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Exploring the Potentials of Campylobacter spp. Chaperone Proteins in the Design of B and T Cell Multi-Epitope Subunit Vaccine: An Immunoinformatics approach

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ABSTRACT

*Campylobacter has been reported to be the cause of community-acquired infection and is often presented in immunocompromised individuals as acute gastrointestinal illness. It is also often transmitted through undercooked poultry products. Campylobacter also causes significant morbidity, leading to huge cases of disability but limited mortality (there can be about 75,000 annual fatal cases). Heat shock proteins are defined as a group of highly conserved and regulated proteins which are known to play vital roles in enabling diverse organisms cope with physiological stress. Thus, this study used an immunoinformatic approach to design a multi-epitope based vaccine that is targeted against Campylobacter spp. and related infections using Heat shock protein sequences. Designed subunit vaccine was evaluated for its antigenicity, immunogenicity, allergenicity and physicochemical parameters. A total of 7200 CTL epitopes (9-mer) were predicted using NetCTL 1.2 server, among them only 97 epitopes with highranked binding affinity score were chosen as final CTL epitopes. Similarly, the HTL epitopes were identified using IEDB MHC-II prediction module based on the higher binding affinity with MHC- II, the mouse alleles used for the prediction were HLA-DRB1*01:01, HLA-DRB1*01:02, HLA-DRB1* 01:03, HLA-DRB1*01:04. A maximum immune response TLR-5 agonist (Accession number: P04949) was used as an adjuvant and CTL epitopes were combined together by EAAAK linker, intra-CTL and intra-HTL epitopes joint by AAY and GPGPG linker to make a final vaccine construct of 1723 amino acid residues designed using 97 CTL and 3 HTL epitopes Collectively, this research provides novel candidates for epitope-based peptide vaccine design against Campylobacter spp.*

Keywords: *Campylobacter* spp., Chaperone proteins, Vaccine, Immunoinformatics.

INTRODUCTION

Campylobacter has been reported to be the vital cause of gastrointestinal bacterial pathogen in human beings [1]. *C. jejuni* is responsible for approximately 90% of infective cases while *C. coli* is responsible for the remaining 10%. Other species responsible for human campylobacteriosis are however, more rarely involved. Campylobacteriosis is generally a self-limited disease inducing symptoms such as abdominal pain, diarrhea and fever, but it can also lead to gastrointestinal manifestations such as bacteremia and neurological disorders [2]. Human infections by Campylobacter are majorly caused by handling and/or the consumption of raw or undercooked poultry meat. Birds are the main reservoir of the pathogen as they carry it in a commensal relationship within their intestines. There have been alarming issues regarding antibiotic resistance in *Campylobacter* spp., as well as the decreasing effectiveness of commonly used antibacterials against the pathogen [3]. In order to address this problem, a multifaceted approach is needed to combat the prevalence and these include improvements in sanitation, availability of safe water, food safety and security, increased breast-feeding, adequate nutrition, and required vaccination [4].

Despite studies by scientists over the years, the conventional method of vaccine production against *Campylobacter* in poultry has not led to the development of vaccine in terms of immunogenicity. It is important to intensify efforts to test and validate new vaccine antigens, hence, reverse vaccinology is a suitable strategy to this end [5].

PEB1 and flagellin- two major *Campylobacter* antigens, have been evaluated as vaccine candidates. However, there are varying levels of glycosylation and high antigenic diversity in *Campylobacter*, making the development of a flagellin-based vaccine difficult [6]. Also, in a recent study, it was observed that significant levels of anti-PEB serum IgG was unable to serve as protection against *C. jejuni* after an oral challenge in mice [7]. Despite the fact that the full elucidation of the molecular basis of pathogenicity of *Campylobacter* has not been done, through the use of *in vivo* and *in vitro* studies, various virulence factors have been identified. Examples are: flaA, cadF, CsrA (which function in adhesion; iam, virB11, ciaB and pldA (which function in invasion); CDT (CdtA, CdtB and CdtC)(which function in cytotoxicity) and dnaJ (which function as heat shock protein). These Heat-shock proteins (HSPs) function importantly in thermotolerance [8]. They also function effectively in response to diverse stresses by acting as chaperones (promoting the folding of cellular proteins) and in the proteolysis of deleterious, misfolded proteins. Furthermore, various HSPs have also been identified in *C. jejuni*, and they include the GroESL, DnaJ, DnaK and ClpB proteins [9].

