	0.23597	Non-allergen	Non-toxin
114	LTARYIHDM	0.7773	0.4453	0.22672	Non-allergen	Non-toxin
521	YTDGLRLRQ	1.3067	1.6702	0.1	Non-allergen	Non-toxin

Table 2. The selected HTL epitopes for the final vaccine construction

Position	Epitopes	Antigenicity score	Percentile rank	IFN score	IF4-inducer	IF410-inducer	Allergenicity	Toxicity
521	YTDGLRLRQILINLL	1.1147	0.15	Positive	Inducer	Inducer	Non-allergen	Non-toxin
22	PMSRHIWRTSLSFRL	0.9142	0.85	Positive	Inducer	Inducer	Non-allergen	Non-toxin
375	LLSAVKAQYAQLEAR	0.6968	0.76	Positive	Inducer	Inducer	Non-allergen	Non-toxin

Table 3. The selected LBL epitopes for the final vaccine construction

Position	Epitopes	Prediction score	Antigenicity score	Allergenicity	Toxicity
56	LSSAGGQPTDTP	0.8695	1.11427	Non-allergen	Non-toxin
74	IWSKPYRSVGGN	0.5112	0.9142	Non-allergen	Non-toxin
109	REMESNFDEVFS	0.5718	0.4532	Non-allergen	Non-toxin

The secondary structural features of the $EsrA_{mev}$ were assessed using SOPMA and PSIPRED v 3.3 servers. The SOPMA estimated secondary structure of the vaccine construct showed 59.63% of Alpha helixes, 8.53% of extended strands, 9.63% of beta turns along with 22.22% of random coils (Supplementary File 5). On the other hand, the PSIPRED server predicted 52.29%-Alpha helix, 7.03%-strands, 9.63%-beta turn, and 40.37% coils (Figure 5).

Prediction, Confirmation, and Refinement of the Tertiary Structure

The constructed $EsrA_{mev}$ was submitted to Robetta protein modeling server. Among the top 5 protein models produced, the 3D model with highest confidence score (GDT score of 0.63) was selected as the potential $EsrA_{mev}$ candidate model. Before the initial refinement, Ramachandran plot produced through PROCHECK server showed a total of 93.9% (217AA) residues in the most favored region. The initial and secondary refinements through MoD refiner and Galaxy refines, provided a total of 5 models with GDT-HA, RMSD, MolProbity, Clash scores, and poor rotamer values ranging from 0.9741-0.9815, 0.307-0.367, 1.303-1.477, 4.8-6.5 and 0-1 for each parameter respectively (Supplementary Table 6). Among them, model 1 was selected as the best model, as indicated by the following parameters: GDT-HA (0.9731), RMSD (0.335), MolProbity (1.448), clash score (6), poor rotamers (0), and Rama favored region (97.4). Again, the validation of this refined model was conducted with PROCHECK server and it showed the improved 3D model had 97.4% of residues in the most favored region of Ramachandran plot (Table 5). To further ensure the quality of the refined model ProSA server and ERRAT servers were utilized. The refined $EsrA_{mev}$ model showed a Z-score of -7.29 and a total quality factor of 99.21 for ProSA and ERRAT server analysis, indicating that it has high structural properties (Figure 6).

Furthermore, the flexibility of the refined $EsrA_{mev}$ construct was determined by the online tool of CABS-flex 2.0 with 50 rounds of stimulations. In comparison, the collective model of 10 structures showed fewer fluctuations around the N terminal region compared to the C terminal region. The generated contact map revealed possible contacts among different residues of all the 10 final retrieved structures (Supplementary file 7).

Prediction of Discontinuous and Linear B Cell Epitopes

The presence of discontinuous and linear B cell epitopes was analyzed using the Ellipro server with default settings for the refined $EsrA_{mev}$ construct. A total of 4 linear epitopes and 5 discontinuous epitopes were predicted by the server with a total of 267 residues and a score ranging from 0.504-0.737 (Figure 7). The antigenic score of the linear epitopes ranged from 1.7475-0.425 (Supplementary Table 8).

Molecular Docking

To observe the potential binding affinity and interactions, the $EsrA_{mev}$ construct (as the ligand) and TLR4 (as the receptor) were docked. As the result, ClusPro v2.0 server produced 9 docked complexes in various positions. Among them, model 1 which had the lowest energy score (–864.9) and the binding posture with functional interactions was evaluated as the potential docked model. Moreover, the residues implicated in active site residues and the binding

MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAAGAAPAGAAVEA
AEEQSEFDVILEAAGDKKIGVIKVVREIVSGLGLKEAKDLVDGAPKPLLEKVAKEAA
DEAKAKLEAAGATVTVK**EAAAK**LTATVAETV**AAY**LTARYIHDM**AAY**YTDGLRLR
Q**GPGPG**PMSRHIWRTSLSFRL**GPGPG**LLSAVKAQYAQLEAR**GPGPG**YTDGLRLRQI
LINLL**KK**LSSAGGQPTDTP**KK**IWSKPYRSVGGN**KK**REMESNFDEVFS

Figure.2. The constructed vaccine sequence

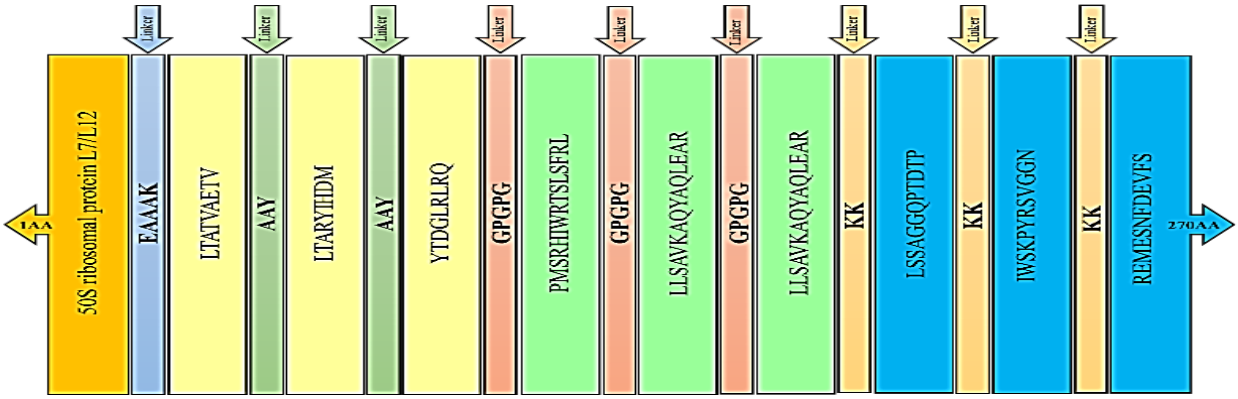

Figure.3. The Illustration final vaccine design. Herein, the adjuvant and the first CTL epitope were linked by EAAAK linker, CTL epitopes (Yellow) were added together by AYY linkers, HTL epitopes (Green) by GPGPG linkers, and LBL epitopes (Blue) by KK linkers

