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An immunoinformatics approach for design and validation of multi-subunit vaccine against *Cryptosporidium parvum*

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ABSTRACT

An immunoinformatics-based approach is explored for potential multi-subunit vaccine candidates against *Cryptosporidium parvum*. We performed protein structure based systematic methodology for the development of a proficient multi-subunit vaccine candidate against *C. parvum* based on their probability of antigenicity, allergenicity and transmembrane helices as the screening criteria. The best-screened epitopes like B-cell epitopes (BCL), Helper T-lymphocytes (HTL) and cytotoxic T- lymphocytes (CTL) were joined by using the appropriate linkers to intensify and develop the presentation and processing of the antigenic molecules. Modeller software was used to generate the best 3D model of the subunit protein. RAMPAGE and other web servers were employed for the validation of the modeled protein. Furthermore, the predicted modeled structure was docked with the two known receptors like TLR2 and TLR4 through ClusPro web server. Based on the docking score, the multi-subunit vaccine docked with TLR2 was subjected to energy minimization by molecular dynamics (MD) simulation to examine their stability within a solvent system. From the simulation study, we found that the residue Glu-107 of subunit vaccine formed a hydrogen bond interaction with Arg-299 of the TLR2 receptor throughout the time frame of the MD simulation. The overall results showed that the multi-subunit vaccine could be an efficient vaccine candidate against *C. parvum*.

1. Introduction

Cryptosporidium a genus belonging to apicomplexan parasites and representing the order Eucoccidiorida, are known to be one of the main cause for diarrhoea in children and also a chief cause of early childhood mortality globally (Certad et al., 2017; Checkley et al., 2015; Kotloff et al., 2013; Ryan and Hijjawi, 2015; Striepen, 2013). Cryptosporidia are also significantly challenging in livestock, causing profuse diarrhoea and substantial economic losses due to fatalities in young lambs and calves in industrialized agriculture (O'Handley and Olson, 2006). The pathway for *Cryptosporidium* infection starts with exposure to oocysts from faeces shed by an infected host (generally either by humans or other animals). Transmission can also occur through contaminated water and to a lesser degree through contaminated food. *Cryptosporidium hominis* and *Cryptosporidium parvum* are the main causes of human clinical infections out of the different species. *C. hominis* causes an anthroponotic infection, while *C. parvum* can cause zoonotic infections. The host ingested oocysts release infectious sporozoites that anchor themselves to the epithelial lining of the intestine. These sporozoites are protected from the host cell cytoplasm through a membrane layer which is highly rich in actin (Ehrenman et al., 2013). After maturation and multiplication through both sexual and asexual cycles they release merozoites as well as oocysts which in turn infect neighbouring intestinal epithelial cells or infect other hosts after exiting. Interestingly, only thin-walled oocysts represent auto-infection risk, however, thick-walled oocysts are shed in the feces.

Previous studies have made it evident that the factors responsible for the *in vitro* proliferation and development of *Cryptosporidium* species includes the host cell type, excystation protocol, stage and size of inoculum, age and strain of the parasite, maturity and culture conditions such as atmosphere, pH, and medium supplements (Hijjawi et al., 2010; Karanis and Aldeyarbi, 2011). Most of *in-vitro* cultivation studies till now used human adenocarcinoma (HCT-8) cells, which support superior development of the parasite in a conventional 5% CO_2 environment in comparison to other cell lines and atmospheres. But, on the downside in conventional 2D cell culture, it suffers from a failure to propagate the parasite for long-term, low yields of oocysts and/or lack of reproducibility (Hijjawi et al., 2010; Karanis and Aldeyarbi, 2011; Morada et al., 2016; Miller et al., 2018).

The increasing *Cryptosporidium*-related fatality of individuals with a

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compromised immune system has raised the criticality of immune response against such disease. As it is with many parasitic disease cases, the development of vaccine against *Cryptosporidium* is still unattainable due to the developmental complexities in its parasitic stages. Intestinal epithelial cells (IECs) are the targets to the freshly excysted (from infective oocysts) sporozoites and thus are critical for *Cryptosporidium* infections. The integrity of these IECs plays a crucial role in naturally preventing the spread of the infection in the host organisms (Laurent and Lacroix-Lamandé, 2017). Supporting the IECs, several other factors such as mucous secretions, cytokines, chemokines andantimicrobial peptides located in intestinal lumen, sub-mucosa and bloodstream are also essential in controlling cryptosporidial infection at an early stage. IECs are well known to express many toll-likereceptors (TLR) such as TLR2, 4, 5, and 9, reported as essentialimmune effectors which efficiently modulate the host immunity against parasitic infections (Fereig et al., 2018).

Vaccination includes inducing protective immunity against the microbial pathogen through exposure to highly immunogenic immunestimulatory antigenic agent-specific components. It serves as an efficient method to decrease morbidity rates of infectious diseases. The execution of vaccination programs countrywide in a comprehensively large-scale scenario, as well as the immense achievement in the purging of diseases like smallpox, measles, polio, and meningitis are amidst well-known milestones of the 20th century (Kieny et al., 2004). So far, there are no known precautionary treatments or therapies accessible for a cryptosporidial diarrhea. Therefore, clinical administration and proper care become crucial to cure this type of disease. Vaccines act as a two way beneficial commodity by providing immunity against pathogens and controls vector-borne diseases as well. It becomes advantageous over drug-based approaches due to associated drug toxicity effects of the latter. Immunoinformatics, a successful *in silico* strategy to design multi-epitope vaccine candidates which are stable in a relatively shorter amount of time is considered cost-effective than traditional methods (Pandey et al., 2018b; Rana and Akhter, 2016; Saadi et al., 2017). In our 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 (Depla et al., 2008; Hajighahramani et al., 2017). The multi-epitope subunit vaccine was evaluated for further evaluation based on its physiochemical parameters, antigenicity and allergenicity (Meza et al., 2017; Nezafat et al., 2014). The major advantage for this type of subunit vaccine is that the low risk of disease transmission by the vaccine as no live parasite components are included. Furthermore, the production of this type of vaccine doesn't require any type of pathogen involvement. Finally, the 3D modeled structured vaccine was docked with TLR2 and TLR4 receptor (Botos et al., 2011; Chen et al., 2005; Testa and Philip, 2012) and the complex structure was validated by MD simulation studies.

