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Designing multi-epitope vaccines against *Echinococcus granulosus*: an in-silico study using immuno-informatics

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Abstract

Cystic echinococcosis (CE) is a worldwide zoonotic public health issue. The reasons for this include a lack of specific therapy options, increasing antiparasitic drug resistance, a lack of control strategies, and the absence of an approved vaccine. The aim of the current study is to develop a multiepitope vaccine against CE by in-silico identification and using different Antigen B subunits. The five *Echinococcus granulosus* antigen B (EgAgB) subunits were examined for eminent antigenic epitopes, and then the best B-cell and Major Histocompatibility Complex MHC-binding epitopes were predicted. Most significant epitopes were combined to create an effective multi-epitope vaccine, which was then validated by testing its secondary and tertiary structures, physicochemical properties, and molecular dynamics (MD) modelling. A multi-epitope vaccine construct of 483 amino acid sequences was designed. It contains B-cell, Helper T Lymphocyte (HTL), and Cytotoxic T Lymphocyte (CTL) epitopes as well as the appropriate adjuvant and linker molecules. The resultant vaccinal construct had a GDT-HA value of 0.9725, RMSD of 0.299, MolProbity of 1.891, Clash score of 13.1, Poor rotamers of 0.9, and qualifying features with Rama favoured of 89.9. It was also highly immunogenic and less allergic. The majority of the amino acids were positioned in the Ramachandran plot's favourable area, and during the molecular dynamic simulation at 100 ns, no notable structural abnormalities were noticed. The resultant construct was significantly expressed and received good endorsement in the pIB2-SEC13-mEGFP expressional vector. In conclusion, the current in-silico multi-epitope vaccine may be evaluated in-vitro, in-vivo, and in clinical trials as an immunogenic vaccine model. It can also play a vital role in preventing this zoonotic parasite infection.

Keywords Cystic echinococcosis, Multi-epitope vaccine, *Echinococcus granulosus Antigen B* (*EgAgB*), Cytotoxic T lymphocytes, B-cell epitopes, pIB2-SEC13-mEGFP expression vector

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Introduction

Cystic Hydated Disease (CHD) is a zoonotic, neglected tropical and subtropical infection caused by multiple species/genotypes of Echinococcus granulosus sensu lato (s.l.), which belongs to the Taeniidae family in class Cestoda [1]. Although CHD is absent in Antarctica, Greenland, and Iceland, it is endemic in many parts of the world, including Europe, Australia, Africa, America, Asia, and the Mediterranean [1]. Cystic echinococcosis (CE) causes substantial economic/financial losses such as expensive diagnostic and treatment costs, decreased meat and milk output, and health losses due to increased morbidity and mortality in the livestock business [2]. The global burden was estimated to be 184,000 disability adjusted life years (DALYs) due to cystic echinococcosis each year, resulting in a loss of 760 million dollars a year [1]. The financial cost of CHD varies by country, however in Pakistan it is estimated to be around US$ 0.94 million/year, in Turkey is US$ 7.708/year, in India it is US$ 212.35 million/year, and in Iran it is US$ 232.3 million/year [3].

Intermediate hosts including Human or cattle can contract *E. granulosus* when they consume eggs that are expelled in the feces of definitive hosts, usually dogs [1]. The eggs hatch in the colon, producing larvae of E. granulosus (oncospheres) that pierce the intestinal wall, enter the bloodstream, and move to the liver and lungs, where they develop into hydatid cysts, creating a safe environment for the growth of parasites [2]. Because of their multiple layer nature, such cysts enable the parasite to elude the immune system of the host, which can lead to organ damage and persistent infection [3]. Due to its latent and chronic behaviour, CE might be regarded a dangerous illness. Additionally, the lack of or limitations in the current diagnostic assays cause instances of CE to go unreported in a given community [1]. The current therapeutic alternatives are either insufficient and ineffectual or carry the risk of negative and harmful effects [2]. A long-term clinical follow-up is additionally necessary once the cyst is surgically removed, and this follow-up is typically insufficient [3]. So, employing immunoprophylaxis would be a rational and effective strategy to stop CE infection [4]. Several diagnostic techniques used for CE includes ultrasonography, x-rays, computed tomography, and immunodiagnostic tests like slide agglutination tests, echinococcus specific antibodies or antigens in the serum of the patients [5].

Several parasite antigens are required for the pathogenesis, development, and commencement of helminth-causing infections [6]. *E. granulosus* AgB (EgAgB) is one of the important components of the hydatid cysts fluid, exhibits significant levels of gene family conservation among E. granulosus, as demonstrated by the alignment of deposited DNA sequences [7]. Antigen B (AgB) encoded by a polymorphic multigenic family with five AgB gene products named *AgB1* to *AgB5* [1]. EgAgB is the most suitable, more specific, potent immunogenic and most abundantly present antigen in the hydatid cyst fluid (HCF) of *E. granulosus* metacestode. *E. granulosus* cannot produce lipids and fatty acids on its own; these are provided by EgAgB, which has a molecular weight of 230 kDa and carries a significant number of polar and neutral lipids (about 50% in mass), including sterol and fatty acid (FA) molecules [8]. By interacting with secreted EgAgB, the neutrophilic granulocytes secrete elastase molecules that control neutrophil activity and aid the parasite in evading the host immune response [9]. Moreover, EgAgB is also involved in regulating and modifying the host immune system by decreasing effective cytokine production and modification in the activity of macrophages [10].

Due to the diverse host range of *E. granulosus*, the best antigen must be chosen in order to create a more effective vaccine to enhance immunization [2]. This is why choosing different immunodominant epitopes of important antigenic molecules for the *E. granulosus* life cycle and using them for vaccine development would be a potential strategy for controlling CE [3]. Designing and developing a suitable vaccine candidate is a time-consuming, expensive and complex process that needs a lot of preclinical and clinical assessment. In addition, the in-vitro and in-vivo efficacy evaluation require natural or experimental/artificial disease conditions, safety for human use, adequate formulation and standard manufacturing practices. Conventional vaccine production frequently uses entire pathogen or single antigens, which can be ineffective in producing long-lasting immunity against complicated and quickly evolving pathogens. Reduced efficacy, failure to elicit a balanced immune response (cellular and humoral immunity), and the requirement for regular updates, as shown in seasonal influenza vaccinations, are among its problems [3], HIV and *Mycobacterium tuberculosis* [4]. These limitations can be addressed by a multi-epitope vaccine strategy that targets different parts of the pathogen. Additionally, advances in immuno-informatics enable precise, effective epitope selection, which is particularly beneficial in the face of novel infectious diseases [5]. In-silico vaccine design is time-efficient, economical, and enables very precise epitope targeting, which may lower side effects and improve safety. However, due to their limited predictive ability, potential inability to properly capture biological complexity, and reliance on the quality of input data, these models raise ethical questions about informed consent and possible hazards to human subjects. There are many obstacles to overcome when transferring in-silico results to actual vaccine research and execution, including clinical trial validation, approval

from regulatory agencies, mass production, public acceptability, and guaranteeing fair access.

Big genomics datasets could be analyzed by artificial intelligence (AI) and machine learning algorithms to find pathogen-specific biomarkers for precise and early diagnosis of infections or diseases [9], minimizing the necessity for manual detection by using medical imaging to interpret respiratory diseases, including tuberculosis [11]. High-throughput virtual screening and molecular docking are widely used in drug discovery to forecast how drug candidates would interact with their target molecules, greatly speeding up the selection of possible treatments, such as those employed during the COVID-19 pandemic [12]. Reverse vaccinology and immuno-informatics are frequently used in the designing of multi-epitope vaccines, avoiding the need for conventional culture-based vaccine production through the pathogen genome examination to find potent immunoepitopes producing high immune responses [10]. This method allowed the researchers to quickly and precisely create candidate vaccines against diseases including *Mycobacterium tuberculosis and Plasmodium falciparum* [13]. Advancements in bioinformatics and computational biology have paved the way for the creation of innovative tools. These advancements have empowered scientists to perform more efficient analyses of proposed vaccination candidates [4, 5]. The current in-silico research offers a viable substitute for conventional therapies, expedites the creation of vaccines, and makes it possible to precisely and effectively combat the disease at a reasonable cost. The current study characterizes the various parts of Antigen B (AgB1/8, AgB2/8, AgB3/8, AgB4/8 and AgB5/8) by predicting and combining with T-cell and B-Cell-specific epitopes using an in-silico vaccinomic approach to construct a multiepitope vaccine against Cystic Echinococcosis.