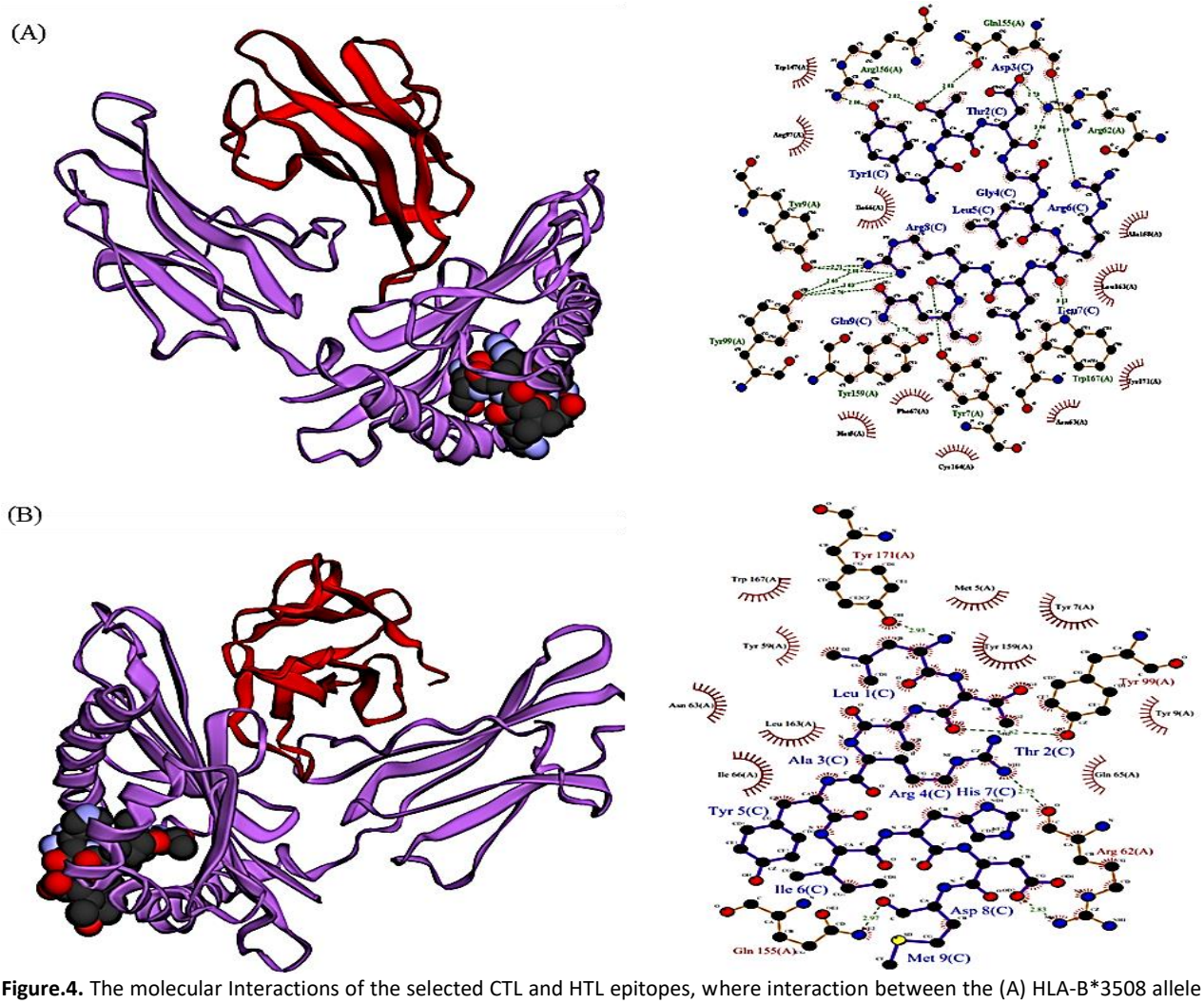

Figure.4. The molecular Interactions of the selected CTL and HTL epitopes, where interaction between the (A) HLA-B*3508 allele and the CTL epitope (YTDGLRLRQ) and (B) between the DRB1*1101 allele and HTL epitope LLSAVKAQYAQLEAR. CTL, cytotoxic T lymphocyte, HTL-helper T-lymphocyte**.**

Table.4. The immunological and physicochemical characteristics of the $EsrA_{mev}$

Characteristics	Finding	Remark
No. of amino acids	270	Suitable
Molecular weight	28701.97	Average
Theoretical pI	5.79	Slightly acidic
Extinction coefficient (at 280 nm in H2O)	21430	-
Estimated half-life (mammalian reticulocytes, in vitro)	30 hours	-
Estimated half-life (yeast-cells, in vivo)	>20 hours	-
Estimated half-life (Escherichia coli, in vivo)	>10 hours	-
Instability index of vaccine	27.81	Stable
Aliphatic index of vaccine	89.78	Thermostable
Grand average of hydropathicity (GRAVY)	-0.147	Hydrophobic
Antigenicity	0.5966	Antigenic
Immunogenicity	0.4836	Immunogenic
Allergenicity	Non-allergen	-
Toxicity	Non-toxic	-
Solubility	Soluble	0.823563

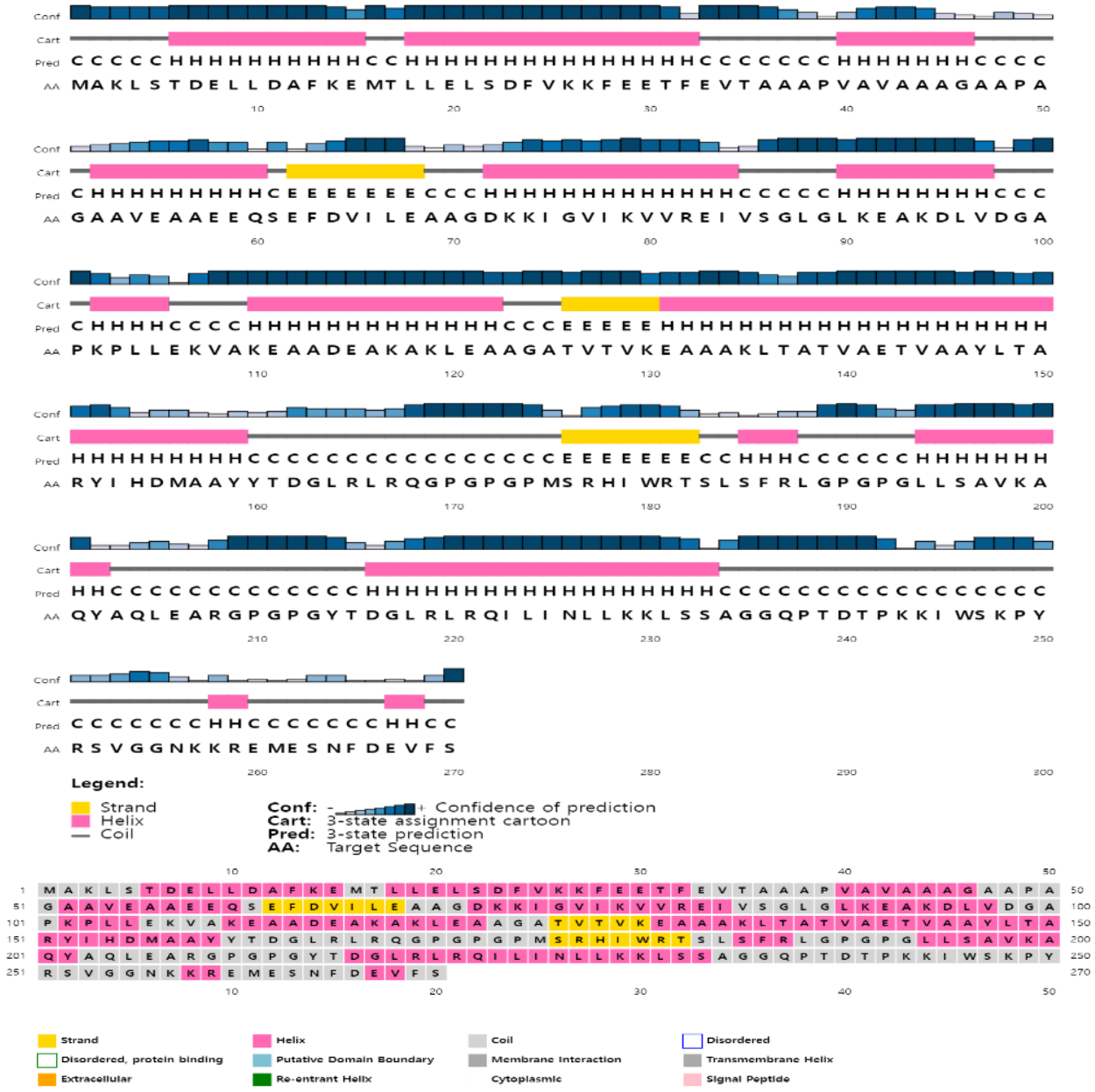

Figure.5. The secondary structures predicted by the PESIPRED server for the designed multiepitope vaccine $EsrA_{mev}$.

interactions were investigated in the chosen complex. A total of 13 hydrogen bonds were found on the interaction surface. Among them interacting residues with $EsrA_{mev}$ were Glu42, Pro28, Lys47, Tyr48, Arg67, Tyr22, and Lys20. Furthermore, there were 2 electrostatic salt bridges (Figure 8).

Simulation of Molecular Dynamics (MDs)

The stimulation of MDs study was conducted by iMODS server to check the physical movement of atoms and the stability in the docking complex. The simulation was performed in normal mode analysis (NMA) and obtained results of TLR4 docking complexes were depicted in Figure 9. As shown in the deformability graph there was a minimal distortion in the complex, as indicated by the hinges highlighted in the deformability regions in the complex. This was further supported by the B-factor plot which calculates the root mean square value (RMS) and represents the uncertainty of each atom in the docking complex. Moreover, a higher Eigenvalues value of 2.247×10^{-6} was observed for the $EsrA_{mev}$-TLR4 docking complex, which showed the energy required to deform the construct, and thus its stability. Also, the covariance matrix is indicated by coupling between pairs of residue experience, where red, blue, and white represents correlated, uncorrelated, and anti-correlated motions of atoms respectively. The elastic network model explains the degree of stiffness of the $EsrA_{mev}$ -TLR4 docking complex (darker gray). All the above results suggested that the interactive $EsrA_{mev}$ -TLR4 complex has molecular stability (Figure 9).

Immune Simulation Analysis

The result of the immune stimulation after injection of $EsrA_{mev}$ is presented in Figure 10. In response to several exposures of $EsrA_{mev}$, the host immune system shows a significantly high level of the secondary and tertiary antibodies (IgG1 + IgG2, IgM, and IgG + IgM) when compared to the primary antibodies. This response may be associated with the host feedback to antigen invasion, showing the establishment of memory cells, resulting an increase in antigen clearance after subsequent exposures. Moreover, the different immune cell population/state counts among B-cells, helper T cells, and cytotoxic T cells had a long time of survival, suggesting class flipping between immune cells and IgM memory development. Furthermore, these immune cells were also significantly increased with memory cell development. Similarly, an increase in the levels of cytokines was observed for IFN-ϒ (>400,000 ng/ml). Expanded macro phage mobility and dendritic cell movement were seen upon stimulation with $EsrA_{mev}$ construct. These observations suggest that the present vaccine construct can stimulate immune memory development and, therefore may confer immunity against the fish pathogenic *Edwardsiella tarda*.