2. Materials and method

2.1. Selection of proteins

For the construction of an effective multivalent potential vaccine, a total of 38 reported *C. parvum* protein sequences [Supplementary Table 1] were obtained from the protein database of NCBI (Ifeonu et al., 2016; Lippuner et al., 2018). Out of 38 protein sequences, only seven proteins were chosen for further analysis based on their probability of antigenicity, allergenicity, and prediction of transmembrane helices. The allergenicity prediction of the protein was calculated through online tools AlgPred (Saha and Raghava, 2006) and AllerTOP v.2.0 (Dimitrov et al., 2013), respectively. AlgPred uses all the default parameters combined to predict the allergenicity of the amino acid sequence. AllerTOP server is based on the ACC (Auto Cross-Covariance) transformation of amino acid sequences into uniform equal-length vectors. The antigenicity prediction was done through ANTIGENpro web server. The prediction was generated by SVM classifier which signifies the probability of the peptide sequences carrying characteristics of antigen (Magnan and Baldi, 2014). The number of proteome transmembrane helices were calculated by TMHMM server v.2.0 (Krogh et al., 2001). The proteins with more than one transmembrane helix were discarded due to its lower probability of success in downstream expression experiments.

2.2. Prediction of helper T-lymphocyte (HTL) epitope

The most important factor of cell-mediated immunity is the helper T-cell immune response which assists in pathogen approval with the help of immune cells and cytokines (Ahluwalia et al., 2017; Pandey et al., 2016a). They are also employed to induce humoral immune response (i.e. secretion of GM-CSF (Granulocyte-macrophage colony-stimulating factor) and lymphokines) and CTL (Dhanda et al., 2013). Thus, HTL epitopes are most likely to play a vital role in the development of immunotherapeutic and prophylactic vaccines. The Immuno Epitope Database (IEDB) MHC II epitope prediction tool was employed for speculating the potential HTL epitopes of the seven proteins which were selected on the basis of their screening criteria (Wang et al., 2010, 2008). Based on the percentile rank score, the output epitopes were ranked. The epitopes having lower percentile rank will have higher binding affinity for the HTL receptor.

2.3. Prediction of cytotoxic T-lymphocytes (CTL) epitope

The CTLPred is a technique which was evaluated for the prediction of CTL epitopes. It incorporates machine learning principles, like artificial neural network (ANN) and support vector machine (SVM) (Bhasin and Raghava, 2004). This technique also uses consensus and combined prediction based approach. The default cut-off score keeps the specificity and sensitivity of the prediction method unchanged.

2.4. Prediction of B-cell (BC) epitope

BCPRED online server was used to recognize the conformational and linear B-cell epitopes from the final subunit vaccine candidate. The BCPRED uses a subsequence kernel (EL-Manzalawy et al., 2008) which is a novel method for the prediction of linear B-cell epitopes. The length of epitope was evaluated as 20-mer (20 amino acids long) and the default value for specificity was set at 75% to get the result from BCPred server.

2.5. Multi-epitope based sub-unit vaccine construction

For the construction of a suitable multi-epitope vaccine candidate, it should preferably induce both HTL and CTL immune responses. Therefore, a multi-subunit vaccine candidate has to have both epitopes along with appropriate linkers. The subunit vaccine is playing a major role in activating both adaptive and innate immune response effectively. In the previous studies, Toll-like receptor (TLR) agonists play a major role as a component of peptide-based subunit vaccines and also have a functional selection role in modern immunotherapy (Black et al., 2010). Linkers are supposed to be a necessary part in simulating the vaccine candidate to work as a self-regulating immunogen and also has a higher antibody titer production compared to single immunogen (Pentel and LeSage, 2014). For constructing final subunit vaccine candidate, we used three important linkers such as EAAAK, AAY, and GPGPG in our study. To connect the HTL and CTL epitopes, AAY and GPGPG out of these three linkers were added at the intra-epitope position.

2.6. Physicochemical properties of vaccine protein

The rationale of immunization is to stimulate immunity post injection of the vaccine into the body. For that reason, it is relevant to characterize the physicochemical parameters linked with the multi-subunit vaccine candidate. The web server ProtParam (Gasteiger et al., 2005) was utilized for understanding the physical and chemical properties of our predicted subunit vaccine protein. The parameters including grand average of hydropathy (GRAVY), estimated half-life, aliphatic index, theoretical pI, molecular weight (kDa), etc were predicted using the amino acid residues of the multi-epitope subunit vaccine.

2.7. Predicting tertiary structure

The 3D structure of the predicted subunit vaccine candidate was obtained. Utilizing online servers RaptorX (Källberg et al., 2012; Peng and Xu, 2011), Phyre2 (Kelley et al., 2015), I-Tasser (Zhang, 2008; Roy et al., 2010), Lomets (Wu and Zhang, 2007) structure prediction server and also done through Modeller 9.20 software (Šali et al., 1995; Sánchez and Šali, 2000; Eswar et al., 2006; Webb and sali, 2014). Due to the formation of a 3D structure, the protein molecule achieves proper stabilization by appropriate bending and twisting with the lowest energy state. Stabilization of the protein structure was performed through this type of interaction between the amino acid residue side chains.

2.8. Refinement of tertiary structure and validation

The best structure from the output of Modeller software was then advanced to MD simulation to relax and refine the protein. After that, the validation of the protein structure was performed through RAMPAGE (Lovell et al., 2003), PROCHECK (Laskowski et al., 1993), ERRAT (Colovos and Yeates, 1993), Verify 3D (Eisenberg et al., 1997), and ProSA-web (Wiederstein and Sippl, 2007) online servers. The validation score of the multi-subunit vaccine protein through different online servers is listed in the Supplementary Table 2.

2.9. Codon adaptation and subsequent in silico cloning of subunit vaccine protein

The higher expression rates may result from adapting the codon usage to *E. coli* K12 which is the most sequenced prokaryotic organism through a unique codon adaptation algorithm. The technique was used to get the expression of the primary sequence of subunit vaccine protein in a higher rate which was then submitted to the JCAT (JAVA Codon Adaptation Tool) (Grote et al., 2005) in case of host organism *E. coli K12*.The parameters such as prokaryote ribosome binding site, rho independent transcription termination, and cleavage site of restriction enzymes were avoided when we set the options section. After that, the optimized codon sequences of the subunit vaccine construct was evaluated to check for commercially available restriction enzyme sites. Upon observation, the construct vaccine lacked important restriction sites such as *Xho*I and *Bam*HI. Taking this into consideration, we added these two restriction sites at the N-terminal and C-terminal ends respectively to perform restriction cloning. The adapted nucleotide sequences of the designed multi-subunit vaccine was further cloned into the *E. coli* pET-28a(+) vector between the *Xho*I (158) and *Bam*HI (198) sites by taking restriction cloning module of SnapGene tool (GSL Biotech, available at snapgene.com).