Materials and methods

The overall in-silico analysis used in this study for the construction of multi-epitope vaccine against CE is shown in Fig. 1.

Selection of protein sequences for vaccine development

The five Echinococcus antigen B (EgAgB) peptide subunits were obtained through a search of the UniProtKB (Universal Protein Resource) database for this investigation. through an online UniProtKB (Universal Protein Resource) server (https://www.uniprot.org/). UniProt is a

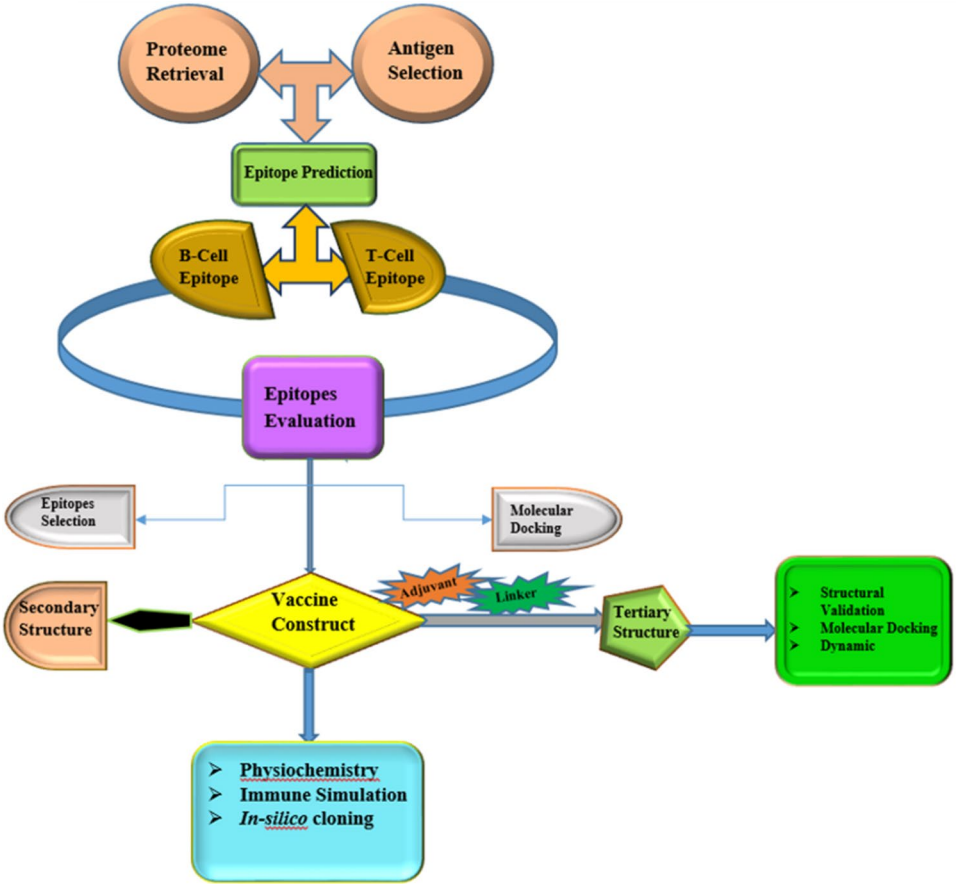

Fig. 1 Methodology for Multiepitope Vaccine against Echinococcosis

freely available, extensive, and stable resource that serves as a principal source of protein sequences and functional information [6]. The *EgAgB* is an α-helix rich polypeptide consisting of five (05) apolipoprotein/subunits (EgAgB8/1 to EgAgB8/5) used for multi-epitope vaccine construction and epitope-mapping. The apolipoproteins of AgB of *Echinococcus granulosus* s.s. EgAgB1 (Accession No: AAW78433.1), EgAgB 2/8 (AAW78459.1), EgAgB3/8 (AAW78445.1), EgAgB4/8 (AAW78449.1) and EgAgB5/8 (Accession No: AY871009.1) were collected and used.

B-cell epitope screening and confirmation

The role of B-cell epitopes in peptide vaccine development, infections diagnosis and allergy research is of vital importance. Various online servers, including Support vector machine Tri-peptide similarity and Propensity score (SVMTriP) accessed through (http://sysbio.unl.edu/SVMTriP/index.php) [7], ABCpred (Prediction of Continuous B-cell Epitopes) accessed at (http://crdd.osdd.net/raghava/abcpred/) and BCPREDS (B-cell epitope prediction server) accessed through (http://ailab.ist.psu.edu/bcpred/predict.html) were applied for the screening and evaluation of B-cell epitope [8]. The critical characteristic of the selected B-cell epitope, including solubility, antigenicity and allergenicity of the high-ranked consensus B-cell epitopes were assessed through peptide calculator (PepCalc) accessed at (https://pepcalc.com/), AllergenFP (Allergenicity prediction by descriptor Fingerprints) version 1.0 accessed through (http://ddg-pharmfac.net/AllergenFP/) [9] and VaxiJen (version 2.0) online server at (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) [10]. The best B-cell epitopes which were non-allergenic, non-toxic, highly immunogenic and maximum antigenic were selected for multi-epitope vaccine development.

Evaluation of MHC-binding epitopes

A multifunctional Immune Epitope Database (IEDB) online server was used for the prediction of specific MHC-II accessed through (http://tools.immuneepitope.org/mhcii), and MHC-I found at (http://tools.immuneepitope.org/mhci) epitopes following a recombinant technique version 2.22 [11]. Further analysis of the predicted epitopes for their affinity characterization was done through inverse correlation by adding percentile rank to them. The binding capacity of the selected epitopes was evaluated for two HLA (human leukocyte antigen) molecules, including (HLA- HLA-A*01:01 and DRB1*07:03) with a binding capacity to CD+8 T-cells (MHC-I) and CD+4 T-cells (MHC-II) respectively. The predicted MHC-I (9-mer) and MHC-II (15-mer) epitopes were used as per the recommendation of IEDB v2.22 and 2020.04 (NetMHCpan EL 4.0) (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/) server were utilized [12]. The various characteristics like solubility, allergenicity and antigenicity of the best selected epitopes were evaluated through VaxiJen (Version 2.0) (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and AllerTOP (Version 2.0) accessed through (https://www.ddg-pharmfac.net/AllerTOP/) server [13]. The best epitopes which were non-allergenic, non-toxic, highly immunogenic and maximum antigenic were selected for multi-epitope vaccine development.

Assembly and engineering of the supposed multiepitope vaccine

The top score immune dominant epitope sequences were selected and used for the organization and assembly of the supposed designed multiepitope vaccinal candidate. The immunogenicity of the selected vaccinal peptide were enhanced by adding a potent adjuvant of mycobacterium tuberculosis the heparin-binding hemagglutinin (hbhA) (Accession No: P9WIP9). The linker EAAAK joined the adjuvant sequence to the first B-cell epitope at the N-terminal of the vaccine sequence. The linker used for epitopes of CD+4 T-cells was AAY, while GPGPG linkers for CD+8 T-cells epitopes. The EAAAK (Alpha helix-forming) linkers was used in this study as it is most commonly in the synthesis of recombinant fusion proteins (Amet et al., 2009; Bai et al., 2006). These linkers possess a stable and rigid α-helical structure, with a closely packed backbone and intra-segment hydrogen bonds (Aurora et al., 1997). Henceforth, this solid α-helical linkers work as stiff spacers between domains of protein.