Codon Adaptation and *in Silico* Cloning

To improve the translation efficiency of the current vaccine, we adjusted the codons according to the E. coli K12 on the Novapro server. The sequence created by the peptide vaccine construct (270AA residues) showed an 810 bp in nucleotide length (Figure 11). Moreover, the modified nucleotide sequence has a GC content of 56.67% and a CAI value of 0.89 which indicates the reliability of the efficient expression in the *E. coli* strain K12. For the insertion of the modified sequence, we employed the pET28a (+) vector *XhoI* and *NdeI* restriction sites as the start and end cut points, which resulted in a 6128 bp long plasmid construct.

Discussion

The utilization of various *in silico* immunoinformatic approaches to construct vaccines against pathogens such as bacteria and viruses are becoming more admirable in modern research (Fatoba et al., 2022; Islam, Mahfuj, et al., 2022). An effective vaccine developed through immunoinformatics has the potential to protect the host against infectious organisms (Islam, Mahfuj, et al., 2022). Among different aquatic pathogens, “edwardsiellosis” causing *E. tarda* is considered as a global threat to world aquaculture because it infects a wide range of economically important fishes including Olive flounder, Sea bream, Sea bass, and Tilapia (T. Xu & Zhang, 2014; Yu et al., 2009). Hence, there is a necessity of effective vaccines against this pathogen and, to develop these vaccines, immunoinformatic approaches can be utilized. In most recent research, a two-component system protein named EsrA, which can manipulate the secretion of other virulent proteins and effectors such as Hemolysin, T3SS1, and T6SS1 was detected in *E. tarda* (Guan et al., 2018; Rogge & Thune, 2011; Yang et al., 2015). Since the Esr genes can stimulate the pathogenesis of *E. tarda*, researchers suggested it as an ideal candidate to develop vaccines (Yang et al., 2015). The present study focused on developing a multi-epitope vaccine by targeting EsrA protein of fish pathogenic *E. tarda* as an effective measure to stimulate the host immune system. Multi-epitope vaccines, based on a single protein, offer targeted immune responses by focusing on specific immunogenic regions, potentially reducing autoimmune risks and enhancing safety (Lucchese et al., 2021). As the initial step, EsrA protein antigenic surface area was assessed to facilitate host cellular and humoral recognition. Then the potential CTL, HTL, and LBL epitopes with antigenic properties were identified by various immunoinformatic tools. In general, the CTL epitopes have the ability to develop durable immunity and eliminate the infected cells whereas, HTL epitopes are associated with eliciting both humoral and cellular immune responses (Alexander et al., 1998; Zhang et al., 2022). Collectively, these cells are also capable of stimulating the activation of B cells (Islam, Mahfuj, et al.,

Table.5. The Ramachandran plot statistics. The analysis indicates a total of 99.6% atoms in the most favorable and additionally allowed RAMA regions.

Plot statistics	Region and percentage
Residues in most favored regions	[A, B, L] 97.4%
Residues in additional allowed regions	[a, b, l, p] 2.2%
Residues in generously allowed regions	[~a, ~b, ~l, ~p] 0 0.0%
Residues in disallowed regions	0.40%

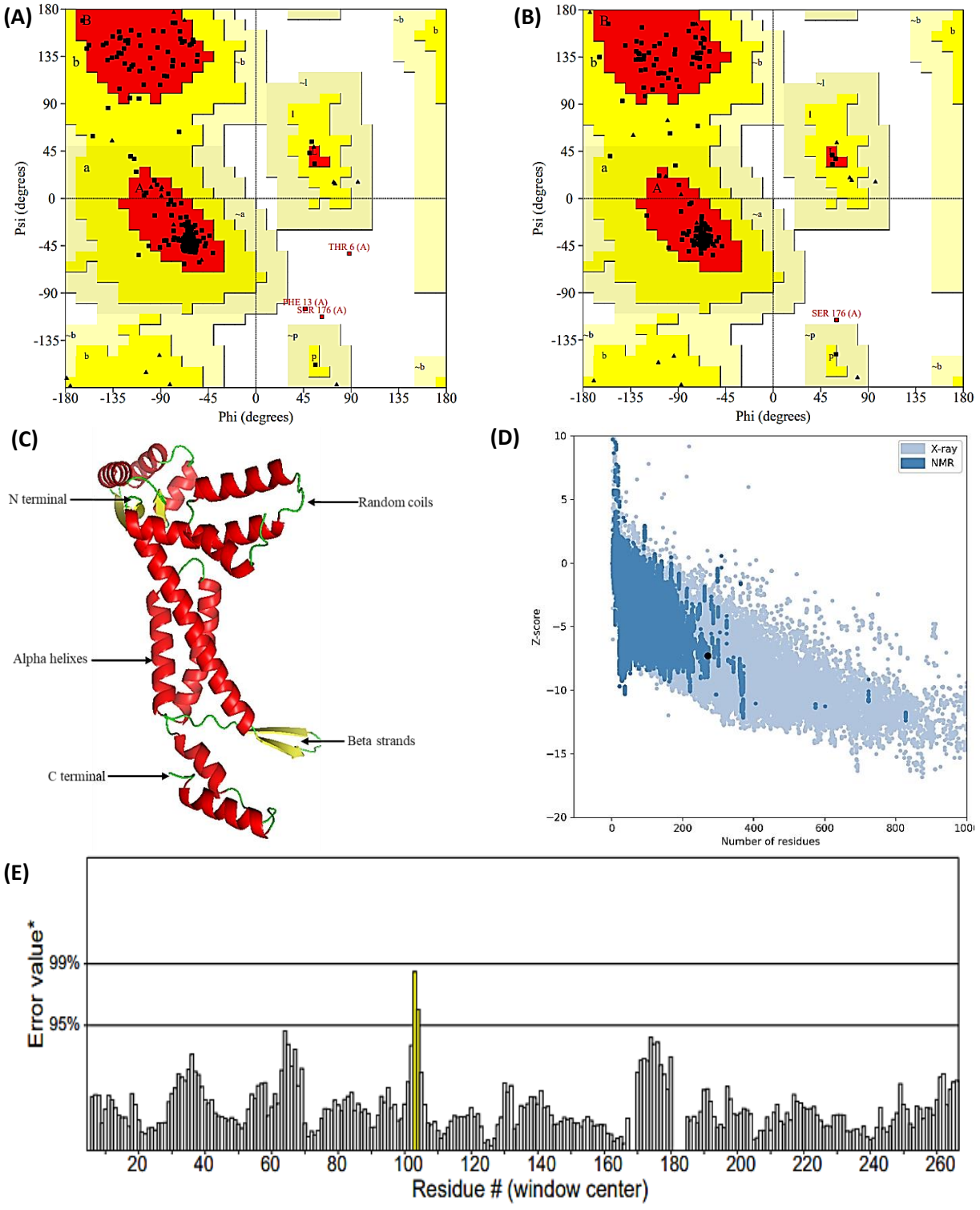

Figure.6. Analysis of Ramachandran plots by PROCHECK server (A) Before refinement, (B) After refinement, (C) Final 3D structure, (D) Validation of model with Pro-SA server, (E) and with ERRAT server