2.10. Molecular dynamics (MD) simulation

The modeled subunit vaccine protein was evaluated for the simulation using GROMACS 2018.3 (Berendsen et al., 1995). The force field 'AMBER99SB' (Hornak et al., 2006) was used for subunit vaccine protein energy minimization. With a defined grid cell of dimensions 20 nm × 20 nm × 20 nm, the multi-subunit vaccine protein centered cubic box was solvated with a total of 38,351 solvent molecules and Na^+ and Cl^- ions for neutralizing the charged protein. Particle–Mesh–Ewald (PME) (Darden et al., 1993) was applied to calculate the long-range electrostatics. The steepest descent integrator was evaluated for the energy minimization process and lowest potential energy of the system was also being calculated. Furthermore, the isothermal and isobaric equilibration was achieved by modified Berendsen thermostat temperature coupling for 200 ps at 299.85 K and Parrinello-Rahman barostat for 300 ps at 0.99 bars respectively. For the integration of Newton's equation in MD simulation, the leap-frog algorithm was performed and the entire bond lengths were limited by the LINCS (Linear constraint solver) algorithm (Hess et al., 1997) for constraining bond lengths. Using Verlet cut-off scheme, with a cut-off of 1.4 nm, Lennard-Jones potential was applied for van der Waals interaction. Finally, a 20 ns production simulation study was performed for the structural refinement of the protein. The GROMACS analysis plots were generated by the Xmgrace software (Vaught, 1996).

2.11. Molecular docking of multi-subunit vaccine candidate with immune receptor

Molecular docking is an in-silico approach, mainly used to predict the stable complex form of the subunit vaccine protein with the receptor molecule in its favored orientation (Pandey et al., 2017). TLR-2 (PDB ID: 2Z80) (Jin et al., 2007) and TLR-4 (PDB ID: 2Z62) (Kim et al., 2007) were selected as receptor and subunit vaccine which was obtained from modeller while the refined model of subunit vaccine candidate was evaluated as a ligand molecule. The docking between protein-protein or receptor-ligand complex was carried out through protein-protein docking server i.e. ClusPro 2.0. This web server was used for checking the binding affinity among proteins (Kozakov et al., 2017).

2.12. Protein-receptor complex MD simulation

MD simulation is an extensively recognized *in-silico* method to determine utmost stability of the receptor-ligand complex (Pandey et al., 2016b). The docked receptor-ligand complex was used as an input to perform the molecular dynamics simulation using GROMACS 2018.3 (Berendsen et al., 1995). The protein topology file was generated using pdb2gmx tool and AMBER99SB force field (Hornak et al., 2006). After that, the protein-receptor complex was defined with a grid cell of dimensions 37 nm × 36 nm × 36 nm, centered in a cubic boundary box, and was solvated with a total number of 48,864 solvent molecules. The SPC (simple point charge) 216 models was used to fill the box by water molecules. The charge of the protein-receptor complex molecule was neutralized by the adding of the sodium and chloride ions. Furthermore, the isothermal and isobaric equilibration was achieved by modified Berendsen thermostat temperature coupling for 200 ps at 300 K and Parrinello-Rahman barostat for 300 ps at 1 bar, respectively. The energy minimization of the complex molecule was performed with a time interval of 100 ps for equilibration. Then the MD simulation of protein-receptor complex molecule was run for a time interval of 100 ns. The analysis of trajectories from different time intervals was done through GROMACS and was visualized using PyMOL (Schrodinger, 2016) and also through LigPlot (Wallace et al., 1995). The RMSD and Rg were determined by the usage of gmx rms and gmx gyrate respectively. Xmgrace utility from Grace Software (Vaught, 1996) was evaluated for GROMACS analysis plot generation.

3. Results and discussion

The work flow chart model is shown in Fig. 1. Out of a total of 38 protein sequences, only seven proteins were chosen for the construction of the multi-subunit vaccine candidate based on their cut-off score for

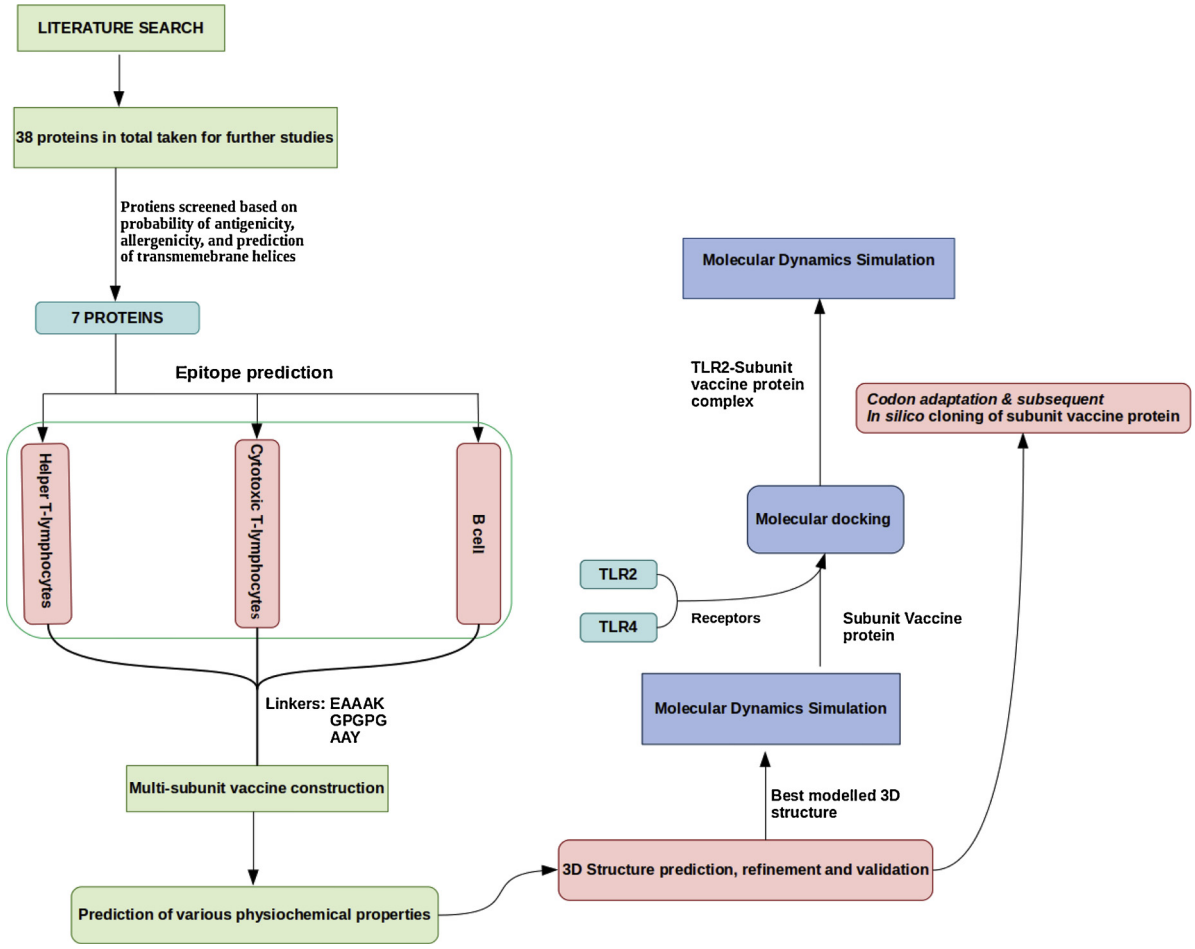

Fig. 1. Illustation of immunoinformatics work flow.

