Exploratory Algorithm of a Multi-epitope-based Subunit Vaccine Candidate Against *Cryptosporidium hominis*: Reverse Vaccinology-Based Immunoinformatic Approach

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Abstract
Cryptosporidiosis is the leading protozoan-induced cause of diarrheal illness in children, and it has been linked to childhood mortality, malnutrition, cognitive development, with retardation of growth. *Cryptosporidium hominis*, the anthroponotically transmitted species within the *Cryptosporidium* genus, contributes significantly to the global burden of infection, accounting for the majority of clinical cases in numerous nations, as well as its emergence in the last decade is largely due to detections obtained through noteworthy epidemiologic research. Nevertheless, there is no vaccine available, and the only licensed medication, nitazoxanide, has been demonstrated to have efficacy limitations in a number of patient groups recognized to be at high risk of complications. Therefore, current study delineates the computational vaccine design for *Cryptosporidium hominis*, the notable pathogen for enteric diarrhea. Firstly, a comprehensive literature search was conducted to identify six proteins based on their toxigenicity, allergenicity, antigenicity, and prediction of transmembrane helices to make up a multi-epitope-based subunit vaccine. Following that, antigenic non-toxic HTL epitope, CTL epitope with B cell epitope were predicted from the selected proteins and construct a vaccine candidate with adding an adjuvant and some linkers with immunologically superior epitopes. Afterwards, the constructed vaccine candidates and TLR2 receptor were put into the ClusPro server for molecular dynamic simulation to know the binding stability of the vaccine-TLR2 complex. Following that, *Escherichia coli* strain K12 was used as a cloning host for the chosen vaccine construct via the JCat server. As a result of the findings, it was resolute that the proposed chimeric peptide vaccine could improve the immune response to *Cryptosporidium hominis*.

Keywords *Cryptosporidium hominis* · Reverse vaccine-approach · Peptide vaccine · Molecular docking

Introduction
Cryptosporidiosis is one of the top listed waterborne diseases leading to severe diarrhea and diarrhea-associated death among young infants in both develop and developing countries (Chen et al. 2019). Malnourished and immune compromised people are particularly prone to this opportunistic foodborne pathogen, which can put a patient’s life in danger (Baptista et al. 2021; Lee et al. 2019). People infected with the Human Immunodeficiency Virus (HIV) from African countries seemed to have a greater prevalence of *Cryptosporidium*, specifically 73.6% in Uganda, 79.0% in Nigeria, and 75.60% in South Africa (Liu et al. 2020). *Cryptosporidium hominis* and *Cryptosporidium parvum* have been recognized as the most imperative sources of cryptosporidiosis in humans among the 38 *Cryptosporidium* species (Costa et al. 2020). Although, some in silico vaccine studies have been progressed in the past few years against *Cryptosporidium parvum*, but shedding light on *Cryptosporidium hominis* remained in dark.

Commencing of *Cryptosporidium* infection in human mainly exposure to oocysts from faeces shed by an infected host and transmitted through direct person to person or animal to person contact, or indirectly through ingestion of contaminated water and food (Lee et al. 2019). Within a single host, the *Cryptosporidium* life cycle alternates between asexual and sexual reproduction. Sexual recombination
produces oocysts, which is necessary for transmission, but recombination may also play a role in the host’s persistent infection (Tandel et al. 2019).

Effective treatment or control measures only can deter the curse of this disease; unfortunately, dearth of these remedy makes the situation more complex for immunocompetent patients (Dayao et al. 2020). Therefore, to protect from the disease, immunization or vaccine could be the best choice and advance technology makes the favorable amid to help with antigenic epitopes prediction, design protein structures, construct vaccine and provide insight into biological characteristics (Faisal et al. 2017). The use of immune-informatics tools can save time, overcome cost hurdles, and improve the possibility of successful vaccine design (Naz et al. 2020). In comparison vaccine design using traditional approaches requires huge time and investment along with probability of inducing allergic responses (Khan et al. 2018). In present study, we mainly focused on designing a novel subunit vaccine candidate to put an end to chances of virulence reversal (Ali et al. 2017) and also reinforce cell-mediated, humoral and innate immune responses by using a combination of B-cell and T-cell epitopes (Hajighahramani et al. 2017). However, we suggest that the modeled vaccine can be accepted and validated in a wet lab setting.