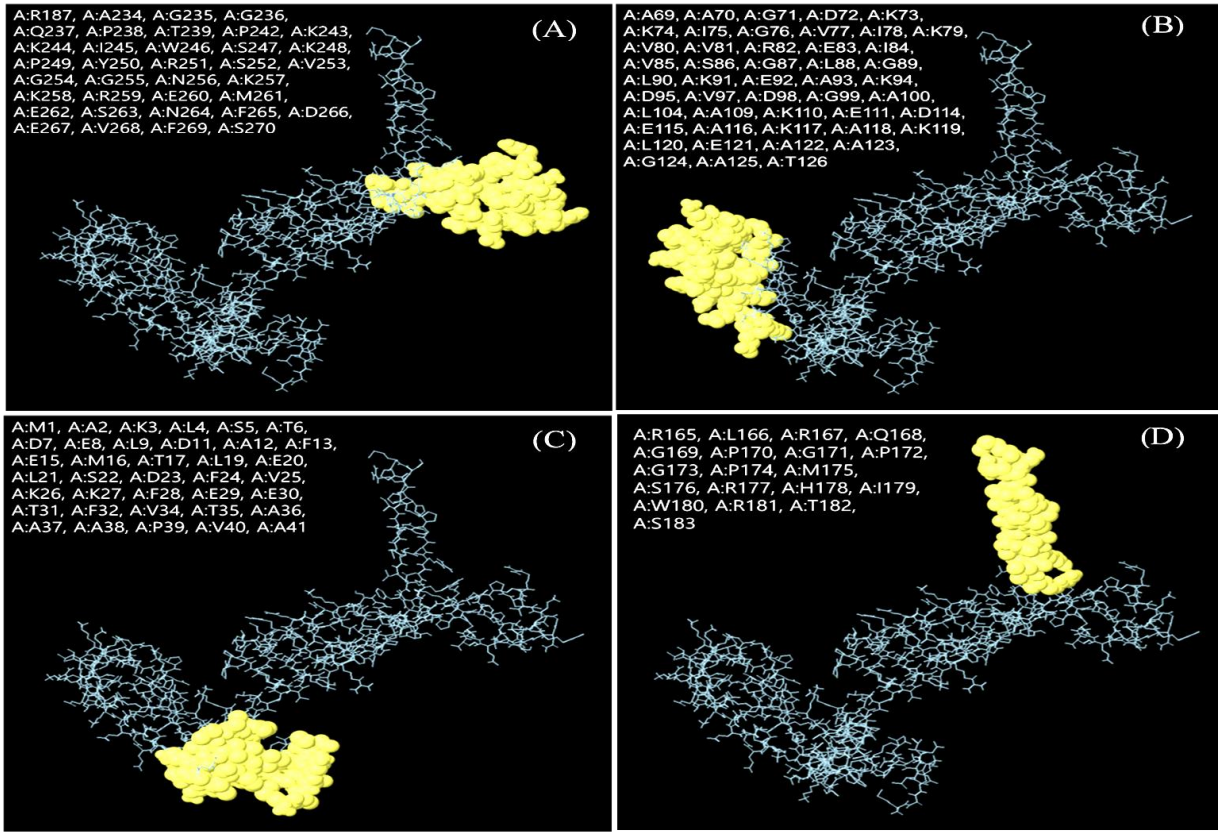

Figure 7. The 3D structure of 4 predicted conformational (discontinuous) B-cell epitopes in the refined $EsrA_{mev}$ construct. The yellow-colored regions are the conformational B-cell epitopes, while the gray regions are the rest of the residues. The residual score for each discontinued epitope was (A) 0.0737 (B) 0.711 (C) 0.696 and (D) 0.646.

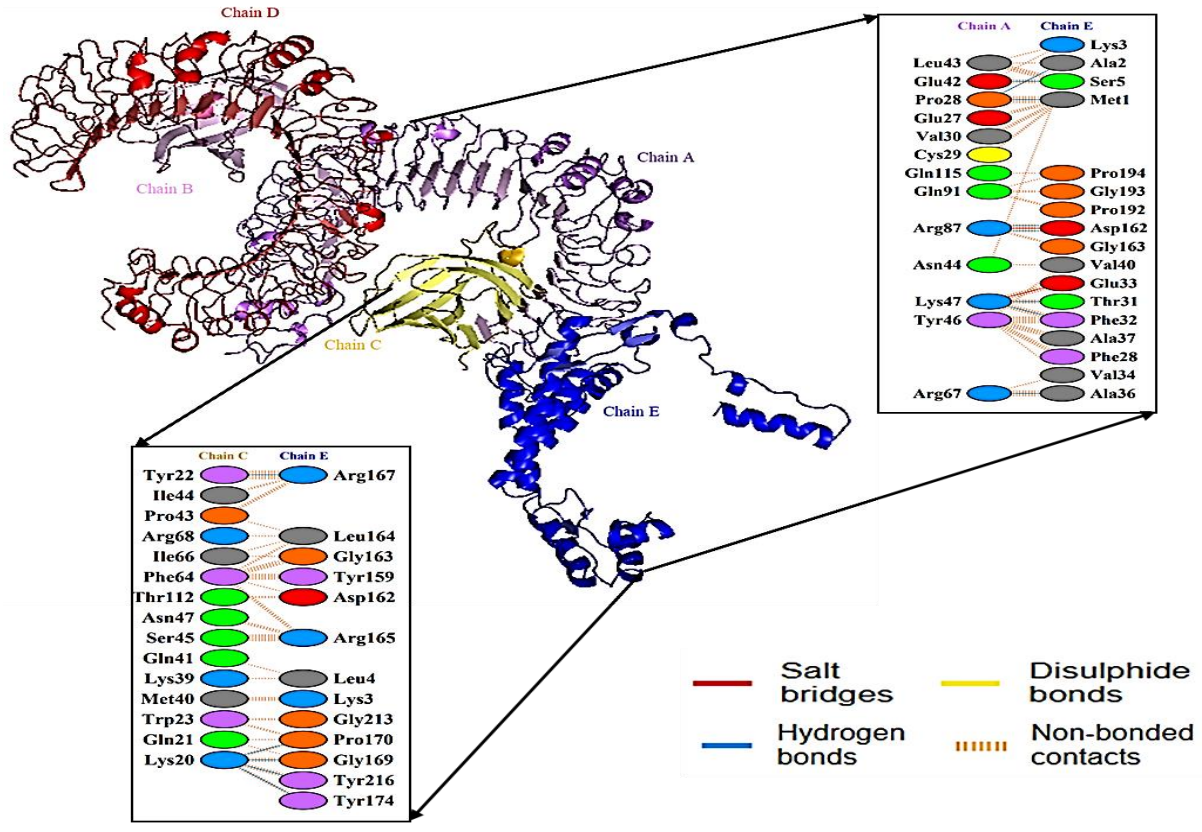

Figure 8. Molecular docking between the $EsrA_{mev}$ construct (Chain E of the docked model) and TLR4 (Chain A, B, C, D in the docked model) as the receptor. The analysis was visualized using the PDBSum online server. Salt, disulfide, and hydrogen bonds between $EsrA_{mev}$ construct and TLR4 are indicated in different colors.

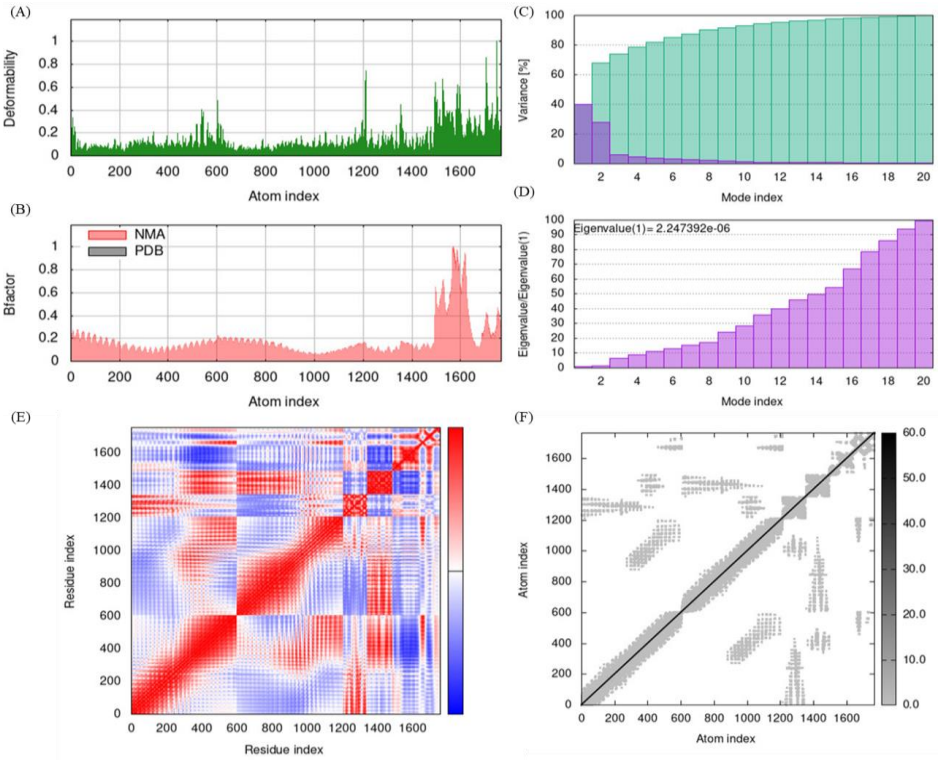

Figure 9. The molecular dynamics simulation of the EsrA$_{mev}$ construct–TLR4 docked complex. (A) Deformability plot (B) Normal mode analysis generates B-factor values, which measure each atoms uncertainty (C) The variance matrix between complex and residue (D) The eigenvalue of the docked complex, showing the energy required to deform the structure (E) The covariance matrix between pairs of residues (red: correlated, white: uncorrelated, blue: anti-correlated) (F) The elastic network model, which suggests that atoms and springs are linked. Stiff springs have a darker grey color.