Table 1
Prediction of final selected proteins of *C. parvum* on the basis of their probability of allergenicity, antigenicity and transmembrane helices. (Cut-off score for Probability of Antigenicity = 0.86, Probability of Allergenicity = −0.10, transmembrane helices ≤ 1).

Serial No.	Name	ID	Probability of Allergenicity	Transmembre helices	Probability of Antigenicity
1	Predicted secreted protein, signal peptide	cgd1_660	−0.2867325	0	0.887414
2	Signal peptide, large secreted protein	cgd7_4500	−1.0586971	0	0.891928
3	Hypothetical secreted protein	cgd7_4680	−1.8145323	1	0.939184
4	Hypothetical protein, signal peptide, predicted secreted protein	cgd8_1800	−1.089302	1	0.905416
5	Hypothetical protein	cgd7_1240	0.060327671	1	0.881925
6	Hypothetical protein	cgd6_710	−0.35610528	1	0.947245
7	Hypothetical protein	cgd5_650	−0.12275991	0	0.873861

antigenicity and allergenicity, and prediction of transmembrane helices. On the basis of these criteria the seven proteins were evaluated for further analysis in our study (Table 1). The final multi-subunit vaccine construct consisted of 250 amino acid residues and these residues were connected to each other with suitable linkers (GPGPG, AYY, and EAAAK). The pictorial representation of the multi-epitope subunit vaccine candidate is depicted in Fig. 2. The vaccine candidate consists of different epitopes like HTL epitopes, CTL epitopes and B-cell epitopes along with proper linkers. Three linkers such as EAAAK, AAY, and GPGPG were evaluated for the construction of the final subunit vaccine (Pandey et al., 2018a). Out of which the GPGPG and AAY linkers were added to the intra-epitope site for linking the CTL epitopes and HTL epitopes, respectively. CTLs are one of the several CD8 + T-cell responses targeting host cells harboring infections (Jordan and Hunter, 2010). During the time of the infection, they encounter the MHC-I antigen which is specific to their receptor and also entered the cell cycle to involve in the mitotic process (Moseman and McGavern, 2013). At this juncture, we mainly tried to predict the CTL receptor epitopes using the CTLPred server and 9mer length of CTL epitopes was obtained from the input of seven protein sequences based on the elegant machine learning approaches such as ANN and SVM. Based on the criterion of immunogenicity a total of 7 epitopes of CTL were chosen to design a potential multi-subunit vaccine (Table 2).

HTL also has an important role to play in both humoral and cell-mediated immune responses (Pross and Lefkowitz, 2007). Thus these epitopes are of utmost importance for the development of a potential immunotherapeutic and prophylactic vaccine (Nezafat et al., 2016). The best seven protein sequences were evaluated for the prediction of the MHC-II epitope from the IEDB web server. These seven epitopes (15mer), based on its lower ranking and IC50 valuation, were selected for the development of a potential multi-subunit vaccine (Ali et al., 2017).

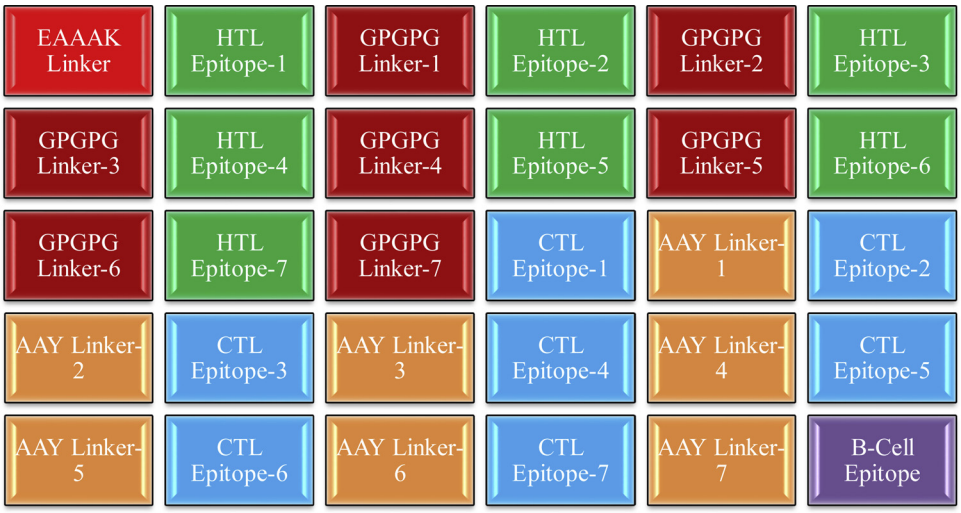

Fig. 2. Pictorial representation of total amino acid residues of the subunit vaccine candidate. Red colour represents EAAAK linker, green colour represents HTL epitopes, Brown colour represents GPGPG linkers, Cyan colour represents CTL epitopes, Orange colour represents AAY linkers and Violet colour represents B-cell epitope. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Prediction of final selected proteins of *C. parvum* on the basis of their HTL epitopes, CTL epitopes, BC epitopes and disorder prediction. **(**Bold font signifies the epitopes which were partially disordered or fully disordered).