Materials and Methods

Selection and Retrieval of Protein Sequences

To identify the proteins that would make up a subunit vaccine, a comprehensive literature search was performed; then, the amino acid sequences of the proteins were obtained in FASTA format from the UniProtKB database (https://www.uniprot.org/) and the NCBI database (https://www.ncbi.nlm.nih.gov/). (Table 1). Following that, total six proteins (UniProt accession ID: Q4VY80, Q5CHW5, A0A0S4TDL2, V9QET8, A0A0S4TJC0 and A0A0S4TIJ0 were chosen based on their toxigenicity, allergenicity, antigenicity and transmembrane helices. Toxigenicity was predicted using the ToxinPred server, while allergenicity was predicted utilizing online tools AllerTOP v.2.0 (Dimitrov et al. 2014). The antigenicity and the transmembrane helices were predicted using the vaxijen web server and TMHMM server v.2.0, respectively (Krogh et al. 2001). Proteins containing more than one transmembrane helix were removed due to a lower likelihood of success in downstream expression experiments.

Predictions of Cytotoxic T Lymphocytes (CTL) Epitopes

The NetCTL 1.2 server was used to detect epitopes of selected proteins because this server relay on artificial neural network (ANN) algorithms and assisted in identifying MHC Class-I supertypes (total of 12: A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, B62) that interact with 9 residues (Joshi et al. 2021), while proteasomal cleavage was predicted by NetChop using the C-terminal end of the CTL epitope and the weight matrix technique to estimate TAP transporter efficiency (Larsen et al. 2007). The default threshold value was 0.75, and three allelic supertypes (A2, A3, and B7) with 88.3% global population coverage were chosen for CTL epitope prediction.

Prediction of Helper T Lymphocytes (HTL) Epitopes

For the selected Cryptosporidium hominis proteins, the Immune Epitopes and Analysis Resource (IEDB) server (http://tools.iedb.org/mhcii/) was applied to predict HTL epitopes contain 15 amino acids using the MHC class II module giving priority on IC50 values and percentile rank (Wang et al. 2010). The default IEDB recommended method was used in this step because it tries to use the best possible method for a given MHC molecule using the combining ANN-align, SMM-align, CombLib, Consensus approach and Sturniolo approach if any corresponding predictor is available for the molecule, otherwise it uses NetMHCIIpan approach (Verma et al. 2018). The IC50 values for peptides with the highest, middle, and lowest affinity for the T-cell receptor are b50 nM, b500 nM, and b 5000 nM.

Table 1

| Serial No | Name                                | UniProt accession ID | Probability of allergenicity | Transmembrane helices | Probability of antigenicity |
|-----------|-------------------------------------|----------------------|----------------------------|-----------------------|-----------------------------|
| 01        | Sporozites glycoprotein antigen      | Q4VY80               | Non-allergen               | 0                     | 0.9347                      |
| 02        | Sporozoite surface Antigen P23      | Q5CHW5               | Non-allergen               | 0                     | 0.7774                      |
| 03        | Proflin                             | A0A0S4TDL2           | Non-allergen               | 0                     | 0.6724                      |
| 04        | 70 kDa heat shock protein           | V9QET8               | Non-allergen               | 0                     | 0.6132                      |
| 05        | Coatomer WDAD domain containing protein | A0A0S4TJC0       | Non-allergen               | 0                     | 0.6089                      |
| 06        | L-lactate dehydrogenase             | A0A0S4TIJ0           | Non-allergen               | 0                     | 0.5992                      |
respectively, and peptides with the lowest percentile rank have high affinity (Dhal et al. 2019). HLA-DRB1*03:01, HLA-DQA1*04:01/DQB1*04:02, HLA-DQA1*04:01/DQB1*04:02, HLA-DRB1*03:01, HLA-DPA1*01:03/ DPB1*04:01 and HLA-DQA1*05:01/DQB1*03:01 alleles were used to identify the HTL epitopes.