Evaluation of physico-chemical characteristics for the designed vaccine

The online server Expert Protein Analysis System (ExPASy-ProtParam), available at (https://web.expasy.org/protparam) was used for the various physico-chemical characteristics of the vaccinal construct [14]. This software evaluated the molecular weight, atomic composition, half-life, GRAVY (grand average hydropathicity), instability index, aliphatic index, and isoelectric point (pI) of the designed vaccinal construct.

Evaluation of solubility, allergenicity and antigenicity of designed vaccinal construct

The solubility of the designed vaccinal construct was evaluated through the SCRATCH server accessed through (http://scratch.proteomics.ics.uci.edu/explanation.html) [15] however, the antigenicity were evaluated through ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) and VaxiJen (Version 2.0) available online at (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server. The vaccine allergenicity was determined through AllerTOP (Version 2.0) available online at (http://www.

ddg-pharmfac.net/AllerTOP, and AllergenFP (Version 1.0) accessed through (http://ddgpharmfac.net/AllergenFP/) servers.

Evaluation and homology modeling of the multiepitope vaccinal construct

Various available webservers from PSI-BLAST (http://bioinf.cs.ucl.ac.uk/psipred/), including Garnier-Osguthorpe-Robson (GOR IV) available at (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and Position Specific Iterated Prediction (PSIPRED) available at (http://bioinf.cs.ucl.ac.uk/psipred/) online server were used for prediction of the secondary structure of the multi-epitope vaccinal candidate [16]. Furthermore, the homology modeling of the multiepitope vaccinal construct was designed by using Iterative Treading ASSEmbly Refinement (I-TASSER) (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) server [17].

Validation and refinement of tertiary model of vaccine

The designed multiepitope vaccinal construct was subsequently validated through the Empirical Atom-Based Method (ERRAT) accessed at (https://saves.mbi.ucla.edu/) server [18]. Prior to validation, the refinement of the 3D model designed through I-TASSER of the vaccinal construct was carried out through GalaxyRefine (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) server. MolProbity server accessed through (http://molprobity.biochem.duke.edu) was used for the construction of Ramachandran plots which gives information about the dihedral angles of the amino acid, including phi (ϕ) and psi (ψ) angles, enable us to predict the energetically allowed and disallowed region and amino acids in the vaccinal construct [19].

Confirmation of the predicted B-cell epitopes

The discontinuous epitopes of B-cells in the designed vaccinal construct were predicted through ElliPro (**Elli**psoid and **Pro**trusion) server accessed through (http://tools.iedb.org/ellipro/), an online tool of Immune Epitope Database (IEDB) accessed through (https://www.iedb.org) [20].

Disulfide engineering of the multiepitope vaccine

The disulfide bridges are of central importance in vaccine development because they strengthen the geometric confirmation and give substantial stability to the vaccinal construct. This was achieved using the DbD2 online server at (http://cptweb.cpt.wayne.edu/DbD2/index.php). Those amino acid residues in which cysteine mutation is found or those capable of disulfide bridge formation can be easily detected through DbD2 server [21].

Molecular docking and molecular dynamics simulation

The vaccine design was subjected to molecular docking with HLA-A*01:01 and HLADRB1*07:01 (Accession No: 2Q6W) utilizing the online server ClusPro 2.0 (https://cluspro.org/help.php). The protein-protein complexes were observed using PyMOL 2.4 (https://pymol.org/2/) [22, 23]. ClusPro is a computational platform used for the prediction of protein protein docking interactions. It has the capability to forecast the binding orientation of two proteins in order to create a stable complex. PyMOL is typically utilized as a molecular visualization system. The evaluation of the binding stability or specificity between the MHC-I and MHC-II and the proposed vaccine was conducted by utilizing online server HADDOCK (https://wenmr.science.uu.nl/haddock2.4/) version 2.4. Molecular dynamics (MD) simulation was carried out in an aqueous solution by using AMBER software (Ver. 16) (https://ambermd.org/) to analyse the system dynamics. The padding distance between the edges of water box and proteins was maintained at 12 Å, heating of 300 K for 20 ps, 50 ps of pressure with production run of 1 ns, 50 ns and 100 ns was proceeded.

Cloning and codon optimization of the vaccinal construct

In-silico expression and cloning of the vaccine model was performed through SnapGene© v5.1.7 software by using a suitable *Escherichia coli* (*E. coli*) expression vector. First, the reverse translation tools of the online server Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/rev_trans.html) was used for the translation of amino acids sequence of the vaccine into nucleotide sequence [24]. Codon adaptations were made through JCat (http://www.jcat.de) online software, followed by the usage of NEBcutter v2.0 (http://nc2.neb.com/NEBcutter2) to identify the various restriction sites for commonly used restriction enzymes in the codon-optimized sequence [25].

Results

Retrieval of Echinococcus Antigen B (EgAgB) amino acid sequences

The successful retrieval of five (05) Echinococcus Antigen B (EgAgB) apolipoproteins/sub-parts (Supplementary Table 1) followed by feeding into DeepLoc 1.0 (https://services.healthtech.dtu.dk/services/DeepLoc-1.0/) showed that EgAgB (EgAgB1/8 to EgAgB5/8) are mostly secretory in nature and dedicated chiefly to the cell membrane and Golgi apparatus.

Screening and confirmation of B-cell epitope

Continuous B-cell epitopes were identified using a crosschecking approach, and the consensus epitopes were tested for solubility, allergenicity, and antigenicity. One continuous epitope of B-cell from each antigen of

Table 1 Screening and confirmation of continuous epitopes of B-cell epitope for MHCI

Peptide	Antigenicity	Immunigenicity
ERDPLGQKV	**0.5708**	-0.20662
YFFERDPLG	**0.6493**	0.19419
DDDDEVTKT	**0.8415**	0.0726
DDEVTKTKK	**0.5094**	-0.1844
EPERCKCLI	**1.1109**	-0.2229
ERCKCLIMR	**0.6679**	-0.28772
NALPFGIPA	**1.2371**	0.23698

convenient features was incorporated in the final multi-epitope vaccine sequence as shown in the (Table 1).

Screening and confirmation of T-cell epitope

The epitopes screening for MHC-II (HLA-DRB1*07:03) and MHC-I (HLA-A*01:01) binding affinity was predicted which showed that non-allergenic, potent antigenic epitopes with high MHC-binding affinity were included in the final vaccine construct as shown in the (Table 2).

Multi-epitope vaccinal construct and their physico-chemical properties

Physicochemical evaluation of the final 483 amino acid containing multiepitope vaccine is shown in Table 3. The designed multiepitope vaccine contains three domains, including both T-cell (CTL and HTL) epitopes and B-cell epitopes linked with HBHA adjuvant through a AAAK linker, as shown in Fig. 2.

Solubility, antigenic and allergenic characteristics of the vaccinal construct

The final multiepitope vaccine was high antigenic and non-allergenic in nature with solubility probability of 0.877210.

Evaluation of 3-D structure of the multiepitope vaccine

The GOR IV software was used for the multiepitope vaccinal construct for geographical representation, which

Table 3 Physico-chemical characteristics of the designed multiepitope vaccine

Characteristics	Score
Number of amino acids	483
GRAVY (Grand average of hydropathicity)	− 0.421
Molecular weight (MW)	40.21 kDa
Aliphatic index	89.93
Positive residues (Arg + Lys)	56
Instability index	36.37
Theoretical isoelectric point (pI)	7.41
In-vivo evaluated half-life (*E. coli*)	> 14 h
Extinction coefficient (at 280 nm in H2O)	17,521 M − 1 cm − 1
In-vitro evaluated half-life (mammalian reticulocytes)	279 h
Negative residues (Asp + Glu)	62
In-vivo evaluated half-life (Yeast cells)	> 19 h

contains 149 (30.84%) alpha helix, 328 (67.91%) random coil, while 6 (1.24%) of extended strand. The RMSD value of the model was12.2±5.5 Å, 0.61±0.18 of estimated TM-score and C-score of 1.49 as shown in the (Fig. 3).