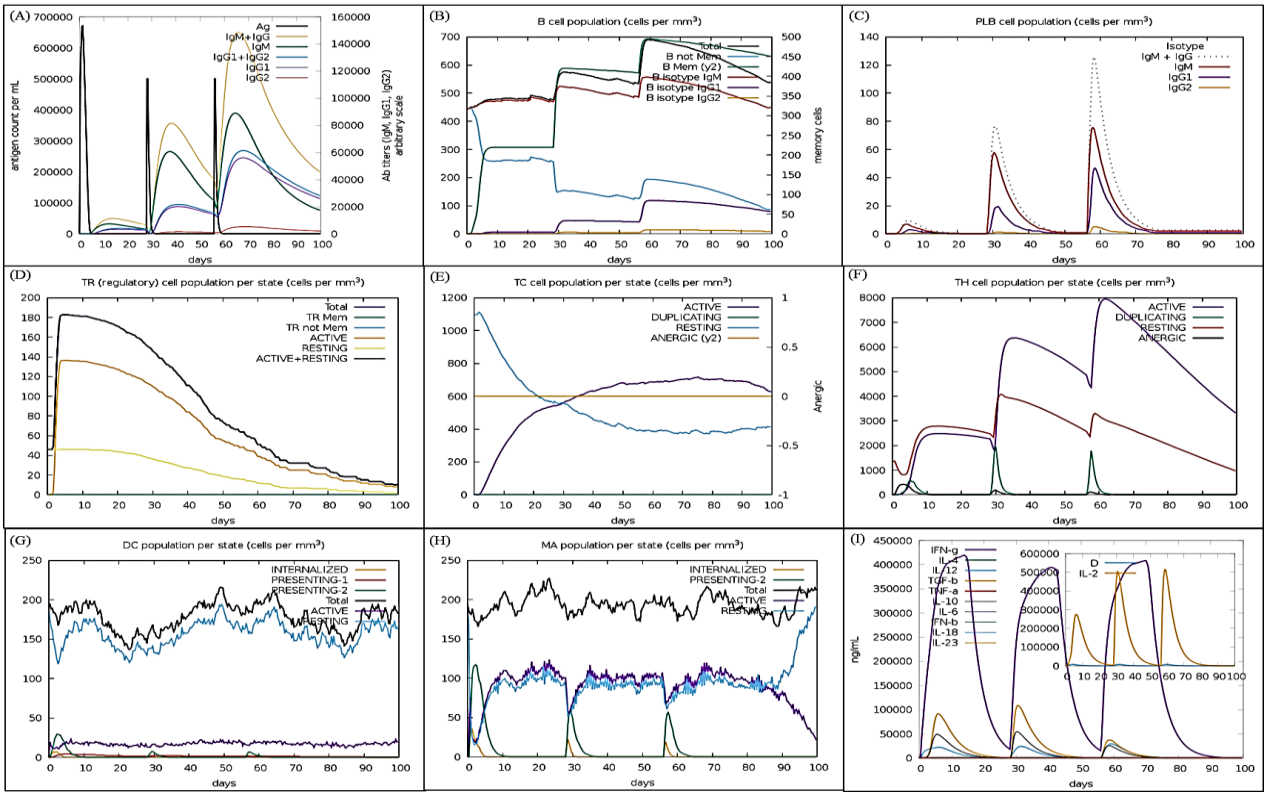

Figure 10. Immunogenic potential of the designed vaccine (EsrA$_{mev}$). In response to the exposure of EsrA$_{mev}$ after three injections, (A) production of immunoglobulins (B) active B-cell populations/state; (C) plasma B-lymphocytes and their isotypes per state; (D) reduction in the level of T regulatory cells; (E) cytotoxic T-cell population/ state; (F) helper T-cell population/state; (G) dendritic cell population per state; (H) activity of macrophage population/state; (I) cytokine level and interleukins (smaller plot) in different states with the Simpson index (dotted line). All units are in cells/mm3 in three subsequent immune responses

2022; Stanekov & Varekov, 2010).

In our study, the multi-epitope vaccine ($EsrA_{mev}$ construct) was developed using specific sequences known as "linkers". The selection of linkers was based on their potential to increase the stability, folding and transcriptional regulation of $EsrA_{mev}$ construct. Several linkers including AAYs, GPGPGs, and KKs were used to combine CTL, HTL, and LBL epitopes to construct the multi-epitope vaccine as reported in previous studies (Fatoba et al., 2022; Khalid et al., 2022). Moreover, an EAAAK linker which can act as a TLR4 agonist was used to connect the selected adjuvant to the initial CTL epitope (Fadaka et al., 2021; Islam, Mahfuj, et al., 2022). Altogether, these selected linkers are also capable of increasing the shelf life and the potency of the vaccine construct (Khalid et al., 2022). Also, the inclusion of multiple epitopes can broaden immune responses, optimize vaccine efficacy, and address challenges such as antigenic variability and cross-reactivity (Alexander et al., 1998). Furthermore, an ideal vaccine candidate should possess different properties to stimulate specific immune response inside the host. Among them, having good thermostability and solubility is very important (Islam, Mahfuj, et al., 2022). In particular, understanding the secondary structures of vaccine constructs are crucial for its antigenicity, stability, epitope accessibility, immunogenicity, and interactions with other molecules, all of which collectively determine its efficacy as a vaccine candidate (S. C. Kim et al., 2022). Recently developed *in silico* vaccine against fish pathogenic *V. harveyi* shown to possess all the above-required characteristics (Islam, Mou, Sanjida, et al., 2022). The $EsrA_{mev}$ construct developed here also carries those "ideal vaccine candidate" properties, including high aliphatic index (89.78) and solubility (0.823).

The different functions (such as molecular signaling and binding, immune response and etc.) of a protein is largely determined by its structure. Hence, designing an accurate 3D structure of a protein is the key to unravel its specific functions. The 3D protein structures are most commonly evaluated using Ramachandran plots. A good quality 3D protein

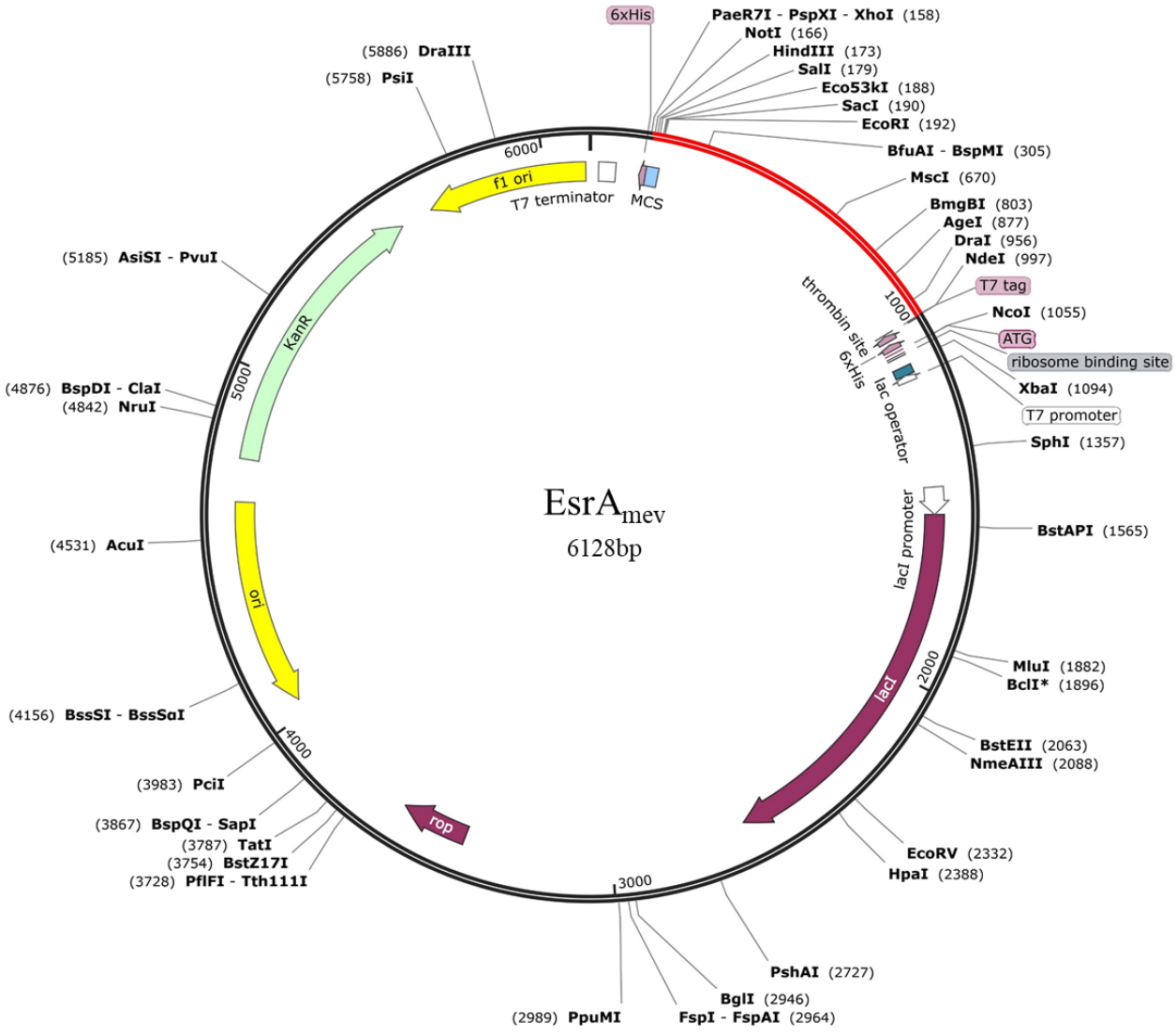

Figure 11. *In silico* restriction cloning of the codon-optimized final designed vaccines in the pET-28a (+) vector between the *XhoI* (158) and *NdeI* (997) restriction enzyme sites (SnapGene software). The red color indicates the cloned region of vaccine construct. The final constructs can further be expressed in *E coli* (strain K12) for efficient vaccine production.

structure should have at least 90% of atoms in the most favored region (RAMA) of the Ramachandran plot (Celik et al., 2022; Tran et al., 2015). In the present study, the refined 3D model of $EsrA_{mev}$ construct showed 97.4% atoms in the RAMA favored region. This structure was further validated by having a Z and quality scores of -7.29 and 99 in ProSA and ERRAT servers suggesting that $EsrA_{mev}$ construct exhibit good properties in comparison to the previously developed *in silico* vaccine models against other fish pathogens such as *V. harveyi*, Tilapia lake virus, and marine birnavirus (Islam, Mahfuj, et al., 2022; Islam, Mou, & Sanjida, 2022; Islam, Mou, Sanjida, et al., 2022).