Prediction of Interferon-γ (IFN-γ)-Inducing Epitopes

The IFN-epitope server (http://crdd.osdd.net/raghava/ifnepitope/predict.php) was used to predict IFN-inducing epitopes using the SVM technique, which categorizes epitopes as either positive or negative inducers. The server is based on a dataset from the IEDB database (Dhanda et al. 2013) that includes 3705 IFN-inducing and 6728 non-inducing MHC class II binders and the accuracy of the server is estimated at 82.2% (Dhanda et al. 2013). For the prediction of IFN-γ inducing epitopes, a hybrid motif and SVM algorithm were used to identify the best epitopes based on their score and likelihood of positivity (Forouharmehr et al. 2022).

B-cell Epitope Predictions

Antigenic regions found on the surface of any pathogen can activate B cells resulting in the production of antigen-specific antibodies. Therefore, when developing effective vaccines against any pathogen, B cell epitopes must be considered (Chakraborty et al. 2021). In the present study, the ABCpred server (http://crdd.osdd.net/raghava/abcpred/) was used to predict the selected six proteins’ 16 amino acid long linear B-cell epitopes from the specialized B-cell epitope database. Then, the epitopes were chosen based on scores ranging from 1 to 0, with higher scores being selected as epitopes. This server, which includes epitopes from bacteria, viruses and parasites, employs artificial neural networks (ANNs) that have been thoroughly tested on hidden units, clean data sets, and fixed-length patterns with an accuracy of 65.9% basis points (Saha and Raghava 2006a).

Toxicity Prediction of the Selected Epitopes

Constructing the multi-epitope subunit vaccine, all of the selected HTL, CTL, and BCL epitopes were put to test for toxicity using the ToxinPred tool, which aids in the identification of toxic and non-toxic peptides (Gupta et al. 2013).

Construction of Multi-epitope-based Subunit Vaccine

The multi-epitope subunit vaccine candidate was constructed by combining an adjuvant, CTL, HTL and B-cell epitopes with appropriate linkers. Vaccine constructed with amino acid residues have the most flexibility in folding into advantageous conformations owing to linkers. The AAY linker enhances epitope partitioning through attempting to make the C-terminus of CTLs more accessible for molecule-mediated binding, leading to increased epitope presentation. Similarly, GPGPG linkers were used to connect the HTLs in order to improve the HTL immune response (Khalid et al. 2022). The best predicted epitopes based on antigenic score, non-allergenicity, and percentile rank were combined with a molecular adjuvant to construct a multi-epitope vaccine that increases the host’s immunogenicity (Forouharmehr 2021). At the N terminal of the construct, a TLR2 agonist Lipoprotein LprA (P9WK55) with 244 amino acids was used as an adjuvant, with the EAAAK helix to aid the interaction of the vaccine protein with the TLR2 receptor in the experimental system and to increase the immunogenicity of the construct (Kalita et al. 2019).

Antigenicity, Allergenicity and Solubility of the Designed Vaccine Construct

The non-allergic activity of the constructed vaccine was determined using AllerTOP v.2.0 (Dimitrov et al. 2014) and AlgPred v.2.0 (Saha and Raghava 2006b) servers. Afterwards, VaxiJen v2.0 (Hebditch et al. 2017) and Protein-sol server (Doytchinova and Flower 2007) were used to find out the antigenic nature and the solubility of the proposed vaccine candidate.

Prediction of Physicochemical Characteristics and Secondary Structure

The ProtParam server (Gasteiger et al. 2005) was used to functionally define physicochemical variables, including molecular weight, isoelectric pH, aliphatic index, estimated half-life, hydropathicity, GRAVY values and instability index of the V1 vaccine antigen. Furthermore, the PSIPRED v3.3 was used to predict the vaccine protein’s beta-sheet, coil structure, and alpha-helix under the secondary structure (Kosciolek and Jones 2014).

Refinement and Validation of the Tertiary Structure

The vaccine sequence’s tertiary structure was predicted using the Raptor X server and refined by using GalaxyWEB server (http://galaxy.seoklab.org/) (Shin et al. 2014). The best-refined model was then screened using Ramachandran plots analysis (Jaydari et al. 2020); additionally, the ProSA-web server has been used to identify potential errors in the
predicted 3-dimensional (3D) structures (Droppa-Almeida et al. 2018; Naz et al. 2020).