Validation, refinement and MD simulation of vaccinal construct

The results of the best model through GalaxyRefine web server of the refined model showed GDT-HA of 0.9725, MolProbity of 1.891, Clash score of 13.1, Poor rotamers of 0.9 and qualifying characteristics with Rama favored of 89.9. The MD simulation of vaccine constructs for 1 ns, 50 ns and 100 ns are shown in Fig. 4 (A). The Ramachandran plot analysis through MolProbity of the refined model with the crude model demonstrated 90.1% of the residues in the favored region (Fig. 4 (B). Molecular dynamic simulation of various variables, including radius of gyration (Rg), root-mean-square fluctuation (RMSF) and root-mean-square deviations (RMSD) revealed RMSD of 0.299 as the mean of 4.7 Å (4.77 Å as the highest value during 130 ns) Fig. 4 (D). In general, the extreme deviation was absent; however, a minute change in RMSD graph going towards stability at the end. The

Table 2 Selected T-cell epitope for multi-epitope vaccine

MHC-1	Allele	Start	End	Length	Peptide	Percentile Rank	Score
	HLA-A*01:01	6	14	9	SSNALPFGI	5.2	0.005578
	HLA-A*01:01	4	12	9	FGSSNALPF	5.7	0.004767
	HLA-A*01:01	15	23	9	PAPLNTDEM	12	0.001516
	HLA-A*01:01	8	16	9	NALPFGIPA	17	0.000808
	HLA-A*01:01	10	18	9	LPFGIPAPL	20	0.000605
MHC-II	**Allele**	**Start**	**End**	**Length**	**Peptide**	**Percentile Rank**	**Score**
	HLA-DRB1*07:03	4	18	15	FGSSNALPFGIPAPL	2.7	0.006618
	HLA-DRB1*07:03	3	17	15	IFGSSNALPFGIPAP	2.7	0.005133
	HLA-DRB1*07:03	1	15	15	KPIFGSSNALPFGIP	2.7	0.002091
	HLA-DRB1*07:03	2	16	15	PIFGSSNALPFGIPA	2.7	0.000799
	HLA-DRB1*01:05	1	15	15	KPIFGSSNALPFGIP	5.7	0.000587

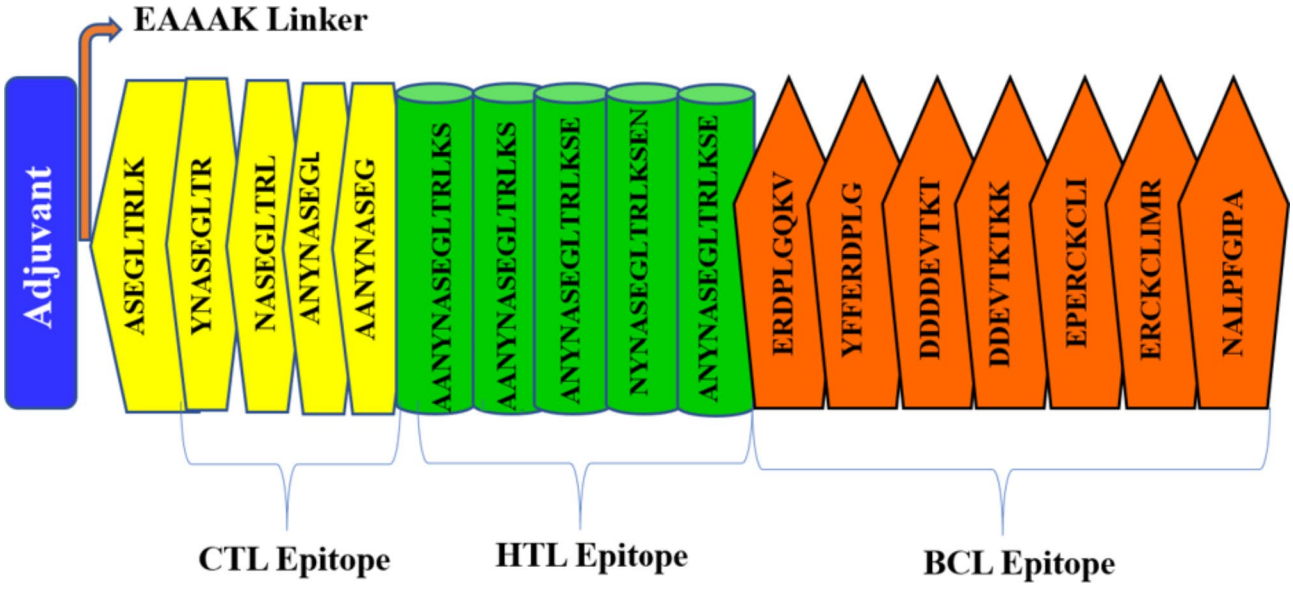

Fig. 2 The conformation of selected B-cell and MHC-binding epitopes in a multi-epitope vaccination design. The resultant chimeric protein contained 483 amino acid residues