Within the host organism, the TLR4 plays a major role in the detection of pathogenic molecules such as gram-negative glycoproteins (Hallman et al., 2001; Mukherjee et al., 2016). Given that, molecular docking was conducted in ClusPro v2.0, by using TLR4 as the receptor for the developed $EsrA_{mev}$. Among the produced models, the one with the lowest energy was selected for MD analysis through iMODS server which indicated high stability and stiffness of the $EsrA_{mev}$- TLR4 complex. In the present study, the eigenvalue required for molecular docking complex of the vaccine construct and receptor was also determined. A higher eigenvalue generally indicates greater stability and binding affinity, essential for effective vaccine activities (Yılmaz Çolak, 2024). The designed construct in present study showed higher eigenvalue that is eseential for its stability, specificity, and biological function. Moreover, a potent vaccine must mimic the natural infection in the host and it should have the capacity to produce long-lasting adaptive immunity (Kang & Compans, 2009). Given that, the immune stimulation of EsrAmev construct through C-ImmSim server showed high potential to trigger secondary and tertiary antibodies (IgG, IgM) along with active B-cell, T-cell and cytokines. The final $EsrA_{mev}$ construct was codon-optimized to avoid mRNA codon inconsistency and, cloned to promote a successful expression in *E. coli* K12 by utilizing vector pET+28a (Fadaka et al., 2021; Islam, Mou, Sanjida, et al., 2022). In the future, this $EsrA_{mev}$ construct can be used for in vitro experiments to assess the efficacy against Fish edwardsiellosis.

Conclusion

Vaccination is one of the most effective ways to control diseases in fish. Currently, there is a necessity for good quality, effective vaccines against “fish edwardsiellosis” caused by *E. tarda*. The multi-epitope vaccine developed in this study is highly immunogenic, antigenic, non-allergen, non-toxic, and capable of eliciting strong humoral and cellular immune responses when used in the *in silico* models. Although the proposed immunoinformatic vaccine here requires further experimental validations, it serves as an important step towards designing a potential vaccine against the etiological agent of edwardsiellosis.

Ethical Statement

Not applicable.

Funding Information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contribution

Gunasekara Chathura Wikumpriya, L.G.T.G. Rajapaksha: Both authors’ equally contributed to the following. Conceptualization, Methodology, Validation, Writing original draft, review & editing. Data curation.

W.S.P. Madhuranga: Writing -review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

References

Alekseenko, A., Kotelnikov, S., Ignatov, M., Egbert, M., Kholodov, Y., Vajda, S., & Kozakov, D. (2020). ClusPro LigTBM: Automated Template-based Small Molecule Docking. *Journal of Molecular Biology*, *432*(11), 3404–3410. https://doi.org/10.1016/J.JMB.2019.12.011

Alexander, J., Fikes, J., Hoffman, S., Franke, E., Sacci, J., Appella, E., Chisari, F. V., Guidotti, L. G., Chesnut, R. W., Livingston, B., & Sette, A. (1998). The optimization of helper T lymphocyte (HTL) function in vaccine development. *Immunologic Research*, *18*(2), 79–92. https://doi.org/10.1007/BF02788751/METRICS

Andersen, M. H., Schrama, D., Thor Straten, P., & Becker, J. C. (2006). Cytotoxic T cells. *The Journal of Investigative Dermatology*, *126*(1), 32–41. https://doi.org/10.1038/SJ.JID.5700001

Antonelli, A. C. B., Almeida, V. P., de Castro, F. O. F., Silva, J. M., Pfrimer, I. A. H., Cunha-Neto, E., Maranhão, A. Q., Brígido, M. M., Resende, R. O., Bocca, A. L., & Fonseca, S. G. (2022). *In silico* construction of a multiepitope Zika virus vaccine using immunoinformatics tools. *Scientific Reports 2022 12:1*, *12*(1), 1–20. https://doi.org/10.1038/s41598-021-03990-6

Bolnick, D. I., Snowberg, L. K., Caporaso, J. G., Lauber, C., Knight, R., & Stutz, W. E. (2014). Major Histocompatibility Complex class IIb polymorphism influences gut microbiota composition and diversity. *Molecular Ecology*, *23*(19), 4831–4845. https://doi.org/10.1111/MEC.12846

Buchan, D. W. A., & Jones, D. T. (2019). The Psipred Protein Analysis Workbench: 20 years on. *Nucleic Acids Research*, *47*(W1), W402–W407. https://doi.org/10.1093/NAR/GKZ297

Calis, J. J. A., Maybeno, M., Greenbaum, J. A., Weiskopf, D., De Silva, A. D., Sette, A., Keşmir, C., & Peters, B. (2013). Properties of MHC Class I Presented Peptides That

Enhance Immunogenicity. *PLOS Computational Biology*, *9*(10), e1003266. https://doi.org/10.1371/JOURNAL.PCBI.1003266

Celik, I., Abdellattif, M. H., & Tallei, T. E. (2022). An Insight Based on Computational Analysis of the Interaction between the Receptor-Binding Domain of the Omicron Variants and Human Angiotensin-Converting Enzyme 2. *Biology*, *11*(5), 797. https://doi.org/10.3390/BIOLOGY11050797/S1

Colovos, C., & Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science : A Publication of the Protein Society*, *2*(9), 1511–1519. https://doi.org/10.1002/PRO.5560020916

Dash, R., Das, R., Junaid, M., Akash, M. F. C., Islam, A., & Hosen, S. M. Z. (2017). *In silico*-based vaccine design against Ebola virus glycoprotein. *Advances and Applications in Bioinformatics and Chemistry*, *10*(1), 11–28. https://doi.org/10.2147/AABC.S115859

Dhanda, S. K., Gupta, S., Vir, P., & Raghava, G. P. (2013). Prediction of IL4 inducing peptides. *Clinical & Developmental Immunology*, *2013*, 263952. https://doi.org/10.1155/2013/263952

Dhanda, S. K., Vir, P., & Raghava, G. P. S. (2013). Designing of interferon-gamma inducing MHC class-II binders. *Biology Direct*, *8*(1), 1–15. https://doi.org/10.1186/1745-6150-8-30/TABLES/9

Dijkstra, J. M., Grimholt, U., Leong, J., Koop, B. F., & Hashimoto, K. (2013). Comprehensive analysis of MHC class II genes in teleost fish genomes reveals dispensability of the peptide-loading DM system in a large part of vertebrates. *BMC Evolutionary Biology*, *13*(1), 1–14. https://doi.org/10.1186/1471-2148-13-260/TABLES/1

Dimitrov, I., Flower, D. R., & Doytchinova, I. (2013). AllerTOP - a server for *in silico* prediction of allergens. *BMC Bioinformatics*, *14*(SUPPL6), 1–9. https://doi.org/10.1186/1471-2105-14-S6-S4/FIGURES/4

Doytchinova, I. A., & Flower, D. R. (2007). VaxiJen: A server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics*, *8*(1), 1–7. https://doi.org/10.1186/1471-2105-8-4/TABLES/2

Du, M., Chen, J., Zhang, X., Li, A., Li, Y., & Wang, Y. (2007). Retention of virulence in a viable but nonculturable *Edwardsiella tarda* isolate. *Applied and Environmental Microbiology*, *73*(4), 1349–1354. https://doi.org/10.1128/aem.02243 06/asset/56f54112-d862-49e5-aea8 c4b1d6d5b685/assets/graphic/zam0040775110003.jpeg

Fadaka, A. O., Sibuyi, N. R. S., Martin, D. R., Goboza, M., Klein, A., Madiehe, A. M., & Meyer, M. (2021). Immunoinformatics design of a novel epitope-based vaccine candidate against dengue virus. *Scientific Reports 2021 11:1*, *11*(1), 1–22. https://doi.org/10.1038/s41598-021-99227-7

Fatoba, A. J., Adeleke, V. T., Maharaj, L., Okpeku, M., Adeniyi, A. A., & Adeleke, M. A. (2022). Design of a Multiepitope Vaccine against Chicken Anemia Virus Disease. *Viruses*, *14*(7), 1456. https://doi.org/10.3390/V14071456/S1

Gao, D., Cheng, J., Zheng, E., Li, Y., Shao, Z., Xu, Z., & Lu, C. (2014). Eha, a transcriptional regulator of hemolytic activity of *Edwardsiella tarda*. *FEMS Microbiology Letters*, *353*(2), 132–140. https://doi.org/10.1111/1574-6968.12420