The synthesis of Heat Shock Proteins (HSPs) occurs virtually in all cells under stress conditions, (i.e temperature or nutrient change). Cells known to react to these chemical and physiological changes include Prokaryotic and Eukarvotic ones and this is caused by the induction of stress or HSPs[10]. Scientific research has shown that these proteins play vital roles as molecular in-vivo chaperones. They also act as immune-dominant antigens during cases of infections by bacteria. Highly immunogenic recombinant Heat shock proteins which include Salmonella Typhi rHsp60 [11], Histoplasma capsulatum rHsp60 [12] and Paracoccidioides brasiliensis rHsp60 [13] have the ability to induce protective immunity against deleterious challenges posed by homologous pathogens.

The most conserved proteins and the best characterized HSPs are those belonging to the 60-kDa (GroEL) and 70-kDa (DnaK) families. In addition, HSPs of bacterial organisms have piqued the interests of researchers, especially microbiologists for various years, as they constitute the major targets of the immune response of hosts (Kaufmann and Schoel, 1994). DnaK homologues of diverse bacterial pathogens have been observed to be immunogenic in humans/animals It has also been shown by an experiment involving the infection of mice with *Borrelia burgdorferi*, that protection against microbial infections may occur as a result of immunization with proteins containing DnaK-specific sequences.

Subunit vaccines are produced from parts of the microorganisms and are known to be safe and effective for human and animals. These vaccines are also capable of inducing humoral- and cell-mediated immunities against microbial antigens. Thus, in order to develop effective subunit vaccines, the identification and prediction of the antigenic epitopes by bioinformatics tools is imperative [14,15]. Furthermore, the application of Bioinformatics methods help provide new theoretical approaches for vaccine design based on immunological databases including but not limited to Epitope form, MHC alleles, molecular interactions, docking pathogens and host cells [16].

Although studies on the evolution of the outer membrane proteins of *C. jejuni* as vaccine candidates have been carried out (Meunier *et al*., 2017), heat shock proteins can be considered for protective vaccine design independentl. Thus, this research was aimed at analyzing heat shock proteins for the epitope-based peptide candidate's identification and evaluation of its proteomic database using In silico tools for a new vaccine candidate development.

Alternative therapies remain an urgent need for patients, hence, the need for vaccine development (Bianconi *et al.*, 2019). Hence, further to the above, this study is aimed at exploring the potentials of *Campylobacter* species heat shock proteins in designing a multi-epitope subunit vaccine using the immunoinformatics approach.

METHODOLOGY

Retrieval of *Campylobacter spp.* protein sequence and antigenic conduct assurance

A total of **58** Essential/Chaperonin proteins (DnaJ, DnaK, GrpE, groL, groS, HtpG, Lon Protease) of ***Campylobacter spp.*** were obtained from the National Center for Biotechnology Information (NCBI) protein database (Retrieval date April, 2020) and subjected to multi-epitope vaccine designing. The induction of a significant immunogenic response in host system is the major purpose of vaccination, thus, all protein sequences that had been retrieved were subjected to ANTIGENpro web server in order to predict their antigenicity. Based on the antigenicity result, only **19** proteins were found to have an antigenic probability of ≥0.8 were selected and used in the next step.