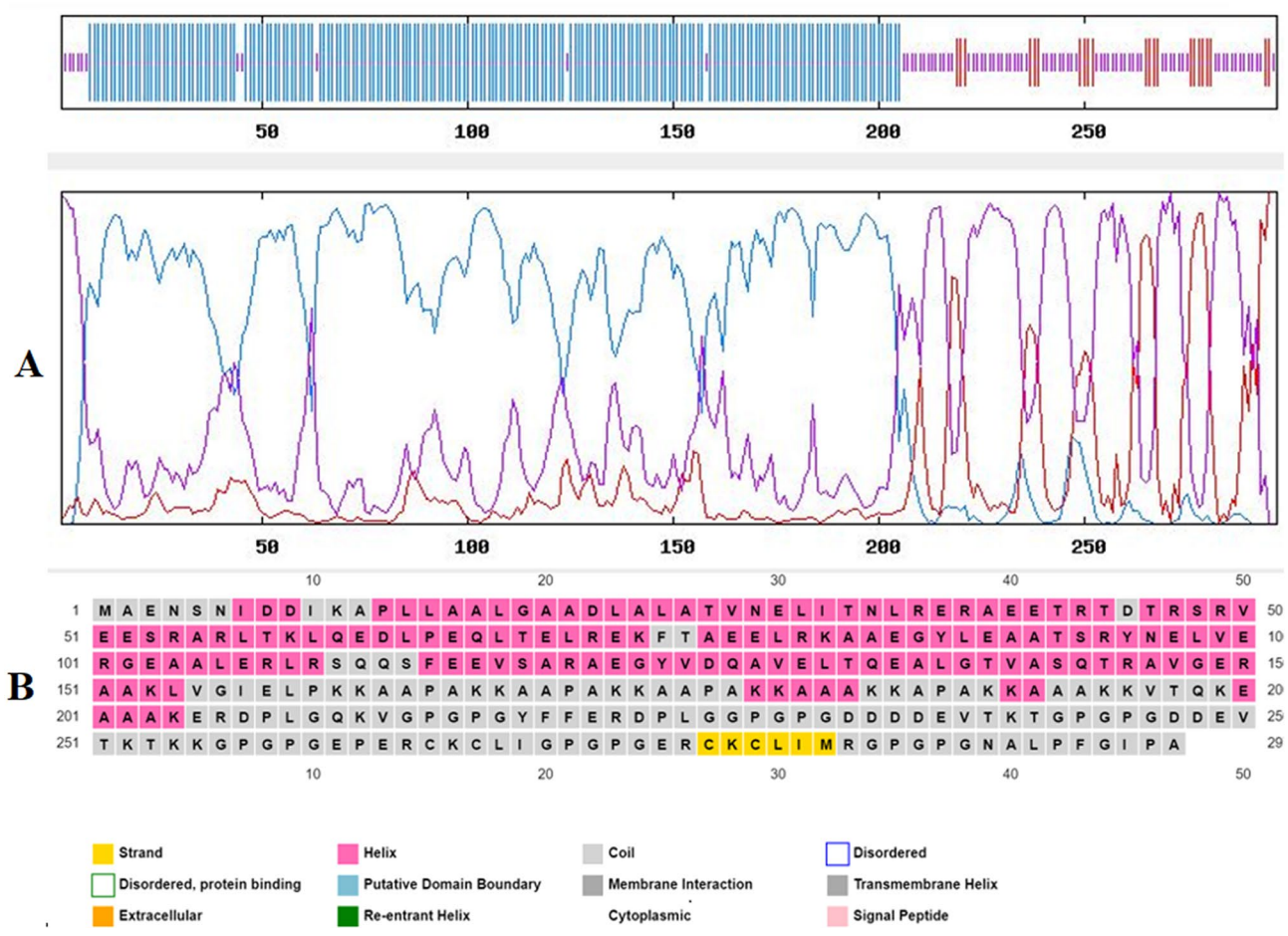

Fig. 3A GOR IV software used for the multiepitope vaccinal construct which contains 149 (30.84%) alpha helix (blue H), 328 (67.91%) random coil (yellow C), while 6 (1.24%) of extended strand (red E), respectively. **3B.** Graphical illustration of the secondary structure

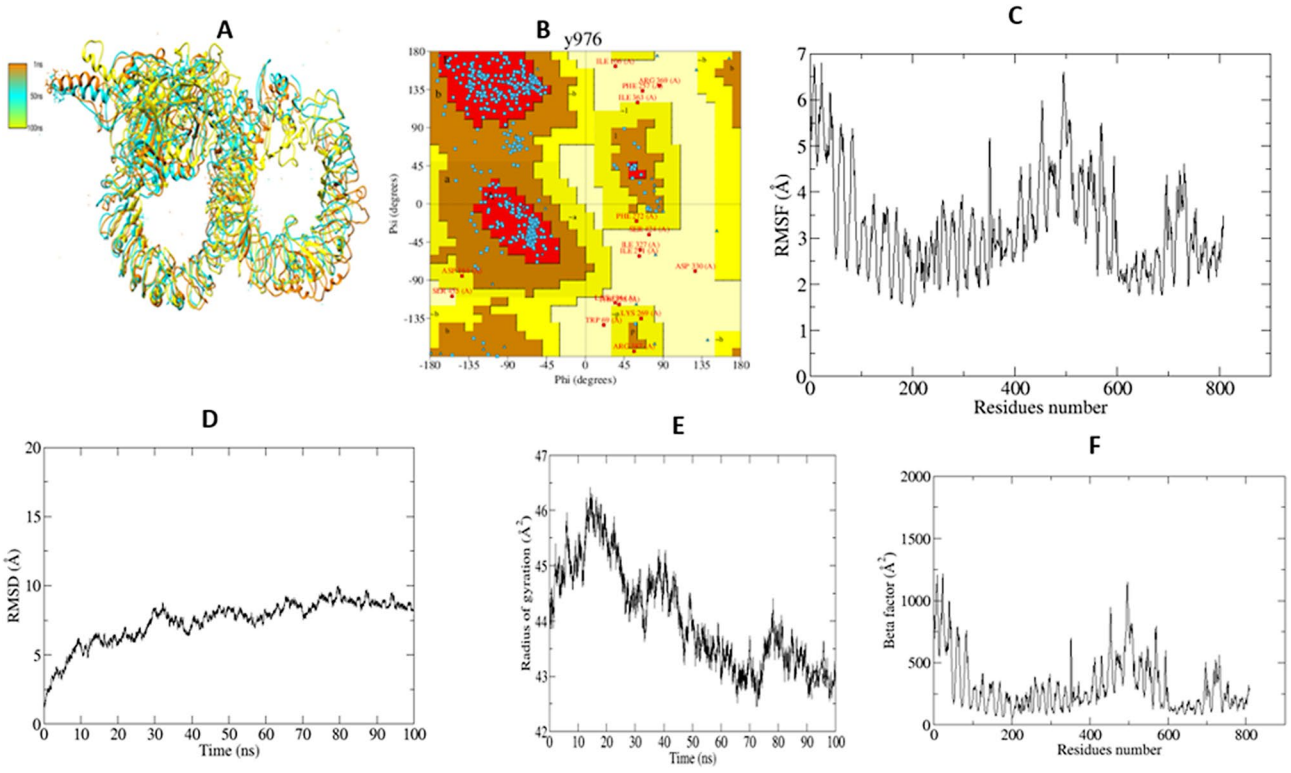

Fig. 4 **(A)** The MD simulation of vaccine constructs. **(B)** The Ramachandran plot analysis through MolProbity of the refined model with the crude model demonstrated 90.1% of the residues in the favored region. **(C)** The RMSF (fluctuations in residues) showed mean residue fluctuation value of 3.1 Å (maximum of 3.9 Å) **(E)** a RMSD graph showing minute change going towards stability at the end **(F)** The beta factor plot showed that the vaccinal construct has flexible binding characteristics. (D) Molecular dynamic simulation of multiple variables

Rg (shows the protein compactness and relaxation) of the system was noted as 39.91 Å (highest variation value of 41.30 Å) with no maximum deviations in plot, which revealed that inside the vaccine molecule, the TLR-4 will present a compact behavior (Fig. 4 (E). The RMSF (fluctuations in residues) showed mean residue fluctuation value of 3.1 Å (maximum of 3.9 Å) (Fig. 4 (C). As a measure of residue flexibility, RMSF (root mean square fluctuation) is stable within the allowed 4 Å range, indicating few variations and steady structural integrity (Ullah et al., 2024). Similarly, the beta factor plot showed that the vaccinal construct comes under flexible binding properties (Fig. 4 (F).

Conformational prediction of B-cell epitopes

According to the IEDB server of the ElliPro tool, the conformational B-cell epitopes in the engineered refined vaccine model are illustrated in Fig. 5 (A, B, C, D). The IEDB and ElliPro server identified four conformational B-cell epitopes in the final vaccine construct, where the respective residues with their score were (I). 33 residues (Score=0.742) (Fig. 5 (A) (II). 42 residues (Score=0.724) (Fig. 5 (B) (III). 46 residues (Score=0.701) (Fig. 5 (C) (IV). 41 residues (Score=0.621) (Fig. 5 (D).

Molecular docking and types of interaction

The molecular docking of multiepitope vaccinal construct with HLA-A*01:01 and HLADRB1*07:01 (Accession No: 2Q6W) has been shown in Fig. 6 (A). The molecular docking of among both molecules showed three types of interactions including; Hydrogen bonds (n=26), Non-bonded contacts (n=168) and salt bridges (n=04) which is shown in Fig. 6 (B) and Supplementary Table 2. In order to optimize the vaccination for improved immune recognition and efficacy, researchers can use molecular docking in vaccine design to anticipate the binding affinity between vaccine antigens and immune receptors [8]. By identifying potential vaccine candidates with high immune elicitation, this computational method expedites vaccine development and allows for quick and accurate reactions to new infections [7].