Molecular Docking of Multi-subunit Vaccine Candidate with Immune Receptor

Molecular docking is an in-silico method for predicting and visualizing the preferred orientation of a peptide-based subunit vaccine and receptor molecule complex (Pandey et al. 2017). As a result, the ClusPro 2.0 (Kozakov et al. 2017), a protein–protein docking server was used to dock the vaccine and TLR2 complex, with TLR2 (PDB ID: 2Z80) acting as the receptor and the revised vaccine candidate representing as the ligand molecule. It should be acknowledged that both proteins' PDB files were submitted to the server, and the best docking model was ultimately chosen based on the largest cluster size and lowest energy (Jaydari et al. 2020).

Molecular Dynamics Simulation and Disulfide Bonds Engineering of the Selected Vaccine Candidate

Molecular dynamics (MD) simulation is a popular technique for evaluating protein structural reliability in a simulated environment that seems to be similar to plausible systems (Khalid et al. 2022). Thus, normal modes analysis (NMA) method of iMOMDS server was used to elucidate the overall motion of proteins (López-Blanco et al. 2014). In terms of eigenvalue, covariance, deformability, and B-factors, the server predicted the trajectory and measure of complex dynamics (Awan et al. 2017; Mia et al. 2021). Besides, the Disulfide by Design 2 (DbD2) tool was used to increase protein stability and decrease conformational entropy (Craig and Dombkowski 2013); this calculation is a measurement of uncertainty or randomness by the addition of new disulfide bonds, and it changes the 3D conformations of the modeled vaccine construct (Bhattacharya et al. 2021; Craig and Dombkowski 2013).

Adaptation of Codon, Cloning, and Similarity Analysis

The Java codon adaptation tool (JCat) was used for codon optimization, expression, and reverse translation (Grote et al. 2005). Moreover, the expression of vaccine structure in *Escherichia coli* (strain K12) as a host requires optimization and the three additional options were considered when optimizing rho-independent transcription, restriction cleavage sites, and prokaryotic ribosome binding sites. JCat output in terms of codon adaptation index (CAI) and percent GC content confirmed a high level of protein expression (Khan et al. 2021). *Escherichia coli* strain K12 was used as a cloning host for the selected vaccine construct. Because human and *E. coli* codons are different, JCAT was utilized to apply the codon to *E. coli* in order to boost expression. Rho-independent transcription termination, the binding site of the prokaryotic ribosome, and the restriction sites of the XhoI and BamH1 endonucleases were all neglected during the process (Grote et al. 2005; Jaydari et al. 2020). The N-terminal of the transcribed sequence was then ligated with the XhoI restriction site, whereas the C-terminal was ligated with the BamH1 site. The altered sequence was loaded between the XhoI (158) and BamH1 (198) locations in the pET28a(+) vector using SnapGene (Solanki and Tiwari 2013), a restriction cloning tool.

Result and Discussion

Protein Sequence Retrieval

The sequences of six proteins of Cryptosporidium hominis were chosen from a literature review and their FASTA sequences were obtained. To comprehend their ability to create an immunogenic reaction, the antigenic propensity of the individual proteins was estimated using the Vaxijen server, which was then confirmed with a threshold value greater than 0.5 (Table 1). Result showed that each of the six proteins had an average antigenic propensity indicating their immunogenic nature; Fig. 1 visualized the all steps of vaccine construction at a glance.

CTL Epitope Prediction

The immune system is made up of a variety of cells and chemicals that work together to remove foreign particles while maintaining cellular integrity. Likely, CTLs are CD8+ T cells that identify foreign antigen fragments on MHC-I molecules and kill target cells by releasing perforin, granzymes, and granulysin, or by Fas ligand binding to Fas receptors on target cells providing protection against both intracellular and extracellular infectious agents. Following that, using the NetCTL 1.2 server, MHC-I specific 13 CTL epitopes from the selected protein sequences were retrieved by using three MHC-I supertypes A2, A3 and B7 with the goal of covering 88.3% of the world’s population (Table 2).