Prediction of CTL & HTL Epitopes

The retrieved antigenic protein sequences were subjected to NetCTL and IEDB server to predict CTL and HTL epitopes respectively. In observing the binding predictions of MHCII, the web-based IEDB analysis resource was used as a Consensus tool. High comb scores, length, and immunogenicity scores were considered.

Construction of multi-epitope subunit vaccine

Linkers are viatl in the simulation of the vaccine construct and function as independent immunogen, producing higher antibodies than those of single immunogens. Three major linkers, as previously used in literature (EAAAK, AAY, and GPGPG) were made use of in the final vaccine construction, with the AAY and GPGPG linkers included at the intra-epitope position to join the CTL and HTL epitopes, consecutively.

B-cell epitope, Antigenicity and allergenicity prediction of designed vaccine

B-cells epitope mapping/prediction was performed using the ElliPro (IEDB Analysis Result) server. In order to validate the allergenicity of the vaccine construct, a web tool, AllerTOP v. 2.0, was used. This was based on the auto cross-covariance (ACC) principle that serves to transform protein sequences into similar equal-length vectors (Dimitrov *et al*., 2013).

Physiochemical properties assessment.

ProtParam web server, a part of Expert Protein Analysis System (EXPASY), was used to define various physicochemical properties of predicted vaccine construct. Prediction of important parameters such as molecular weight (kDa), half-life, theoretical pI, aliphatic index, grand average of hydropathy (GRAVY), and so on was carried out using the primary protein sequence of the vaccine.

Prediction of 3D model of vaccine and structure refinement

Vaccine 3D model and the secondary structure was predicted using the RaptorX Server. The 3D protein structure was further refined using GalaxyRefine.

Disulfide engineering for vaccine stability

Disulfide engineering was carried out using Disulfide by design v2.0 to obtain stability of the final vaccine construct's modeled structure (Craig *et al*., 2013).

RESULTS AND DISCUSSION

Collection of *Campylobacter spp.* protein sequences for Vaccine Construct and Assurance of Antigenic Conduct

Among the 58 protein sequences, only 19 proteins were found to be antigenic as predicted by ANTIGENpro. These 19 sequences were selected based on their score obtained for the probability of antigenicity and all these proteins having a score of ≥08. They are as shown in (Table 1) Obtained score for antigenicity probability clearly denoting the antigenic nature of selected protein sequences which can be used for the subunit vaccine designing.

Cytotoxic T-Lymphocytes (CTL) and Helper T Lymphocytes (HTL) Epitopes Prediction and Immunogenicity assessment

A total of 7200 CTL epitopes (9-mer) were predicted by using NetCTL 1.2 server, among them only 97 epitopes with high ranked binding affinity score were chosen as final CTL epitopes for the input of *Campylobacter spp.* protein sequences. Therefore a total of 97 CTL epitopes with high immunogenicity score were selected and subjected to the vaccine designing. Similarly, the HTL epitopes were identified using IEDB MHC-II prediction module based on the higher binding affinity with MHC- II, the human alleles used for the prediction were HLA-DRB1*01:01, HLA-DRB1*01:02, HLA-DRB1* 01:03, HLA-DRB1*01:04 and the method used was NetMHCIIpan. To become the highest immunogenic epitopes, they must have a lower percentile rank and IC50 value. Only **3** epitopes with lowest percentile rank ranging from 0.03–0.3 were selected for the vaccine designing (Table 2). These 8 were used for the vaccine construction (Table 3).