Cloning and codon optimization for the vaccinal construct

The vaccinal candidate sequences were reversely transcribed into nucleotide sequences for Insilco cloning and codon adaptation. *E. coli* K12 strain were used for codon adaptation. The GC% and CAI-value of the initial sequence were 61.51% and 0.63, respectively, which were improved through codon adaptation as 52.03% and 1.0, respectively. The current study revealed that we can

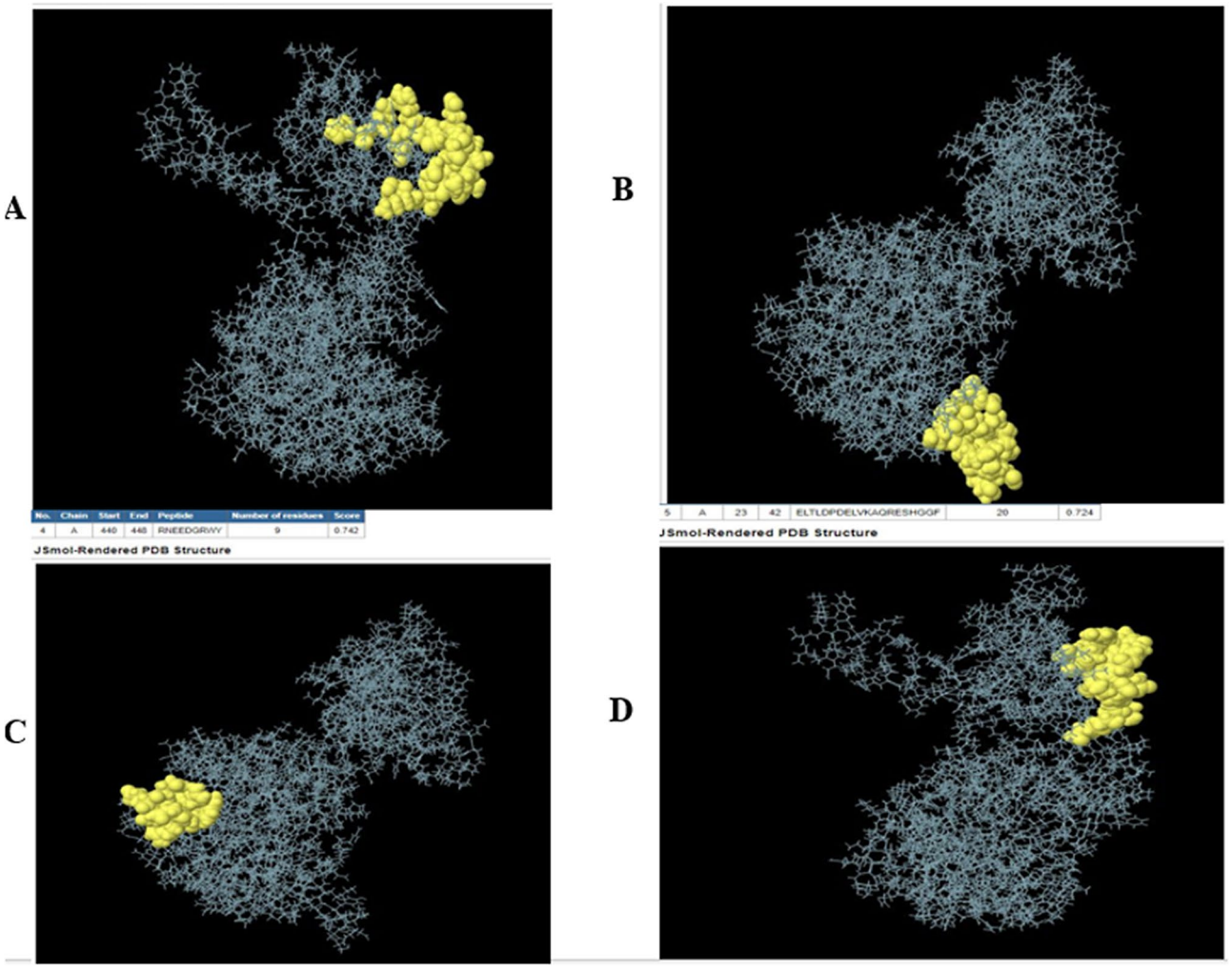

Fig. 5 Analysis of Confirmed epitopes of B-cell through ElliPro Online Server. **(A).** 33 residues (Score = 0.742) **(B)**. 42 residues (Score = 0.724) **(C).** 46 residues (Score = 0.701) **(D).** 41 residues (Score = 0.621)

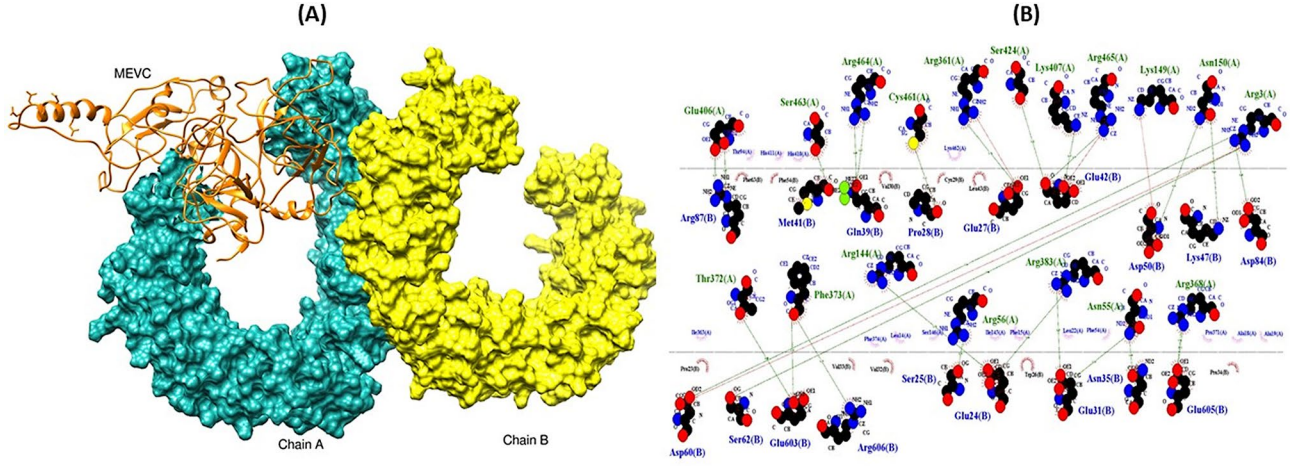

Fig. 6 Molecular Docking of the vaccinal construct **(A).** Molecular docking of Multiepitope vaccinal construct with HLA molecules **(B).** Types of molecular interaction (bonds) of docking molecules

use EcoRV and Eco53KI restrictions enzyme during in-silico cloning because there was no restriction site for this enzyme. So, the cutting sequences with H6-tag and start/stop codon and Shine-Dalgarno (AGGAGG) were embedded in the vaccine sequence. Finally, a successful clone of 8126 bp was obtained following the insertion of the fragment into the pIB2-SEC13-mEGFP(+) vector (Fig. 7).

Discussion

The history of cystic echinococcosis/hydatidosis goes beyond the 17th century [33]. It has been documented all throughout the world, with increased prevalence in developing nations. It poses a serious threat to the populations of people, domestic dogs, and animals where they coexist [26]. The immunization of susceptible hosts is essential for preventing and managing CE. To date, CE has been fought off using three different vaccination strategies: live vaccines or crude antigens, DNA vaccines, and recombinant peptide vaccines [2]. The live vaccination approach utilised hydated cyst fluid, a carbohydrate-rich fraction, or whole-body homogenate of *E. granulosus* s.s [27, 28]. However, this strategy has some significant limitations, including (I) safety concerns and the short shelf life of live vaccines. (II) insufficient synthesis of defending antibodies, and (III) differences in the level of immunity that has been acquired [29, 30]. The development of DNA-based vaccines has mostly resolved the issues listed above. The DNA vaccine technique promotes the development of more effective and long-lasting immunity within the host, activating both the humoral and cell-mediated arms of immunity. Additionally, it can be produced quickly and easily using a biotechnologically developed technique that produces an antigen in its original form [31, 32].