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. In *The Proteomics Protocols Handbook* (pp. 571–607). Humana Press. https://doi.org/10.1385/1-59259-890-0:571

Geourjon, C., & Deléage, G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics*, *11*(6), 681–684. https://doi.org/10.1093/BIOINFORMATICS/11.6.681

Grimholt, U., & Dixon, B. (2016). MHC and Evolution in Teleosts. *Biology 2016, Vol. 5, Page 6*, *5*(1), 6. https://doi.org/10.3390/BIOLOGY5010006

Guan, Y., Yin, K., Zhou, M., Yang, M., Zhang, Y., Liu, X., & Wang, Q. (2018). EsrB negatively regulates expression of the glutamine sythetase GlnA in the fish pathogen *Edwardsiella piscicida*. *FEMS Microbiology Letters*, *365*(4), 7. https://doi.org/10.1093/FEMSLE/FNY007

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., & Raghava, G. P. S. (2013). *In Silico* Approach for Predicting Toxicity of Peptides and Proteins. *PLOS ONE*, *8*(9), e73957. https://doi.org/10.1371/JOURNAL.PONE.0073957

Hallman, M., RÄmet, M., & Ezekowitz, R. A. (2001). Toll-like Receptors as Sensors of Pathogens. *Pediatric Research 2001 50:3*, *50*(3), 315–321. https://doi.org/10.1203/00006450-200109000-00004

Hammed-Akanmu, M., Mim, M., Osman, A. Y., Sheikh, A. M., Behmard, E., Rabaan, A. A., Suppain, R., & Hajissa, K. (2022). Designing a Multi-Epitope Vaccine against *Toxoplasma gondii*: An Immunoinformatics Approach. *Vaccines 2022, Vol. 10, Page 1389*, *10*(9), 1389. https://doi.org/10.3390/VACCINES10091389

Heo, L., Park, H., & Seok, C. (2013). GalaxyRefine: protein structure refinement driven by side-chain repacking. *Nucleic Acids Research*, *41*(W1), W384–W388. https://doi.org/10.1093/NAR/GKT458

Islam, S. I., Mahfuj, S., Alam, M. A., Ara, Y., Sanjida, S., & Mou, M. J. (2022). Immunoinformatic Approaches to Identify Immune Epitopes and Design an Epitope-Based Subunit Vaccine against Emerging Tilapia Lake Virus (TiLV). *Aquaculture Journal 2022, Vol. 2, Pages 186-202*, *2*(2), 186–202. https://doi.org/10.3390/AQUACJ2020010

Islam, S. I., Mou, M. J., & Sanjida, S. (2022). Application of reverse vaccinology for designing of an mRNA vaccine against re-emerging marine birnavirus affecting fish species. *Informatics in Medicine Unlocked*, *30*, 100948. https://doi.org/10.1016/J.IMU.2022.100948

Islam, S. I., Mou, M. J., Sanjida, S., Tariq, M., Nasir, S., & Mahfuj, S. (2022). Designing a novel mRNA vaccine against *Vibrio harveyi* infection in fish: an immunoinformatics approach. *Genomics & Informatics*, *20*(1). https://doi.org/10.5808/GI.21065

Jaan, S., Zaman, A., Ahmed, S., Shah, M., & Ojha, S. C. (2022). mRNA Vaccine Designing Using Chikungunya Virus E Glycoprotein through Immunoinformatics-Guided Approaches. *Vaccines*, *10*(9), 1476. https://doi.org/10.3390/VACCINES10091476/S1

Jos´, J., Ramón, J., Ramón, R., Opez-Blanco, L. ´, Aliaga, J. I., Quintana-Ortí, E. S., Chacón, P., & Chacón, C. (2014). iMODS: internal coordinates normal mode analysis server. *Nucleic Acids Research*, *42*(W1), W271–W276. https://doi.org/10.1093/NAR/GKU339

Joshi, A., Pathak, D. C., Mannan, M. A. ul, & Kaushik, V. (2021). *In-silico* designing of epitope-based vaccine against the seven banded grouper nervous necrosis virus affecting

fish species. *Network Modeling Analysis in Health Informatics and Bioinformatics*, *10*(1), 1–12. https://doi.org/10.1007/S13721-021-00315-5/FIGURES/9

Kang, S. M., & Compans, R. W. (2009). Host responses from innate to adaptive immunity after vaccination: Molecular and cellular events. *Molecules and Cells*, *27*(1), 5–14. https://doi.org/10.1007/S10059-009-0015-1/METRICS

Kerie, Y., Nuru, A., & Abayneh, T. (2019). Edwardsiella Species Infection in Fish Population and Its Status in Ethiopia. *Fisheries and Aquaculture Journal*, *10*(2). https://doi.org/10.35248/2150-3508.19.10.266

Khalid, K., Irum, S., Ullah, S. R., & Andleeb, S. (2022). *In-Silico* Vaccine Design Based on a Novel Vaccine Candidate Against Infections Caused by *Acinetobacter baumannii*. *International Journal of Peptide Research and Therapeutics*, *28*(1), 1–17. https://doi.org/10.1007/S10989-021-10316-7/FIGURES/3

Kim, D. E., Chivian, D., & Baker, D. (2004). Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Research*, *32*(suppl_2), W526–W531. https://doi.org/10.1093/NAR/GKH468

Kim, S. C., Sekhon, S. S., Shin, W. R., Ahn, G., Cho, B. K., Ahn, J. Y., & Kim, Y. H. (2022). Modifications of mRNA vaccine structural elements for improving mRNA stability and translation efficiency. *Molecular and Cellular Toxicology*, *18*(1), 1–8. https://doi.org/10.1007/S13273-021-00171-4/FIGURES/2

Krzyżek, P., Biernat, M. M., & Gościniak, G. (2019). Intensive formation of coccoid forms as a feature strongly associated with highly pathogenic *Helicobacter pylori* strains. *Folia Microbiologica*, *64*(3), 273. https://doi.org/10.1007/S12223-018-0665-5

Kuriata, A., Gierut, A. M., Oleniecki, T., Ciemny, M. P., Kolinski, A., Kurcinski, M., & Kmiecik, S. (2018). CABS-flex 2.0: a web server for fast simulations of flexibility of protein structures. *Nucleic Acids Research*, *46*(W1), W338–W343. https://doi.org/10.1093/NAR/GKY356

Lamiable, A., Thevenet, P., Rey, J., Vavrusa, M., Derreumaux, P., & Tuffery, P. (2016). PEP-FOLD3: faster *de novo* structure prediction for linear peptides in solution and in complex. *Nucleic Acids Research*, *44*(W1), W449–W454. https://doi.org/10.1093/NAR/GKW329

Larsen, M. V., Lundegaard, C., Lamberth, K., Buus, S., Lund, O., & Nielsen, M. (2007). Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. *BMC Bioinformatics*, *8*(1), 1–12. https://doi.org/10.1186/1471-2105-8-424/TABLES/3

Laskowski, R. A., Jabłońska, J., Pravda, L., Vařeková, R. S., & Thornton, J. M. (2018). PDBsum: Structural summaries of PDB entries. *Protein Science : A Publication of the Protein Society*, *27*(1), 129–134. https://doi.org/10.1002/PRO.3289

Laskowski, R. A., MacArthur, M. W., Moss, D. S., & Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography*, *26*(2), 283–291. https://doi.org/10.1107/S0021889892009944

Li, M. F., & Sun, L. (2018). *Edwardsiella tarda* Sip2: A serum-induced protein that is essential to serum survival, acid resistance, intracellular replication, and host infection. *Frontiers in Microbiology*, *9*(MAY), 367404. https://doi.org/10.3389/FMICB.2018.01084/BIBTEX

Lucchese, G., Jahantigh, H. R., De Benedictis, L., Lovreglio, P., & Stufano, A. (2021). An Epitope Platform for Safe and Effective HTLV-1-Immunization: Potential Applications for mRNA and Peptide-Based Vaccines. *Viruses*, *13*(8). https://doi.org/10.3390/V13081461

Magnan, C. N., Randall, A., & Baldi, P. (2009). SOLpro: accurate sequence-based prediction of protein solubility. *Bioinformatics*, *25*(17), 2200–2207. https://doi.org/10.1093/BIOINFORMATICS/BTP386

Magnan, C. N., Zeller, M., Kayala, M. A., Vigil, A., Randall, A., Felgner, P. L., & Baldi, P. (2010). High-throughput prediction of protein antigenicity using protein microarray data. *Bioinformatics*, *26*(23), 2936–2943. https://doi.org/10.1093/BIOINFORMATICS/BTQ551

Mahendran, R., Jeyabaskar, S., Sitharaman, G., Michael, R. D., & Paul, A. V. (2016). Computer-aided vaccine designing approach against fish pathogens *Edwardsiella tarda* and *Flavobacterium columnare* using bioinformatics softwares. *Drug Design, Development and Therapy*, *10*, 1703–1714. https://doi.org/10.2147/DDDT.S95691