Table 1: *Campylobacter spp.* protein sequences used for Vaccine Construct

S/N	Accession Number	Protein Name	Antigenic score
1	6927	*Campylobacter jejuni* GrpE	0.829
2	A0A0B6UYF7	*Campylobacter jejuni* GrpE	0.829
3	A7I2D5	*Campylobacter hominis* GrpE	0.821
4	A0A1T2	*Campylobacter pinnipediorium* GrpE	0.809
5	A0A0A8H1D0	*Campylobacter insulaenigrae* DnaK	0.854
6	S3XHI2	*Campylobacter ureolyticus* DnaK	0.831
7	Q5HV33	*Campylobacter jejuni* DnaK	0.814
8	A0A381D4R7	*Campylobacter jejuni* DnaK	0.819

9	A0A0E1GIP3	*Campylobacter fetus* DnaK	*0.835*
10	A0A172SRA2	*Campylobacter hyointestinalis* DnaK	0.856
11	WODB67	*Campylobacter fetus* DnaK	0.835
12	A0A172SVH5	*Campylobacter hyointestinalis* DnaK	0.865
13	85213	*Campylobacter jejuni* DnaJ	0.844
14	A7H2C0	*Campylobacter jejuni* DnaJ	0.813
15	A8FMW6	*Campylobacter jejuni* DnaJ	0.83
16	A1W0P5	*Campylobacter jejuni* DnaJ	0.853
17	Q5HTK3	*Campylobacter jejuni* DnaJ	0.823
18	25890	*Helicobacter pylori* dnaJ	0.857
19	Q9ZJQ2	*Helicobacter pylori* dnaJ	0.867

Table 2: Some Predicted cytotoxic T-lymphocyte (CTL) specific epitopes and their immunogenicity score obtained from the immune epitope database

S/N	Accession ID	Epitopes	Comb scores	Immunogenicity scores	Selected or non-selected
1	A0A0B6UYF	DYDELKDKY	2.798	0.7599	Selected
2	A7I2D5	AVDYANEDF	2.677	1.0875	Selected
		NIDVKDNEF	2.572	1.0563	Selected
		ESGDIVQVY	2.699	1.5498	Selected
		QVYQKGYMY	3.31	0.9956	Selected
3	A0A0A8H1D	TTNSCVSVY	2.758	2.8311	Selected
			2.615	0.7558	Selected
			0.176	1.6623	Selected

Table 3: Predicted Helper T-lymphocyte (HTL) specific epitopes and their percentile rank obtained from the immune epitope database

S/N	Allele	Epitope	Method	Percentile Score
1	HLA-DRB1*01:03	YMYKGRVLRAAMVV	NetMHCIIpan	**0.3**
2	HLA-DRB1*01:03	GYMYKGRVLRAAMVV	NetMHCIIpan	**0.32**
3	HLA-DRB1*01:04	MYKGRVLRAAMVVA	NetMHCIIpan	**0.38**

Designing of Multi-epitope Subunit Vaccine

A final vaccine construct of 1723 amino acid residues was designed using 97 CTL and 3 HTL epitopes. In order to attain maximum immune response TLR-5 agonist (*Escherichia coli* Flagellin which was retrieved from UNIPROT server and consisting of 498 amino acid residues with Accession number:P04949) was used as an adjuvant and CTL epitopes were combined together by EAAAK linker, intra-CTL and intra-HTL epitopes joint by AAY and GPGPG linkers.

B-Cell Epitope Prediction

BCPREDS and Ellipro (IEDB Analysis Result) server was used to predict the linear B-cell binding epitopes for the final vaccine construct as shown in Plates 1.0 and 2.0. In humoral immunity, B-cells are invaluable; hence, following the production of antibodies, epitopes matching receptors on the B-cell are important in vaccine design. The results revealed how the epitopes will best interact with B-cells. Total 43 residues were predicted as the discontinuous B-cell epitopes that ranging from the residue number 455-497 with a score of 0.8, where the default threshold was 0.5 and the default maximum distance was 6. Moreover, the discontinuous epitope found with start residue Arginine with residue score of 0.524 and end residue was found on Glutathione with residue score of 0.998. Discontinuous epitopes of 89 amino acids long were also predicted from the final 3D model of vaccine construct with the probability scoring of **0.789.** The immunogenic behavior of the designed subunit vaccine was also fully validated using the obtained probability scores.