Serial No.	Name	Epitopes	Disorder Prediction	Selected/Non-selected
1	Predicted secreted protein, signal peptide	LYIPVIFVLLSTLIF (HTL)	Non disordered	Selected
		AILFLFELI (CTL)	Non disordered	Selected
		TKPT**SSSSTTTTSEPIEIMN** (BC)	Partially disordered	Non- Selected
2	Signal peptide, large secreted protein	LTQNYAALSSTLNFS (HTL)	Non disordered	Selected
		TLNLGLPQL (CTL)	Non disordered	Selected
		QSPPPPPPPPPPPPPPPTYP (BC)	Fully disordered	Non- Selected
		SQPGPH**QPPTKPTPPYPLPQ** (BC)	Partially disordered	Non- Selected
		TIPQPIPQPIPQPIPQPISQ (BC)	Partially disordered	Non- Selected
		PTPSSPTPMPMPMPMPTPTP (BC)	Fully disordered	Non- Selected
		VFIIPTNTQGGNSEGSQIVK (BC)	Non disordered	Non- Selected
3	Hypothetical secreted protein	PPQMVKQMRSQLSRK (HTL)	Non disordered	Selected
		DEADIARYF (CTL)	Non disordered	Selected
		QPSITQQQGKQFTWDEESQS (BC)	Non disordered	Selected
4	Hypothetical protein, signal peptide, predicted secreted protein	NHEWYLLAMGQFTQL (HTL)	Non disordered	Selected
		DLDTCKATL (CTL)	Non disordered	Selected
		LNKSIAPGEDTPIPSSVESS (BC)	Non disordered	Non-Selected
5	Hypothetical protein	GISHIYLLLVIILLV(HTL)	Non-disordered	Selected
		RILPPYWYM(CTL)	Non-disordered	Selected
		FPKGDKDTNNNNSVYVPRDE(BC)	Non-disordered	Non-Selected
6	Hypothetical protein	EVVFGYNMVKGKFVV(HTL)	Non-disordered	Selected
		QPDGGLILL(CTL)	Non-disordered	Selected
		STSTTSTSSTSTSTTTTTTT(BC)	Fully disordered	Non-Selected
		SKSFNIPGIPIFGGPRVENR(BC)	Non-disordered	Non-Selected
7	Hypothetical protein	**LKYR**TQP**PEML**SNEN(HTL)	Partially disordered	Selected
		ARQILVFEL(CTL)	Non-disordered	Selected
		FFGANIPDPPPKVPREKCSY(BC)	Non-disordered	Non-Selected
		ESETPTVVSSSETQLELDQS(BC)	Non-disordered	Non-Selected

B-lymphocytes are the kind of white blood cells, which produce antibodies, having a key role in the humoral immune response of the body. The discovery of B-cell receptors is an integral part of vaccine designing (Chan et al., 2014). For that reason, a B-cell epitope prediction server (BCPREDS) was the method of choice. A total number of 13 B-cell epitopes were screened with 20mer length, evaluated for the input sequence for the design of subunit vaccine. From these only 1 epitope was chosen and finalized for the construction of multi-subunit vaccine candidate because of their highest score. The selection of only highest scoring epitopes (B-cell) for the designing of a potential multi-subunit vaccine is done with the expectation of efficient cell-mediated and humoral immune response (Khatoon et al., 2017). For the improvement of a successful standard multi-subunit vaccine, both of the lymphocyte subsets are evaluated into consideration to induce an effective immunological memory which can respond quickly and effectively (Johnston et al., 2010). In general, the multi-subunit vaccine proteins are inadequately immunogenic while administered alone and have need of co-administration with the adjuvant or carrier proteins for the advancement of the immunity or immune-related response. Due to the activation of TLRs, the innate immune and adaptive immunity responses and inflammatory cytokine secretion (IL-1, IL-6, and IL-12) are promoted. They also play a role in generating co-stimulatory signals in Antigen Presenting Cells (APCs) and up-regulation of MHC molecules (Arish et al., 2015; Rana et al., 2015a,b,c).There are reports which discuss about the *in silico* strategy to generate the multivalent vaccine protein that demonstrate efficacy against protozoan parasite like *Plasmodium falciparum* (Pandey et al., 2018a) and *Toxoplasma gondii* (Dodangeh et al., 2019). These reports validate our in silico approach for design and validation of a multi-subunit vaccine candidate against *C. parvum* and paves path for further experimental follow-up in future.

The allergic reaction is a type over-reaction which was previously encountered by our immune system and harmless material that outcome in sneezing, inflammation of the mucous membrane, and skin rash. AllerTOP and AlgPred online server were mainly evaluated for the

prediction of allergenicity and observed that the vaccine proteins are safe for human use and also non-allergic in nature. A vaccine which is given to human host must be potent to generate an efficient humoral immunity that eventually should trigger memory cells formation against pathogenic epitopes leads to the memory cell formation against the target epitopes and also immunogenic in nature. The antigenicity of the screened proteins was performed by using ANTIGENpro web server and observed that it has the probability cut-off score of antigenicity is 0.86. It shows the antigenic nature of the multi-subunit vaccine construct (Ali et al., 2017).

The physico-chemical properties of multi epitope subunit vaccine candidate were performed by using ProtParam web server with the evaluation of different variables. The subunit vaccine of 27,098.31 Da molecular weight favors the antigenic nature of the vaccine candidate. Further, the theoretical pI of the multi-subunit vaccine protein was observed to be 8.51 which showed slightly alkaline in nature and the residues totaled to 18 positively charged and 16 negatively charged. The predicted half-life of the subunit vaccine candidate was approximately 10 and 20 h in *E. coli* and yeast, *in vivo*; while in case of mammalian reticulocytes; it was 30 h, *in vitro*. Assuming reduction in cysteine residues, other factors such as instability index and the extinction coefficient (measured in water) were found to be 36.94 and 38,850 M^{-1} cm^{-1}, at 280 nm respectively. The instability index score actually represents the stability of the as-constructed subunit vaccine. The value of the grand average of hydropathicity (GRAVY) and the aliphatic index was 0.091 and 85.56, respectively. The aliphatic index's approximate value represents the thermal stability of the constructed vaccine. This may be due to the linear relationship between the aliphatic index and thermos-stability. GRAVY's negative value represents the hydrophilicity of the subunit vaccine. Lastly, the designed multi subunit vaccine candidate is immunogenic, hydrophilic and thermo-stable in nature. From previous studies, we found that the three known potential vaccine candidates like Cp15, Profilin and *Cryptosporidium* apyrase (CApy) were used to induce specific and efficient immune responses (humoral and cellular), signifying their potential vaccine source against *Cryptosporidium* (Manque et al., 2011). The physicochemical properties of the above three candidates with our multi-subunit vaccine candidate are listed in Table 3. Molecular weight, pI, instability index, and other factors play a very important role in the design of a multi-subunit vaccine candidate. The subunit vaccine protein sequence was reverse translated and codon optimized to an improved DNA sequence for codon usage in *E. coli* K12 strain (host). The parameters such as cleavage sites of restriction enzymes, prokaryotic ribosome binding sites, and the rho-independent transcription terminators were avoided when we performed the JCAT server for codon adaptation. CAI (Codon Adaptation Index) (Sharp and Li, 1987) values of the obtained protein sequence were found to be 1; which is perfect for expression in the desired host (*E. coli* K12 strains). The GC-content of *E. coli* (strain K12) was found to be 50.73 and GC-content of the subunit vaccine protein sequence was 56.27, which shows that the vaccine protein sequence will be highly expressed in the host organism. After that, the adapted codon sequences along with *Xho*I and *Bam*HI restriction sites were inserted into the pET-28a (+) vector and a clone of 6085 base pairs was created. The resulting cloned vector carrying the subunit vaccine insert is shown in Fig. 3.