Mahmud, S., Rafi, M. O., Paul, G. K., Promi, M. M., Shimu, M. S. S., Biswas, S., Emran, T. Bin, Dhama, K., Alyami, S. A., Moni, M. A., & Saleh, M. A. (2021). Designing a multi-epitope vaccine candidate to combat MERS-CoV by employing an immunoinformatics approach. *Scientific Reports 2021 11:1*, *11*(1), 1–20. https://doi.org/10.1038/s41598-021-92176-1

Manavalan, B., Govindaraj, R. G., Shin, T. H., Kim, M. O., & Lee, G. (2018). iBCE-EL: A New Ensemble Learning Framework for Improved Linear B-Cell Epitope Prediction. *Frontiers in Immunology*, *9*, 388500. https://doi.org/10.3389/FIMMU.2018.01695/BIBTEX

Miniero Davies, Y., Xavier de Oliveira, M. G., Paulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S. L., Zanolli Moreno, L., Túlio de Moura Gomes, V., Zanolli Sato, M. I., Schiavo Nardi, M., Micke Moreno, A., Becker Saidenberg, A., Rose Marques de Sá, L., & Knöbl, T. (2018). *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. *Veterinary Quarterly*, *38*(1), 99–105. https://doi.org/10.1080/01652176.2018.1540070

Mohanty, B. R., & Sahoo, P. K. (2007). Edwardsiellosis in fish: a brief review. *Journal of Biosciences*, *32*(7), 1331–1344. https://doi.org/10.1007/S12038-007-0143-8

Moutaftsi, M., Peters, B., Pasquetto, V., Tscharke, D. C., Sidney, J., Bui, H. H., Grey, H., & Sette, A. (2006). A consensus epitope prediction approach identifies the breadth of murine TCD8+-cell responses to vaccinia virus. *Nature Biotechnology 2006 24:7*, *24*(7), 817–819. https://doi.org/10.1038/nbt1215

Mukherjee, S., Karmakar, S., & Babu, S. P. S. (2016). TLR2 and TLR4 mediated host immune responses in major infectious diseases: a review. *The Brazilian Journal of Infectious Diseases : An Official Publication of the Brazilian Society of Infectious Diseases*, *20*(2), 193–204. https://doi.org/10.1016/J.BJID.2015.10.011

Nagpal, G., Usmani, S. S., Dhanda, S. K., Kaur, H., Singh, S., Sharma, M., & Raghava, G. P. S. (2017). Computer-aided designing of immunosuppressive peptides based on IL-10 inducing potential. *Scientific Reports 2017 7:1*, *7*(1), 1–10. https://doi.org/10.1038/srep42851

Nielsen, M., Lund, O., Buus, S., & Lundegaard, C. (2010). MHC Class II epitope predictive algorithms. *Immunology*, *130*(3), 319–328. https://doi.org/10.1111/J.1365-2567.2010.03268.X

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*, *34*(4), 415–425. https://doi.org/10.1111/J.1574-6976.2009.00200.X

Park, S. Bin, Aoki, T., & Jung, T. S. (2012). Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. In *Veterinary Research* (Vol. 43, Issue 1). https://doi.org/10.1186/1297-9716-43-67

Pereira, U. P., Soares, S. C., Blom, J., Leal, C. A. G., Ramos, R. T. J., Guimarães, L. C., Oliveira, L. C., Almeida, S. S., Hassan, S. S., Santos, A. R., Miyoshi, A., Silva, A., Tauch, A., Barh, D., Azevedo, V., & Figueiredo, H. C. P. (2013). *In silico* prediction of conserved vaccine targets in *Streptococcus agalactiae* strains isolated from fish, cattle, and human samples. *Genetics and Molecular Research : GMR*, *12*(3), 2902–2912. https://doi.org/10.4238/2013.AUGUST.12.6

Plotkin, S., Robinson, J. M., Cunningham, G., Iqbal, R., & Larsen, S. (2017). The complexity and cost of vaccine manufacturing - An overview. *Vaccine*, *35*(33), 4064–4071. https://doi.org/10.1016/J.VACCINE.2017.06.003

Ponomarenko, J., Bui, H. H., Li, W., Fusseder, N., Bourne, P. E., Sette, A., & Peters, B. (2008). ElliPro: A new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics*, *9*(1), 1–8. https://doi.org/10.1186/1471-2105-9-514/FIGURES/3

Pyasi, S., Sharma, V., Dipti, K., Jonniya, N. A., & Nayak, D. (2021). Immunoinformatics approach to design multi-epitope-subunit vaccine against bovine ephemeral fever disease. *Vaccines*, *9*(8), 925. https://doi.org/10.3390/VACCINES9080925/S1

Rapin, N., Lund, O., & Castiglione, F. (2011). Immune system simulation online. *Bioinformatics*, *27*(14), 2013–2014. https://doi.org/10.1093/BIOINFORMATICS/BTR335

Rogge, M. L., & Thune, R. L. (2011). Regulation of the *Edwardsiella ictaluri* type III secretion system by pH and phosphate concentration through EsrA, EsrB, and EsrC. *Applied and Environmental Microbiology*, *77*(13), 4293–4302. https://doi.org/10.1128/AEM.00195-11/ASSET/3F524328-D6C8-4B7D-B64C-352BDBBF9596/ASSETS/GRAPHIC/ ZAM9991022020005.JPEG

Shi, J., Zhang, J., Li, S., Sun, J., Teng, Y., Wu, M., Li, J., Li, Y., Hu, N., Wang, H., & Hu, Y. (2015). Epitope-Based Vaccine Target Screening against Highly Pathogenic MERS-CoV: An *In Silico* Approach Applied to Emerging Infectious Diseases. *PLOS ONE*, *10*(12), e0144475. https://doi.org/10.1371/JOURNAL.PONE.0144475

Stanekov, Z., & Varekov, E. (2010). Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. *Virology Journal*, *7*(1), 1–13. https://doi.org/10.1186/1743-422X-7-351/FIGURES/3

Tran, N. T., Jakovlić, I., & Wang, W.-M. (2015). *In silico* characterisation, homology modelling and structure-based functional annotation of blunt snout bream (*Megalobrama amblycephala*) Hsp70 and Hsc70 proteins. *Journal of Animal Science and Technology 2015 57:1*, *57*(1), 1–9. https://doi.org/10.1186/S40781-015-0077-X

Wang, X., Wang, Q., Xiao, J., Liu, Q., Wu, H., & Zhang, Y. (2010). Hemolysin EthA in *Edwardsiella tarda* is essential for fish invasion *in vivo* and *in vitro* and regulated by two-component system EsrA-EsrB and nucleoid protein HhaEt. *Fish & Shellfish Immunology*, *29*(6), 1082–1091. https://doi.org/10.1016/J.FSI.2010.08.025

Xu, D., & Zhang, Y. (2011). Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophysical Journal*, *101*(10), 2525–2534. https://doi.org/10.1016/J.BPJ.2011.10.024

Xu, T., & Zhang, X. H. (2014). *Edwardsiella tarda*: an intriguing problem in aquaculture. *Aquaculture*, *431*, 129–135. https://doi.org/10.1016/J.AQUACULTURE.2013.12.001

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., Liu, S., Zhao, P., Liu, H., Zhu, L., Tai, Y., Bai, C., Gao, T., Song, J., Xia, P., Dong, J., Zhao, J., & Wang, F. S. (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *The Lancet. Respiratory Medicine*, *8*(4), 420–422. https://doi.org/10.1016/S2213-2600(20)30076-X

Yamaguchi, T., & Dijkstra, J. M. (2019). Major Histocompatibility Complex (MHC) Genes and Disease Resistance in Fish. *Cells 2019, Vol. 8, Page 378*, *8*(4), 378. https://doi.org/10.3390/CELLS8040378

Yang, W., Wang, L., Zhang, L., Qu, J., Wang, Q., & Zhang, Y. (2015). An invasive and low virulent *Edwardsiella tarda* esrB mutant promising as live attenuated vaccine in aquaculture. *Applied Microbiology and Biotechnology*, *99*(4), 1765–1777. https://doi.org/10.1007/S00253-014-6214-5/TABLES/4

Yılmaz Çolak, Ç. (2024). *In silico* analysis of virulence factors of *Streptococcus uberis* for a chimeric vaccine design. *In Silico Pharmacology 2024 12:1*, *12*(1), 1–16. https://doi.org/10.1007/S40203-023-00181-1

Yu, J. H., Han, J. J., Park, K. S., Park, K. H., & Park, S. W. (2009). *Edwardsiella tarda* infection in Korean catfish, *Silurus asotus*, in a Korean fish farm. *Aquaculture Research*, *41*(1), 19–26. https://doi.org/10.1111/J.1365-2109.2009.02296.X

Zhang, Y., Yang, Z., Tang, M., Li, H., Tang, T., Li, G., Zhong, Y., Zhang, X., Wang, X., & Wang, C. (2022). Three Specific Potential Epitopes That Could Be Recognized by T Cells of Convalescent COVID-19 Patients Were Identified From Spike Protein. *Frontiers in Immunology*, *13*, 752622. https://doi.org/10.3389/FIMMU.2022.752622/BIBTEX