Plates 1.0 and 2.0 show the representations of the predicted B-cell epitopes (in number) and their highlighted position sequences respectively; while Plates 3a and 3b prove to reveal how the epitopes will best be in interaction with the B-cells.

BCPREDS Server 1.0

Submitted sequence: 395 amino acids
Epitope length: 20 amino acids
Classifier Specificty: 75%
Prediction method: bcpred
Use overlap filter: yes

BCPred Predictions

Position	Epitope	Score
148	MQRLREAAGPGPGTKDKMAM	1
269	MQRLREAGPGPGDLTKDKMA	1
108	MQRLREAAGPGPGLTKDKMA	1
309	MQRLREAGPGPGKDKMAMQR	1
227	MQRLREAAEGPGPGDLTKDK	1
351	EAAEKGPGPGKDKMAMQRLR	1
248	AMQRLREAGPGPGDLTKDKM	1
330	REAAEKGPGPGKDKMAMQRL	1
87	AMQRLREAAGPGPGLTKDKM	1
372	AAEKGPGPGKDKMAMQRLRE	1
187	MQRLREAAEGPGPGTKDKMA	1
7	SEAAKSIEIDGKKYAAYVVD	0.758

Plate 1.0: Tabular Representation of the predicted B-Cell Epitopes

```
1         11        21        31        41        51        60
|         |         |         |         |         |         |
APHALLSEAAKSIEIDGKKYAAYVVDWLVDKFAAYQSTSINLPYAAYDADKNPLFLAAYL 60
......EEEEEEEEEEEEEEEEEEE.................................
LDVTPLSLAAYSQALGQAIYLTKDKMAMQRLREAAGPGPGLTKDKMAMQRLREAAGPGPG 120
.........................EEEEEEEEEEEEEEEEEEE.EEEEEEEEEEEEE
LTKDKMAMQRLREAAGPGPGLTKDKMAMQRLREAAGPGPGTKDKMAMQRLREAAEGPGPG 180
EEEEEEE...................EEEEEEEEEEEEEEEEEEE.............
TKDKMAMQRLREAAEGPGPGTKDKMAMQRLREAAEGPGPGTKDKMAMQRLREAAEGPGPG 240
......EEEEEEEEEEEEEEEEEEE.....................EEEEEEEEEEEEE
DLTKDKMAMQRLREAGPGPGDLTKDKMAMQRLREAGPGPGDLTKDKMAMQRLREAGPGPG 300
EEEEEE.EEEEEEEEEEEEEEEEEEE.EEEEEEEEEEEEEEEEEEE............
DLTKDKMAMQRLREAGPGPGKDKMAMQRLREAAEKGPGPGKDKMAMQRLREAAEKGPGPG 360
........EEEEEEEEEEEEEEEEEEE.EEEEEEEEEEEEEEEEEEE.EEEEEEEEEE
KDKMAMQRLREAAEKGPGPGKDKMAMQRLREAAEK 395
EEEEEEEEEE.EEEEEEEEEEEEEEEEEEE....
```

Plate 2.0: Pictorial Representation of the B-cell epitopes and their highlighted Position Sequence

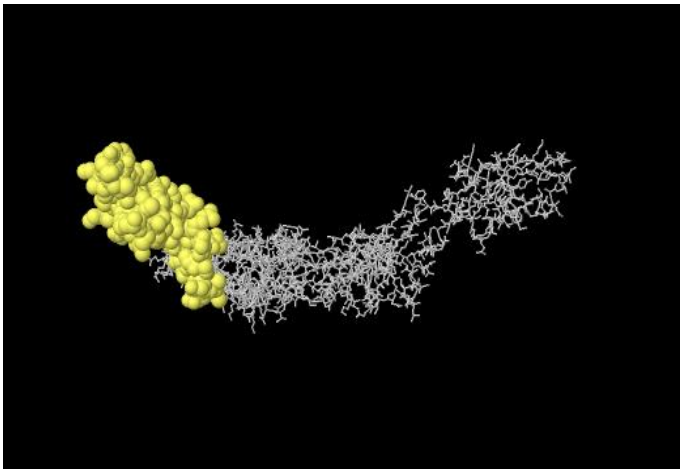

Plate 3(a): Humoral epitope prediction for subunit vaccine showing the discontinuous B-cell epitopes among the 3D structure of the final vaccine construct.