HTL and IFN-γ Inducing Epitopes Prediction

The construction of preemptive and immunotherapeutic vaccine requires the prediction of HTL epitopes because they are important members of the adaptive immune system, as T cells release cytokines which govern almost all adaptive immunological responses. Activation of B-cells to release antibodies, macrophage activation for microbial destruction, and CTL activation to kill infected target cells are some of their roles. They also aid in the release
of lymphokines, GM-CSF, and IFN, enhancing both CTL and humoral immune responses. Therefore, the IEDB web server was used to predict MHC-II-specific HTL epitopes, with the alleles HLA-DRB1*03:01, HLA-DQA1*04:01, DQB1*04:02, HLA-DPA1*01:03, and HLA-DQA1*05:01 spanning more than 90% of the world’s population. After that, the HTL epitopes were sorted by lowest percentile rank and IC50 value (Tables 3 and 4). In the case of interferon (IFN) induction, whenever naive HTLs are activated by antigen presenting cells, T cells are differentiated into Th1 or Th2 helper cells, which secrete cytokines. When Th1 cells are differentiated, IFN is released, which is a type II interferon. In both the innate and adaptive immune responses, Th1 cells release IFN and it causes macrophages to become activated, which are important for
managing intracellular infections and the production of IFN was shown to be induced by all of the epitopes tested.

B-cell Epitope Prediction

Epitopes that are similar to the B-cell receptor are not only important in vaccine development and antibody generation (Sanchez-Trincado et al. 2017) but also responsible for the release of humoral or cellular immunity antibodies (Naz et al. 2020). Thus, the ABCpred server was used to anticipate B-cell epitopes, and six epitopes for each of the six proteins with the highest score were chosen for the subunit vaccine design, along with appropriate linkers and an adjuvant (Table 5).

Antigenicity, Allergenicity and Toxicity Prediction of the Selected Epitopes and Designing Multi-epitope Subunit Vaccine Construct

To determine if the selected epitopes were hazardous or non-toxic in nature, the ToxinPred tool was employed. Then, antigenicity, non-allergenicity and non-toxicity properties were predicted for all six HTL epitopes, six B cell epitopes, and thirteen CTL epitopes. Multi-epitope peptide vaccines are a promising way to elicit a specific immune response and also have an antigen-shift flexibility. As a result, the adjuvant, B-cell, CTL, and HTL epitopes were combined to create the multi-epitope subunit vaccine candidate (Table 6). An adjuvant improves the vaccine-mediated immune response by promoting B cell mediated antibody release and long-lasting immunity. In the constructed vaccine Lipoprotein LprA (P9WK55), a TLR-2 agonist was employed. Consequently, each epitope was successfully separated using the EAAAK,
KK, AAY and GPGPG linkers, meanwhile, the adjuvant and B cell epitopes are separated by the EAAAK linker. Likely, a bi-lysine (KK) linker was used between the B-cell epitopes to reserve the independent immunogenic activity. Similarly, The AAY and GPGPG linkers were employed to segregate CTL and HTL epitopes, which improved the subunit vaccine's recognition. There are 631 amino acid residues in the final subunit vaccine, with 1 EAAAK linker, 5 KK linkers, 13 AAY linkers, and 6 GPGPG linkers.

### Antigenicity, Allergenicity and Solubility of the Designed Vaccine Constructs with the Analysis of Secondary Structure and Physicochemical Characteristics

Both AlgPred and AllerTOP v.2.0 proclaimed that the vaccine construct comprising TLR2 agonist Lipoprotein LprA (P9WK55) adjuvant was non-allergic as well as non-allergic, highly soluble and antigenic in nature (Table 6). The half-life in mammalian reticulocytes in vitro was estimated to be 1 h, but it was estimated to be >10 h in Escherichia coli in vivo. Furthermore, the predicted GRAVY value and aliphatic index for the designed vaccine were –0.031 and 75.94, respectively (GRAVY value and aliphatic index are the key factors to determine the protein's hydrophilic behavior and thermostability). In the term of secondary structure prediction, the chosen vaccine candidate had 27.83% alpha-helix, 23.45% sheet and 48.72% coil structure (Fig. 2). Thus, it is concluded that the engineered vaccine was stable and capable of eliciting a significant immune response in the body.