The tertiary structure of the design multi-subunit vaccine was predicted by using the online servers like I-TASSER (Supplementary Fig. 1), Phyre2 (Supplementary Fig. 2), RaptorX (Supplementary Fig. 3), Lomets (Supplementary Fig. 4) and also through Modeller 9.20 software. Each model was evaluated through a Ramachandran plot analysis. The best 3D model has obtained through Modeller 9.20 software on the basis of their Ramachandran-plot analysis. The predicted 3D structure used the best template (PDB ID: 4FYE) i.e. crystal structure of *Legionella* phosphoinositide phosphatase, with a resolution of 2.413 Å for homology modeling. A total of 250 residues were modeled. The pictorial depiction of the best-modeled structure with proper linkers is shown in Fig. 4. When we compared the Ramachandran plot of the modeled structure before refinement and after refinement, we found that initially 89.2% of residues were in the most-favored region, 9.8% additional allowed region, 0.5% in generously allowed region and 0.5% in disallowed regions (Supplementary Fig. 5). But, after protein structure refinement through MD simulation, the number of amino acid residues in the most-favored region is 82%, 18% residues in the additional allowed area or in the part of the favored region and no residue were found in the disallowed area (Supplementary Fig. 6). The validation score of the multi-subunit vaccine before and after simulation through different online servers (ERRAT, Verify 3D, and ProSA-web) is listed in the Supplementary Table 2. From ERRAT server and ProSA analysis we found that the overall quality factor and Z-score of the final multi-subunit vaccine protein were 78.50 and -1.88, respectively. The RMSDs and RMSFs were used to evaluate the most flexible parts of the target vaccine protein. The RMSD plot [Fig. 5 (A)] reported that the system is at equilibrium and almost stable up to 20 ns run. The RMSF plot [Fig. 5 (B)] showed maximum fluctuation in the range of residues between 30–35, which can be attributed to the structural dynamics of the protein. The radius of gyration (Rg) of the target vaccine protein was fairly stable throughout the 20 ns simulation and seemed converged at the end [Fig. 5 (C)]. The stability of the complex during the simulation run is indicated by its potential energy plot [Fig. 5 (D)]. The pictorial depiction of the super-imposed structure of multi-subunit protein before simulation and after simulation is shown in Fig. 6. Afterwards, the refined multi-subunit vaccine protein was considered for docking studies using ClusPro2.0 online server. The molecular docking of the protein with both TLR2 receptor and TLR4 receptor were performed and a total of 60 models were generated [Supplementary Table 3 & 4]. Out of these models, the best-docked complex was chosen based on their lowest energy score and proper occupancy of the receptor. The TLR2 complex reported the highest probable binding affinity with the model having a lowest energy score of -966.9 kcal/mol. The best-docked complex of the model with TLR2 receptor is shown in the Fig. 7. Furthermore, the best complex was considered for Molecular Dynamics (MD) study for suitable timeframes. The simulations study used for the determination of the atoms interaction in the system under the Newtonian motion of equation where the forces between the particles and potential energies were evaluated using molecular force field at a specific time period. For the identification of the potential flexible part in the refined subunit vaccine model, MD simulations were performed for 100 ns time-frames. During the MD simulations period, the RMSD plot reported that the maximum RMSD value of approximately 0.65 nm between 20 ns–25 ns range [Fig. 8 (A)]. The RMSF plot represents a slight fluctuation in the positioning of the amino acid side chains; this reflects the continual interaction between the multi-subunit

Table 3
Comparison of physicochemical properties of various vaccine canidates with modeled protein.

Protein Protein name	Molecular weight	Theoretical pI	Estimated Aliphatic index	Grand average of hydropathicity (GRAVY)	Instability index	Extinction coefficient value
Modeled protein	27098.31Da	8.51	85.56	−0.091	36.94	38,850
Cp15	13258.84Da	10.33	88.40	−0.178	40.91	6085
Profilin	17606.48Da	4.34	77.59	−0.311	29.86	29825
CApy	39368.01Da	5.15	87.57	−0.260	28.27	57995

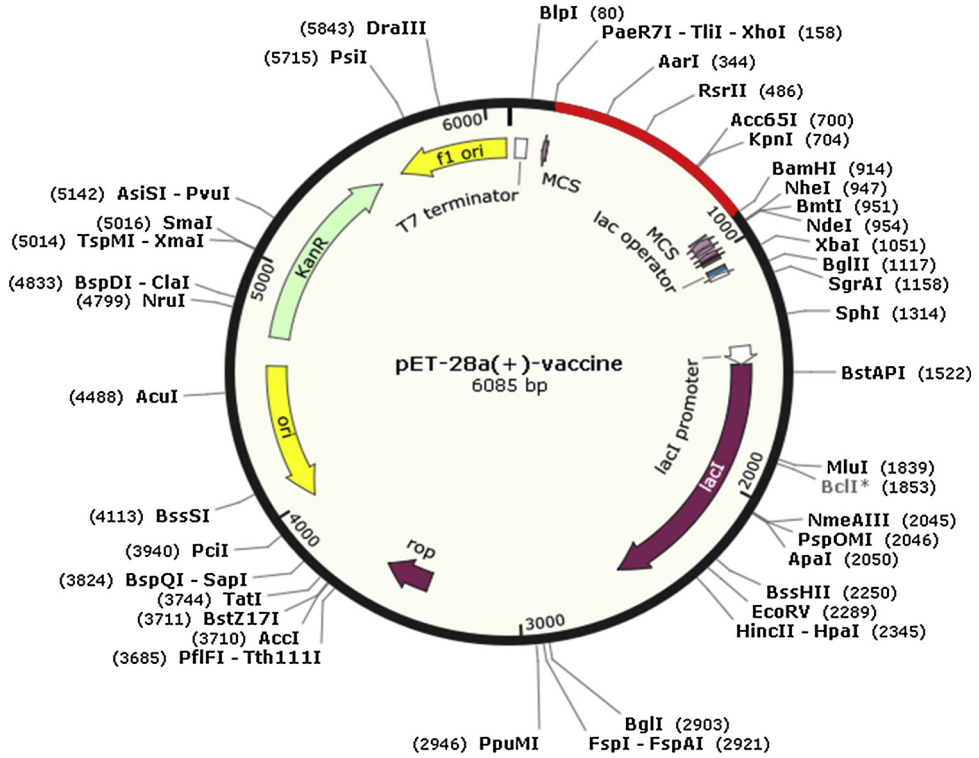

Fig. 3. *In silico* cloning of optimized codons encoding subunit vaccine protein into the pET-28a(+) vector showing the region of choice in red in color and the vector has show in black color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