There have been reports of the development of a vaccine employing different recombinant echinococcus immunoprotective antigens [33–35]. During experimental trials, the EG95 vaccination against echinococcosis produced great results in sheep in Iran, Romania, Chile,

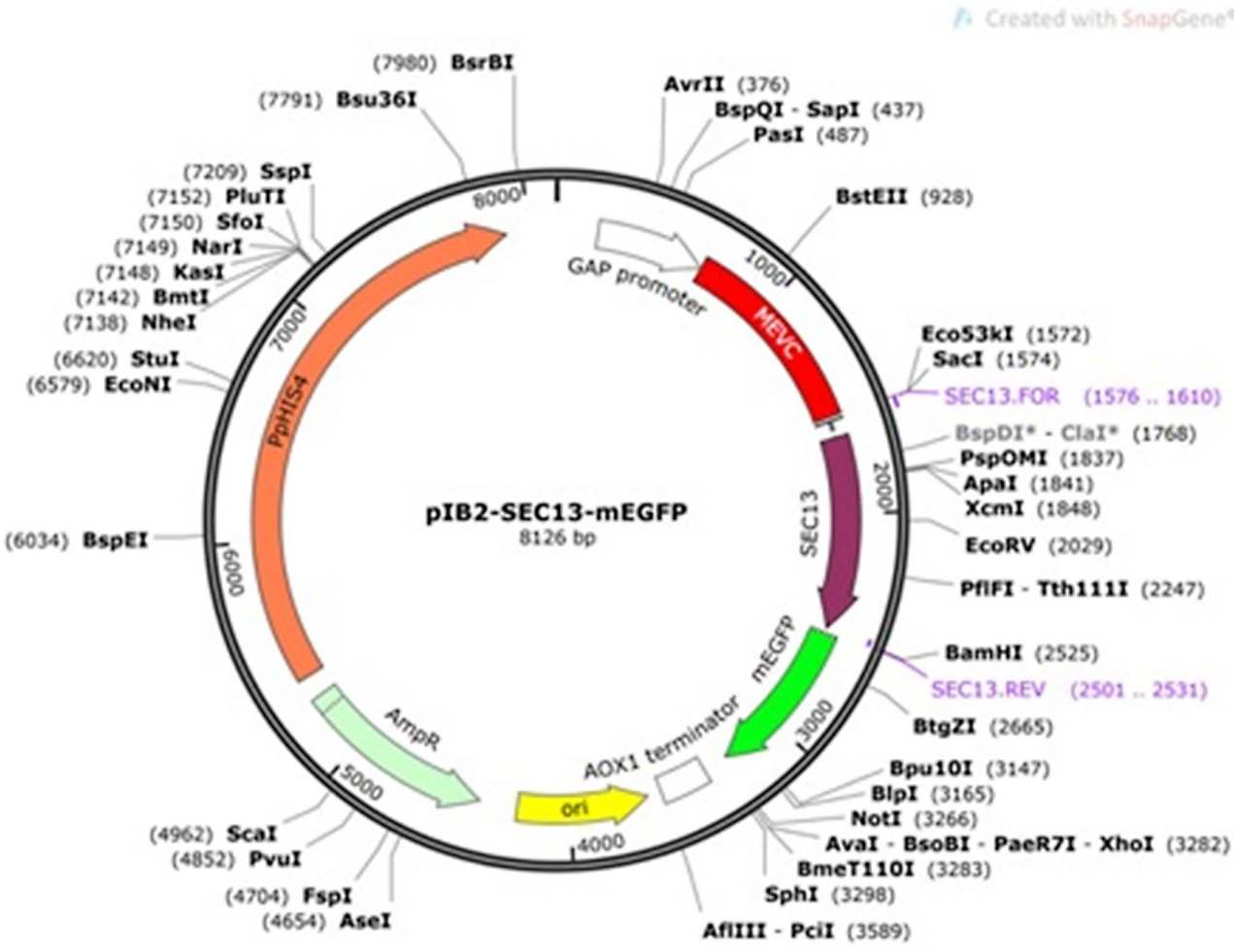

Fig. 7 Computer-aided Cloning and Codon Optimization of the designed vaccinal construct into pIB2-SEC13-mEGFP expression vector

China, Australia, New Zealand, and Argentina [36]. A number of drawbacks of EG95 vaccine, including a different response in sheep than in cattle, a dosage-dependent response, the requirement for an adjuvant [37], cost, scarcity, inability to activate the humoral and cellular arms of immunity, and lack of approval for use in humans [38, 39], rendering it unfit for use. It is strongly advised to use/combine multiple amino acid antigenic sequences in order to elicit the necessary immunological responses because the amino acid sequence/unit of a single antigen cannot provide an adequate immune response. Recent advances in bioinformatics allow researchers to pinpoint the immune-dominant epitopes in a given antigenic peptide, which can be exploited to create a CE vaccine with exceptional efficacy. For this kind of reverse vaccinology method, using immunoinformatics algorithms by utilising various web-based software and standalone servers will be ideal [2, 40]. In the last two decades, there has been a lot of new research and information reported on the immunological mechanism of *E. granulosus* using innovative omics data. The humoral response completes around IgM, IgG (IgG1 and IgG4), and IgE antibodies, while the cellular immunity against CE circulates around the Th2 and Th1 helper cells. The Th1 response against CE inhibits cyst formation, which is advantageous for the host; however, the Th2 response results in greater IL-10 production, which establishes an active, chronic cyst with potential treatment resistance. Therefore, from vulnerability (rapid host death) to resistance (self-cure), the variation in the composition and type of immune response play a crucial role in the progression and outcome of parasite disease [41, 42]. This suggests that development of and a balance between Th1/Th2 responses play a crucial role in CE's immunopathogenesis and effective management. A number of antigenic peptides that have been previously characterised for use in various diagnostic assays and as prospective CE vaccine candidates are present in the excretory and secretory fluids of hydated cysts of *E. granulosus* [47, 48]. However, the development of an in-silico based multi-epitope vaccination cannot proceed without knowledge of their immunodominant epitopes [43].

The advantages of computational vaccinology approach include virtual screening of every antigen, approaching non-cultivable microorganisms, determining non-profuse antigens, identifying nonimmunogenic antigens, knowing antigens expressed during infectious stages, finding antigens not expressed in vitro, and implementing non-structural proteins in the prediction. However, there still exist some disadvantages such as difficulty in predicting non-proteinous antigens and the requirement of biological determination for the prediction of antigenic proteins [44, 45]. On the other hand, computational vaccinology is yet to be the primary procedure followed for vaccine development in this era, despite the significant advantage of least time taken in mining the antigenic protein of a pathogen [44]. As proteins exist in four distinct structures, the multiepitope vaccine developed exhibited good physicochemical properties and stable homology [43]. The vaccinal construct candidate has a molecular weight of 40.21 kDa and can be used as an indicator in protein blot and SDS-PAGE analysis using the ProtParam server. The current results were inconsistent with the results of Nourmohammadi and colleagues [46]. Aside from other aspects, the developed vaccine's theoretical pI (7.41) was acidic, as reported by Nourmohammadi and colleagues and it could be easily purified using isoelectric focusing and ion-exchange chromatography [46]. The MEV of this investigation reveals that the most stable vaccine identified elsewhere has an instability score of 36.37 [46]. The current vaccinal construct was highly thermotolerant with an aliphatic index of 89.93 and a hydrophilic nature having a negative GRAVY value of −0.421 as similarly found in the study of [46]. The vaccinal construct's negative GRAVY rating indicates that it is hydrophilic. The molecule had a very high thermotolerance based on the aliphatic index (89.93). Another study found a similar result for an in-silico multiepitope vaccination against *Echinococcus granulosus* using peptides other than AgB. Overall, these biochemical features are extremely important for future purification/extraction processes in experimental research [46]. Two different softwares, VaxiJen v2.0 and ANTIGENpro, offer us the probability of the intended vaccinal build as 0.8988 and 0.5892, respectively, indicating that the MEV will have high antigenicity. The allergenicity of the vaccinal construct was assessed using AllergenFP v1.0 and AllerTOP v2.0, which revealed that the vaccine will not cause an allergic reaction. Another study [48] found similar programme utilisation and outcomes. The projected solubility of the expressed protein in the *E. coli* host as 0.877321% likelihood implies that this protein was soluble, as previously reported in comparable [46]. According to the PSIPRED and GOR IV web server results, the vaccination had 149 (30.84%) alpha helix, 328 (67.91%) random coil, and 6 (1.24%) extended strand. The model's RMSD was 12.2 5.5, with an estimated TM-score of 0.61 0.18 and a C-score of 1.49, which was relatively consistent with the results of [46]. Strict hydrogen bonding within the protein would maintain the secondary structure of the protein's -sheets and alpha helix, whereas -turns and random coils are typically projecting structures on the protein's surface [47]. The suggested vaccine's tertiary structure was predicted using I-TASSER web server, the best homology modelling software. GalaxyRefine, another internet server, improved the vaccine's tertiary structure and achieved a high-quality 3D structure. The vaccine created in this study has GDT-HA of 0.9725,