### Tertiary Structure Modeling, Refinement and Validation

The vaccine’s 3D structure was predicted using the Raptor X server and refined using the Galaxy Refine server, with a total of 631 amino acid residues (Fig. 3). The revised model was then exposed to ProSA-web testing to assess its quality and the created model had a z score – 6.46 (Fig. 4). To verify the overall quality of the simulated tertiary structure, a Ramachandran plot was created. According to the 0.1 plot analysis 79.8% of residues are found in favored locations, whereas 17.7% are found in additional allowed regions. The ClusPro server was used to calculate the binding energy of the designed subunit vaccine to the TLR2 immunological receptor and a total of 30 clusters were predicted.
Fig. 2  Secondary structure prediction of designed vaccine V1 using PESIPRED server

Fig. 3  3D modelled structure of vaccine protein (A) and (B) generated via Raptor-X server and Galaxy refine server
each with its own binding energy. Analyzing the result, the final selected model had a −826.0 kcal/mol center and the lowest binding energy (Table 8 and Fig. 6). After that, selected model was used in MD simulation experiments and the internal coordinates of the protein–protein complex are used by the iMODS server for this study. According to the prediction, V1 and TLR 2 are focused on each

**Table 8** Binding energy of predicted vaccine with selected TLR 2 and TLR 4 molecules generated from molecular docking

| Vaccine construct | Receptor | PDB ID | ClusPro 2.0 server | Energy score |
|-------------------|----------|--------|--------------------|--------------|
| V1                | TLR 2    | 2Z80   | Center             | −826.6       |
|                   |          |        | Lowest             | −826.6       |

**Fig. 4** Vaccine 1-TLR 2 complex validations using ProSAweb tool, revealing a Z-score of −6.46

**Fig. 5** Tertiary structure prediction and validation of vaccine protein V1. A Verify3D shows a score of 87.80% for our selected vaccine mode. B Validation of the 3D structure of vaccine protein V1 by Ramachandran plot analysis
other and the presence of chain hinges suggests that the
docked complex may be deformable (Fig. 7A). The NMA
provided the B-factor value (Fig. 7C) and the eigenvalue
of the complex was 2.637191e-08 (Fig. 7B). The bigger
the eigenvalue, the less the variance associated with each
NMA investigation, and vice versa (Hasan et al. 2019).
Then, the coupling of residues was visualized using a
covariance matrix, with red, blue, and white colors denot-
ing clustered, anti-correlated, and uncorrelated motions,
respectively (Fig. 7E). Similarly, an elastic network model
(Fig. 7F) and variance are also used to investigate the pairs
of atoms connected by springs (Fig. 7D).

Fig. 6 Docked complex of vaccine construct V1 with human TLR2; A spacefill format and B structure by ClusPro server

Fig. 7 Molecular dynamics simulation of vaccine protein V1-TLR2 complex. Stability of the protein–protein complex was investigated through deformability (A), eigenvalue (B), B-factor (C), variance (D), covariance (E), and elastic network (F), analysis
Disulfide Bonds Engineering of the Selected Vaccine Candidate

Using the DbD2 server, a total of 55 amino acid pairings with the capacity to form disulfide bonds were discovered. Only three sequences (ASP45-MET48, GLY133-THR262, ASP452-SER 461, PRO554-PHE580, and MET557-SER577) were exposed to form disulfide bonds efficiently after considering the energy, chi3, and B-factor criteria. A chi3 value of 87 to +97 and an energy of 2.5 were used in residue screening. Then, to create a mutant model, all of these residues were substituted with cysteine residues (Fig. 8).

Adaptation of Codon, Cloning, and Similarity Analysis

The Codon Adaptation Index (CAI) was 1.0, indicating that the most common codons were employed in the most cases. The GC level was satisfactory (53.24%) after correction, and the series lacked the Xhol and BamHI restriction sites, making cloning more appealing. The adopted sequence was then inserted between the BgIII and Apal restriction sites in the pET28a(+) vector. The final clone was 7228 bp long, with a 1899 bp embedded segment and the rest being vector (Fig. 9). The red hue is used to draw attention to the extracted area.