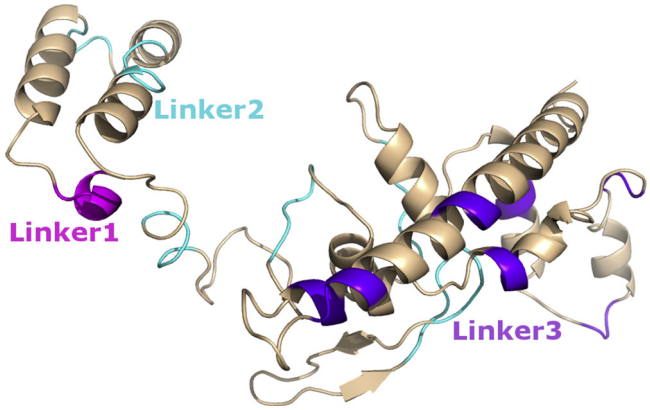

Fig. 4. Cartoon representation of subunit vaccine candidate modeled through modeller. Magenta colour represents EAAAK linker, Cyan colour represents GPGPG linkers, and purple blue colour represents AAY linkers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vaccine and receptor, whereas regions with heavy fluctuations represent highly flexible regions in the protein-receptor complex [Supplementary Fig. 7]. The compactness of protein and receptor complex was evaluated through the radius of gyration (Rg). The Rg plot of the complex was observed to be stable during the simulation timeframes and converged by the end of the 100 ns simulation [Fig. 8(B)]. From MD simulation study, we observed that the residue Glu-107 of subunit vaccine formed a hydrogen bond interaction with Arg-299 throughout the MD simulation in the time-frame of 0 ns, 25 ns, 50 ns, 75 ns and 100 ns [Supplementary Fig. 8 (A–E)]. Similarly, another residue Tyr158 also has interaction with the Glu215 of the TLR2 receptor in the time frame of 25 ns, 50 ns and 75 ns throughout the MD simulations (Table 4). Thus, the MD simulation study confirms as a piece of imperative evidence to the binding stability of the protein-receptor complex structure. Previous studies have shown that normal human cholangiocytes indicate all known TLRs. *C. parvum* infection of cultured cholangiocytes influence the selective induction of TLR2 and TLR4 to the infection sites. Activation of several downstream effectors of TLRs including NF-kB, IL-1R-associated kinase, and p-38, was observed in infected cells. These findings indicate that cholangiocytes show a variety of TLRs, and demonstrate that TLR2 and TLR4 interpose cholangiocytes defence responses to *C. parvum* via activation of NF-kB (Chen et al., 2013). So, in this study we have mainly focused on the two important receptors TLR2 and TLR4 and docked with the refined multi-subunit vaccine protein. Finally, we have designed a multi-subunit vaccine capable of interlinking a cellular and humoral immune response against cryptosporidial infection. The combined immunoinformatics approach and structure-based technique make possible a rational selection of the potential multi-subunit peptide vaccine candidates. The success rate of the *in-silico* studies may be relatively higher than its experimental counterparts (Pandey et al., 2018c; Rana and Akhter, 2016). Moreover, it has an added advantage of significantly reducing the sample size of potential vaccine candidates for experimental studies.

4. Conclusion

Vaccination has established itself as an effective medical intervention having a fairly large impact on worldwide public health. Whole cell vaccines including inactivated, attenuated as well as live are part of the modern day conventional vaccine systems. Compared to these whole cell vaccines the unconventional multi epitope based research approach in vaccine development appears most promising. This *in-silico* study was specially designed to forward the development course of subunit vaccines. Here, the previously established data of protein sequence of *C. parvum* was utilized to develop a design of the potential subunit vaccine. The resulting subunit vaccine construct was found to have epitopes (i.e. CTL, HTL and BCL epitopes) of different lengths with added stability and binding affinity to the TLR2 receptor as compared to TLR4.