RMSD of 0.299, MolProbity of 1.891, Clash score of 13.1, Poor rotamers of 0.9, and qualifying features with Rama favoured of 89.9. The Ramachandran plot analysis of the refined model vs. the crude model using MolProbity revealed 90.1% of the residues in the favoured region, demonstrating similarities with the proposed multiepitope vaccination against echinococcus by using two different epitopes of an antigen other than AgB. During MD simulation the average deviations of atomic position from their mean position over time has been evaluated through RMSF value. These RMSF value provide data about the dynamics and flexibility of a protein structure [14]. In the figure C4 the core region showing decreased RMSF value indicating that the region was stable while higher value of RMSF is observed in flexible region as reported somewhere also [15, 16].

Our vaccine, on the other hand, utilised five peptide subunits of AgB, which is continually expressed throughout the whole life cycle, dominating our vaccinal design. The little improvement and refinement of the proposed vaccine via MolProbity and the ERRAT quality actor web server demonstrated an improvement in vaccine quality when compared to the crude model. According to common perception, full pathogen eradication necessitates the correct activation of B-cells and subsequent humoral responses for neutralising antibody against CE. As a result, the prominent and significant B-cell epitopes for our MEV were predicted using the ElliPro tool on the IEDB server. Various 10 immunodominant B-cell epitopes (05 for each of MHCI and MHCII), each consisting of 09 amino acids for MHCI and 15 amino acids for MHCII, with highly qualifying scores. Surprisingly, these complete epitopes with high antigenicity and low allergenicity will have a significant impact on the quality of antibody-vaccine interactions [46]. Despite the common observation that TLR antagonists and/or negative regulator agonists against parasitic infections have an important function and increase immunity, there is a paucity of reliable data on the protective effect of toll-like receptors (TLRs) against CE [48, 49]. With this in mind, we conducted a molecular docking study using human MHC alleles, which revealed that our designed multiepitope vaccine possessed affinity to both HLA-A01.01 (MHC-I) molecules with an energy score of 9939.5 and HLADRB1*07:03 (MHC-II) molecules with a minimum energy score of 985.1, as observed by Nourmohammadi and colleagues [46]. In general, a DNA sequence with a GC content of 30-70% and a CAI value of 0.8-1 is regarded optimal for expression in a specific host [48], which was eventually found in our manufactured vaccine as a CAI of 1.0 with a GC content of 52.38%, indicating improved expression in the E. coli K12 strain expression system. According to [48], the current designed MEV was successfully endorsed into the pIB2-SEC13-mEGFP(+) vector for expression and heterologous cloning and yielded substantial results [46]. The hydated cyst fluid of *E. granulosus* is rich in antigenic peptides that can be exploited for a variety of useful applications, including immunoprophylaxis. A multi-epitope vaccinal construct that incorporates numerous antigenic sections engaged in distinct life stages of the parasite's complex life cycle, such as *E. granulosus*, is highly recommended [46]. According to Nourmohammadi and colleagues, the outstanding results of this work demonstrated that the current multiepitope vaccine may be significantly expressed in multiple eukaryotic/prokaryotic hosts for future in vivo and in vitro studies. In contrast to injectable recombinant protein vaccines, which require numerous additional in-vitro processes, nucleic acid and edible-based vaccine delivery systems can be employed often and simply [46]. Using a non-viral delivery system, such as lipid and polymeric nanoparticles, as well as in vivo transfection systems such as electroporation and gene guns, can improve the cellular absorption effectiveness and durability of vaccination candidates [50]. Multiple epitope-based vaccinations have proven effective in boosting both humoral and cellular immunity simultaneously in immunization campaigns against a variety of diseases [51–55] including CE. The multi-epitope vaccine suggests a wide variety of antigens or epitopes incorporated into a vaccine design. When a vaccine contains a diverse array of antigens, it can potentially stimulate a broader immune response, making it effective against multiple strains or variants of a pathogen.

Translating an in-silico vaccine candidate (a vaccine designed using computational methods) from the research stage to clinical trials and field deployment involves navigating a complex regulatory landscape. This process could be completed in several stages including preclinical testing, regulatory submissions, phase I clinical trials, phase II clinical trials, phase III clinical trials, regulatory approval, manufacturing and scale-up, post-market surveillance and field deployment where the vaccine candidate can be deployed in the field once it has been approved and manufacturing capabilities are established. However, field deployment requires infrastructure, funding, and the logistical challenges of distribution and administration.

Conclusion and limitations

The current study concluded that the current in-silico-based multi-epitope vaccination will result in significant and fruitful results in the control and elimination of CE in both definitive and intermediate hosts in the future. The provided ensemble multiple epitope-based vaccine, consist of multiple antigens, each epitopes represents an independent immunological entity which would elicit both cellular and humoral immunity, required to control

the intracellular and extracellular forms of the parasite. Furthermore, this multi-epitope vaccinal design will reliably and simultaneously activate both innate and adoptive immunity with minimal allergenicity and adverse side effects according to bioinformatics analysis. Additionaly, the validations of multi-epitope platform, which incorporates multiple antigenic determinants (epitopes) from various proteins of a pathogen, serve as a versatile and powerful tool that can significantly impact vaccine design by identifying conserved epitopes, personalize vaccines, stimulate diverse immune responses, provide cross-protective immunity, and leverage computational tools makes it invaluable in advancing our understanding of parasite immunology and accelerating the development of effective vaccines against complex parasitic diseases. Utilizing immunoinformatics and computational modeling, this strategy can improve vaccine efficacy, expedite vaccine development, and ultimately support the global endeavor to eradicate cystic echinococcosis as a public health concern, particularly in environments with limited resources. This study possesses several limitation like, in the case of helminth infections, which include parasitic worms like *Echinococcus* species, cross-reactivity might be possible if the immune system recognizes shared epitopes among different helminth species which will require additional invitro and in-vivo validation and confirmation. Furthermore, using two HLA molecules can provide initial insights into epitope binding capacity, a more comprehensive analysis involving a broader panel of HLA molecules is often necessary for a thorough evaluation. This vaccine will passes through several testing procedures like Immunogenicity and protection assessment in animals appropriate animal species (e.g., mice, rats, non-human primates), formulation of appropriate dosage of vaccine, establishment of control group, serum antibody analysis, analysis of cell-mediated immunity, T-cell proliferation assay, cytokine profiling, flow cytometry for the activation of specific immune cell subsets (e.g., CD4+and CD8+T cells), protection assessment, clinical assessment, and histopathological examination. Additionaly, applying this vaccine in field from computer-aided research stage to clinical trials and field deployment involves navigating a complex regulatory landscape. This process could be completed in several stages including preclinical testing, regulatory submissions, phase I clinical trials, phase II clinical trials, phase III clinical trials, regulatory approval, manufacturing and scale-up, post-market surveillance and field deployment where the vaccine candidate can be deployed in the field once it has been approved and manufacturing capabilities are established. However, field deployment requires infrastructure, funding, and the logistical challenges of distribution and administration.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
Conceptualization, J.K, AS, NB, SNUM did methodology and validation, J.K, AAS, HK did Validation, Formal analysis, Investigation, Writing—original draft preparation, AAS, TAS, IA, and HK did writing—review and editing, J.K, IA, MMA, AAM, and KAA provided supervision, project administration, and funding acquisition.

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Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
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