Discussion

Recently, several pathogen outbreaks have emerged and become undetectable threats both to animals and human. As a consequence, from last couple of years, Cryptosporidium hominis has long attracted the interest of the research community due to its public health importance (Widmer et al. 2020). Moreover, recent study reported that Cryptosporidium hominis is a leading cause of childhood diarrhea associated with impaired physical and cognitive development (Thivierge et al. 2016). Thus, to deter the severity of the infestation, vaccination is considered as the most fundamental and safest way. Recent advances in immunoinformatics and structural vaccinomics have revolutionized antigen screening and aided in the development of vaccines against a diverse range of infectious agents (Capelli et al. 2018; Soltan et al. 2021). As a result, numerous multi-epitope-based-peptide vaccines construction were reported in the previous studies which showed the ability to focus on the humoral immune response on specific antigenic epitopes, resulting in a more safe and effective immune response (Bourdette et al. 2005; Knutson et al. 2001; Lpez et al. 2001; ul Qamar et al. 2020). However, there has been no report of the development of a vaccine candidate against Cryptosporidium hominis using an immunoinformatic approach (Akhtar et al. 2021).

Fig. 8 Disulfide engineering of vaccine protein V1; A initial form, B mutant form
In this study, reverse vaccinology approach, molecular docking and MD simulation were used to identify and assess antigenic peptide proteins in the core proteome of *Cryptosporidium hominis*. The core proteome was subjected to a subtractive proteomics pipeline to identify antigenic, virulent, non-redundant, non-homologous and non-allergenic vaccine candidates. The number of transmembrane helices is another key criterion for excluding proteins because it is very difficult task to purify proteins with more than one transmembrane helix. Thus, it seems wise to exclude these proteins from the selection process (Meunier et al. 2016). The TMHMM server revealed none of our six proteins (Sporozoites glycoprotein antigen, Sporozoite surface Antigen P23, Profilin, 70 kDa heat shock protein, Coatomer WDAD domain containing protein and L-lactate dehydrogenase) contain a transmembrane domain, implying that they are all extracellular proteins that can be fully contacted by antigen-presenting cells to stimulate T and B cell priming and strong immune responses. The selected proteins must be surface-exposed and able to be recognized by the immune system in order to be considered good candidates (Aslam et al. 2021). Then, the six proteins were tested for allergenicity and toxigenicity; additionally, the VaxiJen server investigated all of the retrieved protein sequences to determine the most potent antigenic protein and ability to confer immunity (Mia et al. 2021). Thus, it is concluded that all of these proteins are meaningful in pathogenesis and thus could be good vaccine candidate. When a long-lasting, significant immune response is desired, both B and T cell epitopes must work together to generate both humoral and cellular immunity. Previous research has found that host HLA haplotypes are major determinants of B-cell and T-cell immune response development (Yang et al. 2021). Thus, the current study focuses primarily on screening peptides from selected *Cryptosporidium hominis* proteins (Table 1) that can interact with MHC Class I and II HLA alleles (representing more than 90% of the global population and elicit adaptive immune response in the human host domain). To accomplish this, the IEDB and NETMHC-II server were applied (Joshi et al. 2022b); the IEDB screened out epitope shows good conservancy in fraction of protein sequences and identity the degree of correspondence among strains, and the NetCTL 1.2 server predicts MHC class I binding and proteasomal cleavages using artificial neural networks (ANNs), and TAP transport efficiency predicted using weight matrix (Atapour et al. 2021; Naveed et al. 2021). Besides, vaccines stimulate B cells to produce antibodies and assist to mediate effector actions by particularly engaging with a pathogen. The humoral immune response plays a clear role in vaccine-mediated protection against infections.

![Fig. 9](image-url) Restriction digestion (A) and in silico cloning (B) of the gene sequence of final vaccine construct V1 into pET28a(+) expression vector. Target sequence was inserted between XhoI and BamHI.

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due to its involvement in memory cell retention, longer life, and defense against reinfection (Amanna and Slifka, 2011; Shtykova et al. 2013).