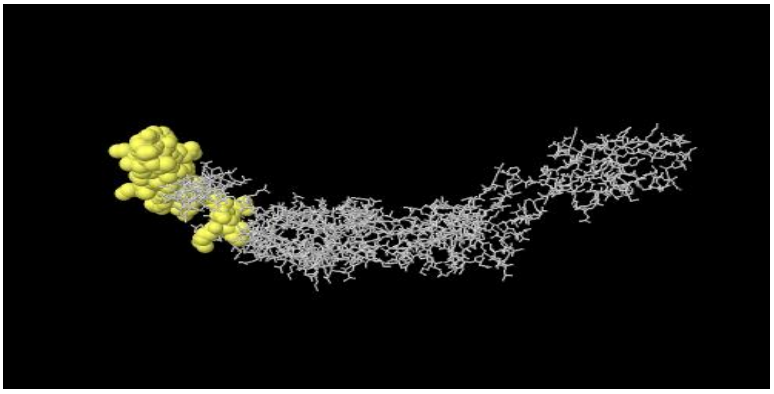

Plate 3(b): Humoral epitope prediction for subunit vaccine showing the linear B-cell epitopes among the 3D structure of the final vaccine construct.

Prediction of Antigenicity, Allergenicity and Physicochemical Parameters of the Vaccine Construct

By making use of the web-based ANTIGENpro server, the antigenic probability of the newly designed vaccine was 0.86. This serves to represent the antigenic nature of thevaccine construct. In the same order, AllerTOP online server was used to predict the allergenicity of the designed vaccine and the protein was determined to be nonallergic in nature, that is, safe for the human use. The molecular weight of vaccine protein was found to be 183371.16 kDa which will favor the antigenicity of the vaccine construct. The instability index (II) is computed to be 27.01 which classifies the protein as stable. The theoretical pI was found to be 4.51 showing its acidic to neutral nature while the total numbers of negative and positive charge residues were 164 and 103, respectively. The value of the aliphatic index and Grand average of hydropathicity (GRAVY) was 85.71 and 0.124, respectively. Furthermore, the thermostability of the vaccine construct was validated based on the results of the aliphatic index according to a rule that states that the value of aliphatic index is directly proportional to the protein thermostability. Conclusively, the designed vaccine is immunogenic and thermostable in nature (Adhikari *et al*., 2018).

Secondary Structure Prediction

The 3D model result of the predicted secondary structure using the **Raptor X** web server is shown in Plate 4a. A total of 1663(96%) amino acid residues were modeled with 100.0% confidence by the single highest scoring template. 5% of sequences were predicted disordered and as such, could not be predicted. Out of the 6 domains predicted, the best template for protein model was (PDB ID: 1ucu: A) with its P-value of 2.59e-07. The obtained score was 223 with a sequence identity of 45%, rest of the templates was found to be less identical.

Secondary Structure Refinement

Using GalaxyRefine- a web server, the vaccine model was refined. Out of all refined models, Model 1 was chosen as the final vaccine for further analysis out of all refined models based on validation by variously predicted parameters such as: GDT-HA (0.9375), RMSD (0.355), MolProbity (2.099), Clash score (14.0), Poor rotamers (0.7) and Ramachandran plot (92.0).

The 3D protein refinement using the **GalaxyRefine** server leads to an increase in the number of residues in the favored region. Plate 4a and 4b show the initial structure and refined structure respectively while Plate 4c shows the visualization of refined models are displayed in rainbow colors and initial models in white.