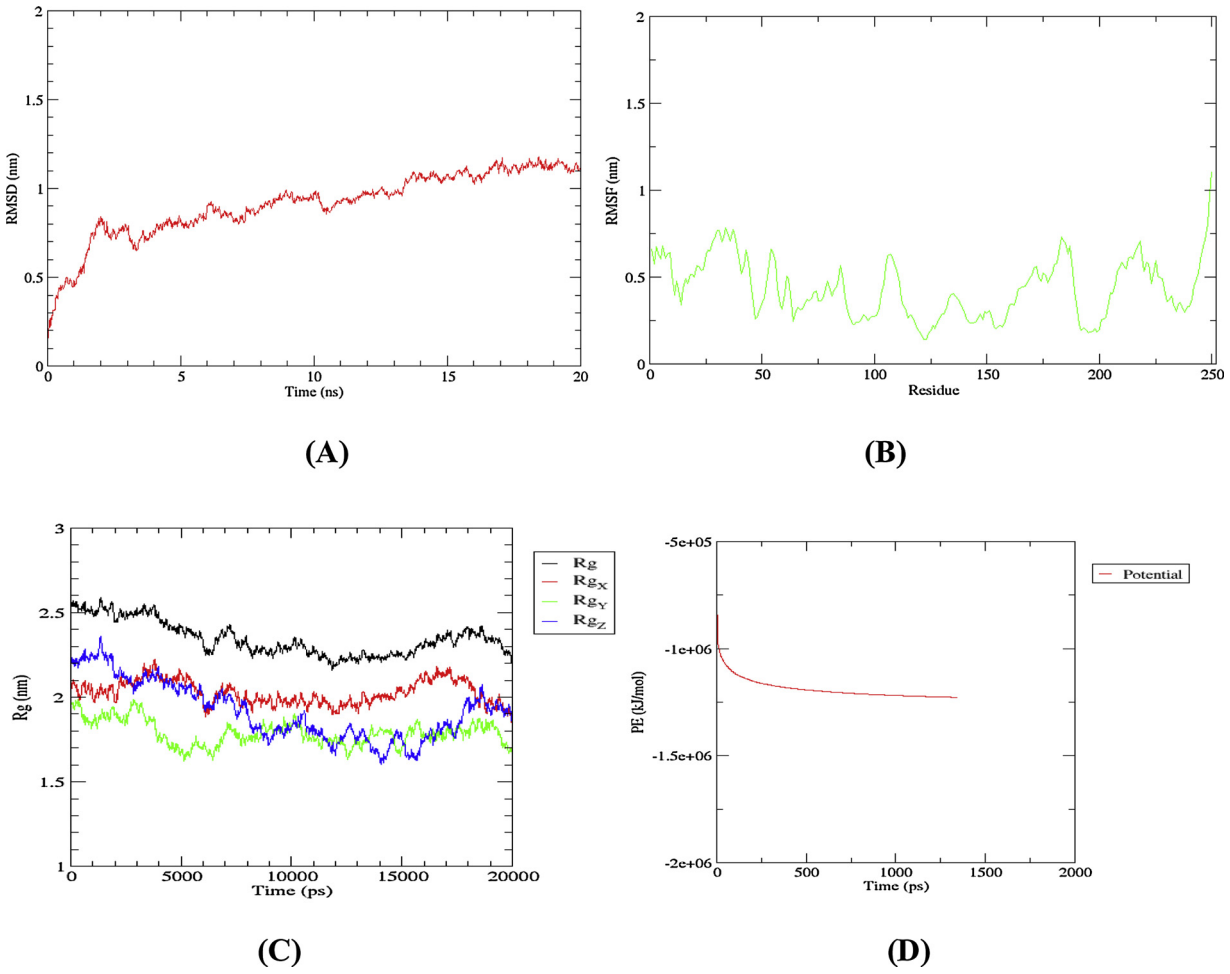

Fig. 5. (A) RMSD of multi-epitope protein is shown in red colour at 20 ns. (B) RMSF of protein residues shown in green colour at 20 ns. (C) Radius of gyration of protein at 20 ns. (D) Potential energy (PE) of system at 20 ns in red colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

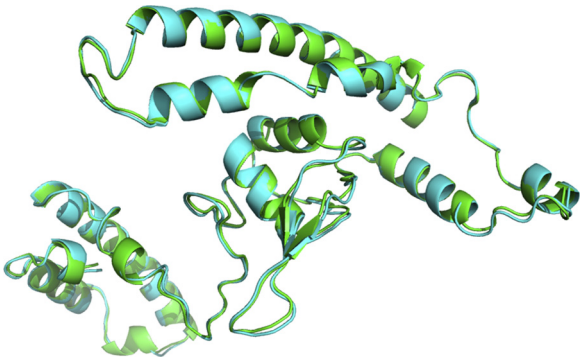

Fig. 6. Cartoon representation of the subunit vaccine protein before simulation (green in colour) and after simulation (cyan in colour). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Molecular docking was performed to study the stable interactions between the multi-subunit vaccines with TLR2 receptor. The obtained vaccine-protein complex was put through MD simulation to further refine the stability of the potential novel vaccine candidate. Finally, a novel immunoinformatics based approach was successfully applied in discovering a potential vaccine candidate against the parasite.

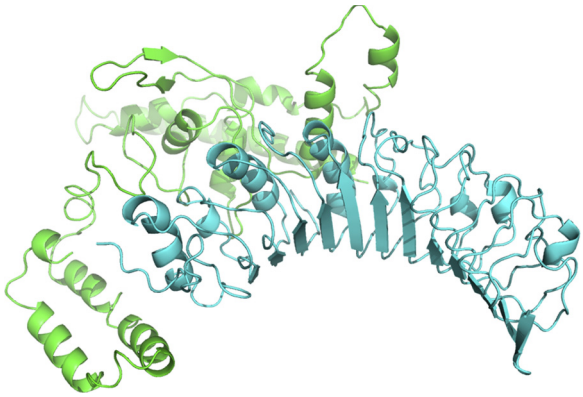

Fig. 7. Cartoon representation of the docked complex of subunit vaccine protein (green in colour) and TRL2 receptor (cyan in colour). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

None declared.

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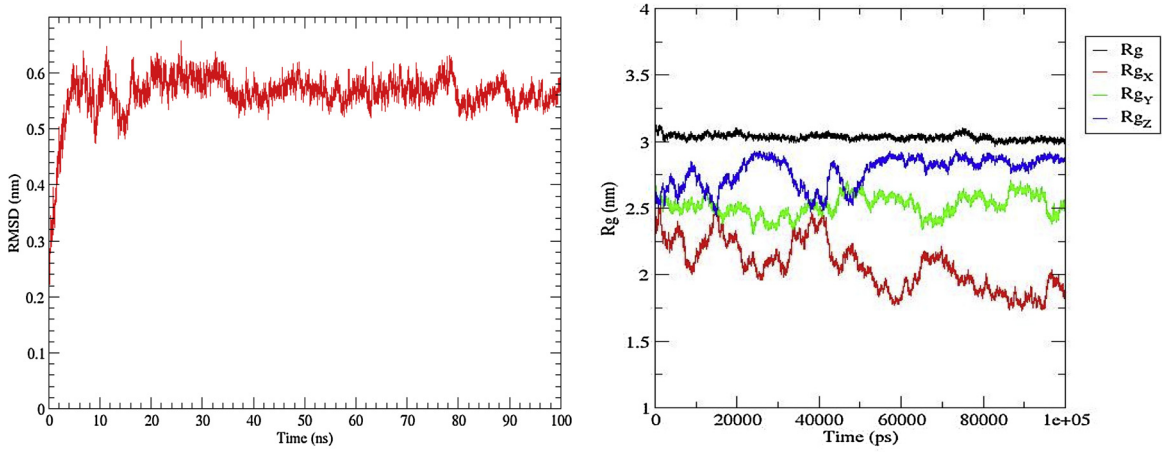

Fig. 8. (A) RMSD of multi-subunit protein with TLR-2 complex is shown in red colour at 100 ns. (B) Radius of gyration (Rg) of protein-protein complex at 100 ns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Hydrogen bond interaction between the multi subunit vaccines with TLR-2 receptor complex in different timeframe of the MD simulations. (Bold font signifies the common H-bond interaction between the multi-subunit vaccine with TLR2 receptor before simulation and after simulation).

erial No.	Timeframe of MD simulations	H-bond interaction between complex structure (subunit vaccine-TLR2)
1	0ns	**Glu107/Pro85-Arg299**, Val 108-Ser302, Gly106-Aln274, Gly102-Lys 271, Leu149-Thr247, Tyr158-Tyr189
2	25ns	Tyr158-Glu215, **Glu107-Arg299**, Arg177-Glu190, Tyr31-Ser331, Glu172-Arg167
3	50ns	Asn30-Arg348/Asn328, **Glu107-Arg299**, Ser248-Lys195, Glu172-Arg167, Ser250-Lys192, Tyr158-Glu215
4	75ns	**Glu107-Arg299**, Tyr-71-Arg319, Tyr158-Glu215, Ser248-Lys195, Glu172-Arg167
5	100ns	**Gly107-Arg299**, Glu172-Arg167, Arg177-Glu215, Tyr71-Val292, Ser248-Lys195, Leu38-Lys329, Thr28-Asn328

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.09.001.

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