To design the vaccine HTL, CTL, and B cell epitopes were bonded to GPGPG, AAY and KK linkers, separately (Aslam et al. 2021), and the use of linkers in vaccine development can improve its stability, expression and folding. The EAAAK linker is a stiff linker that has been used in numerous vaccine construction studies, including bacterial, viral, and protozoal diseases, especially when separate epitopes and adjuvants are required in the design (Arai et al. 2001). Similarly, GPGPG, AAY, and KK linkers are typically composed of hydrophilic, flexible amino acids and can prevent domain function and folding disruption. However, to achieve adequate immunogenicity, an adjuvants must be added in the multi-epitope vaccines (Meza et al. 2017). Adjuvants in vaccine formulations can help protect against infection while also improving immune responses to antigens’ stability and duration (Lee and Nguyen 2015). Thus, Lipoprotein LprA (P9WK55) was used as an adjuvant for the designed vaccine.

Furthermore, the Expasy ProtParam web server also determined the vaccine’s physicochemical properties, such as stability, number of amino acids, molecular weight, isoelectric point, aliphatic index, and number of positively and negatively charged amino acids (Jain et al. 2021) and the webservers predicted that the final vaccine construct would be stable, soluble, antigenic, and nonallergic. Then, PSIPRED web server was used to determine the secondary structure of the vaccine, and the secondary structure of the chosen vaccine candidate was validated using the percent of alpha-helix (27.83%), sheet (23.45%), and coil structure (48.72%). As a result, because a higher aliphatic index value is directly proportional to thermal stability, the proposed vaccine was assumed to be thermostable (Azam and Shamim 2014). Besides, Galaxy Refine was used to refine the 3D structure of a chimeric vaccine construct, and desirable properties were obtained. Ramachandran Plot depicts the necessary properties for a potential vaccine candidate and the results confirmed that the majority of the residues (79.8%) are present in favored areas and only a few residues are present in outlier regions, and previous research stated this range of value as the acceptable intended model quality (Naveed et al. 2021).

In addition, TLR-mediated immune activation is vital in vaccine therapy because TLRs activate several signaling cascades and induce type 3 interferon (INF), which is required for macrophage activation and increased expression of MHC-I and MHC-II molecules (Hajishengallis 2009). Therefore, molecular docking analysis was carried out to predict the binding affinity of the vaccine with the human TLRs (Gorai et al. 2022). Previous study has reported the involvement of TLR particularly TLR2 in generating immune response against Cryptosporidium species (Dhal et al. 2019). After that, the stability of the interactions was then determined using molecular dynamics simulation analysis. The findings revealed several factors, which are detailed in Fig. 3. The figure depicts the complex structure’s MNA mobility, deformability values, B-factor, eigenvalues, c-variance, and elasticity. The constructed complex is shown to be stable and has fewer chances of deformation during immune response based on maximum eigenvalue. The immune simulation of the designed construct was revealed using covariance matrix analysis, and its output matched the immune responses. The humoral response was expected to arise as a result of vaccine administration in the body (Chauhan et al. 2021).

Following that, in silico cloning was performed. In-silico cloning was successful, indicating that the vaccine could be mass-produced in Escherichia coli (Akhtar et al. 2021). Furthermore, the primary goal of codon biasness study is to identify amino acid expression patterns that can be linked to epitope-based vaccine forecasting (Joshi et al. 2022a).

However, there are some drawbacks to the computational vaccinology approach, such as the inability to conduct in vivo and intro tests and the rapid change of software versions. As a result, our projected in silico results were predicted using a variety of immunological databases and computational sequence analysis. More wet lab-based research involving model animals is recommended for experimental validation of the anticipated vaccine candidates.

Conclusion

Cryptosporidiosis, caused by Cryptosporidium hominis and Cryptosporidium parvum, is the most common protozoan-induced cause of diarrhea in children, and it has been linked to childhood mortality, malnutrition, cognitive development and growth retardation. Going deeper, emerging Cryptosporidium hominis now contributes significantly to the global burden of infection worldwide. Despite previous studies describing Cryptosporidium parvum treatment and immunization, little progress has been made in developing recombinant subunit vaccines against Cryptosporidium hominis. As a result, effective therapeutics and preventive strategies are urgently needed. Thus, bioinformatics techniques can help predict the efficacy of a vaccination by identifying antigenic epitopes that are active against this emerging pathogen. As a result, our study identifies new and valuable epitope candidates, paving the way for future Cryptosporidium hominis vaccine development; however, we suggest that the modeled vaccine be accepted and validated in a wet lab setting.
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Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors have no conflict interest to declare that are relevant to the content of this article.

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