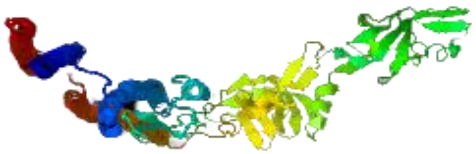

Plate 4a: 3D secondary protein structure of the vaccine construct modeled using the Phyre2 web server.

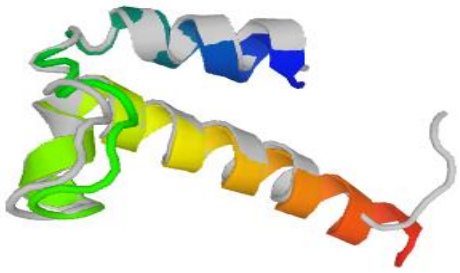

Plate 4b: Showing the Refined protein structure modeled as predicted by GalaxyRefine Server

Disulphide Engineering of Final Vaccine Construct

In order to stabilize the modeled structure of final vaccine constructs disulfide engineering was performed using Disulfide by design v2.036 and found that there are total 27 pairs of residues that can be used for the purpose of disulfide engineering.

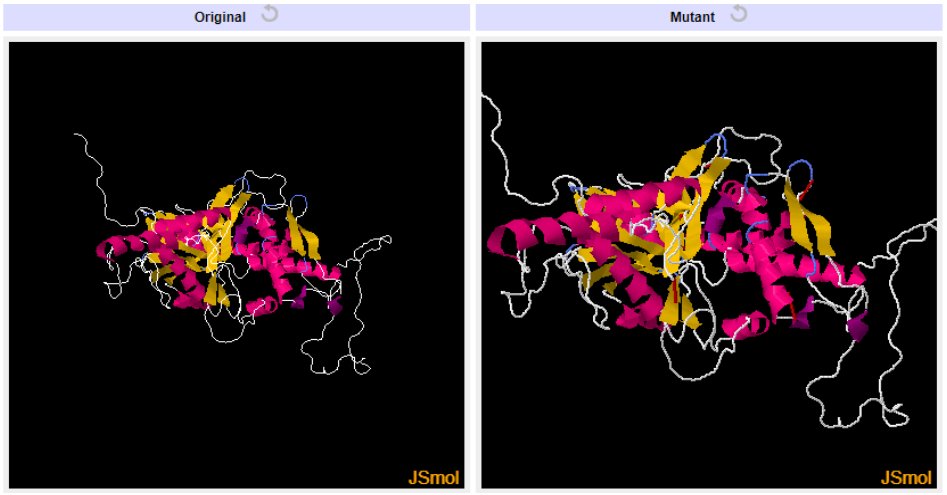

Plate 4c: Disulfide engineering to improve protein stability- Mutant (Disulphide bonds) are shown in RED color.

CONCLUSION

Vaccines are the pharmacological products which can provide the finest cost-benefit ratio in the prevention or treatment of diseases. However, effective vaccine progression and production are costly and can take years to be completed; hence, researchers have tried for many years to minimize the cost and time for the development of vaccines. This has therefore led to different strategies being developed for the design and

development of effective and safe new-generation vaccines based on the Bioinformatics approaches.

This research work has provided a multi-epitope subunit based vaccine having antigenic properties in the absence of allergenic properties against *Campylobacter spp.* using the immunoinformatics approach. Further studies will include an experimental validation of the proposed vaccine to ensure effective control of *Campylobacter spp* infections and resultant diseases. The experimental work may also include the synthesis of designed subunit vaccine followed by the *in vitro* and *in vivo* analysis to determine the immunogenicity and safety concern of the same. Conclusively, in the quest for an epitope-based peptide vaccine for Campylobacter, the findings from this research will serve to provide the preliminary data to be used in observing molecular docking interactions.

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Conflict of interest

All authors declare no conflict of interest.

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