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A NOVEL MULTI-EPITOPE PEPTIDE VACCINE CONSTRUCT AGAINST INFLUENZA A VIRUS AND *STREPTOCOCCUS PNEUMONIAE* CO-INFECTION: AN *IN SILICO* APPROACH

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Abstract

Viral and bacterial respiratory tract co-infections in the same host often result in severity and heightened pathology of illness compared to single infections. This has proven to be true for combined infections with Influenza A virus and the bacterium *Streptococcus pneumoniae*. Separate vaccines do exist for each individual infection but they prove to be ineffective and non-specific when the infection has multiplied in case of co-infection. The study utilised in silico approaches and proposed a structural design for multi-epitope peptide vaccine having the ability to target co-infection caused by A/New York/392/2004 (H3N2) and R6 strains of Influenza A virus (NCBI Accession: PRJNA15622) and *Streptococcus pneumoniae* (NCBI Accession: PRJNA278), respectively. Epitope prediction followed by protein prioritization was performed using the reference sequence of each strain to short list the epitopes that can later be used for constructing multi-epitope structure. The multi-epitope constructs having Cholera Toxin Subunit B as adjuvant and $(Gly_4Ser)_3$ as flexible linker were then analyzed for their ability to induce an effective immune response in human body for which Macrophage receptor with collagenous structure, Toll-like receptor 2, 4 and 5 were taken as Pattern Recognition Receptors. The significant immune response generated through each Pattern Recognition Receptor helped to conclude that multi-epitope peptide structures can be used as probable candidates for the design of vaccine. The combination of the epitopes LWSYNAELL and FTGKQLQVG of Influenza A virus and *Streptococcus pneumoniae*, respectively, induced highly significant immune response in case of each Pattern Recognition Receptor when tested through in-silico predictive tools.

Keywords: Co-infection, Influenza A virus, Multi-epitope, *Streptococcus pneumoniae*.

1. INTRODUCTION

Bacterial-viral co-infections are best described with the case of influenza. However these co-infections are extended to other respiratory viruses, as well, such as Respiratory syncytial virus (RSV), Parainfluenza virus (PIV), Rhinovirus, Adenovirus, and human Metapneumovirus (hMPV) [1]. Several factors including enhanced susceptibility to co-infection with bacterial strains lead to increased virulence [2].Pneumonia and other lower respiratory tract infections are associated with the prevalence of respiratory viruses, particularly Respiratory syncytial virus (RSV) and Influenza [3], [4]. Besides, the bacterial strains in case of positive cultures are almost always *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus influenzae*, or a combination of these bacteria[5]–[8]

Post influenza bacterial infection and combined viral-bacterial pneumonia are the two types of bacterial respiratory infection that occur during influenza virus infection. The former, however, can be recognized more easily compared to the latter in terms of clinical diagnosis mainly because it is during the recovery phase from influenza that this infection tends to take place[9]. The combined viral-bacterial infection involves the interaction of the virus with the host response along with the inflammation induced by bacteria, which leads to increased bacterial colonization and outgrowth followed by viral replication. Hence, both the viral replication and bacterial growth will be affected by the host response[10], [11].On the other hand, virus-induced changes to the host are involved in case of post influenza pneumonia. Besides, the absence of virus makes this infection less complicated[12], [13].

The research carried out over the years is suggestive of the fact that several factors of host, virus and bacteria are involved in viral-bacterial pneumonia[10], [12]–[15]. Mechanisms that play critical role in either post-influenza pneumococcal pneumonia or combined viral-bacterial infection have been identified through mouse studies and are summarized in Table 1[16]–[18]. Currently, the focus of these studies is mainly on combined viral-bacterial pneumonia (bacterial challenges up to 7 days after influenza) [14], [15], [19], [20], while other studies aim to investigate post-influenza pneumonia (bacterial challenges ranging from 14 days up to 35 days after influenza infection) [12]–[14].

Once the influenza virus gains entry in to epithelial cells and replicates, the mucociliary clearance of the virus gets

impaired due to the reduction in mucociliary velocity. Hence the events lead to increased burden of *S. pneumonia* 2 hours after bacterial challenge [14]. It is the role of influenza-induced damage to the airway epithelium that influences the severity of both combined viral-bacterial infection and post-influenza pneumococcal pneumonia. The ultimate effect of the influenza-induced damage to the airway epithelium is the increased colonization of bacteria at the basal membrane[21]. This preferred infection and replication of the influenza virus in the airway epithelial cells leads to induction of an antiviral response that aims to remove the virus through transcription and translation based inhibition of the viral replication [22]. Apoptosis also occurs to eradicate the virus in the form of apoptotic bodies that are subsequently removed by alveolar macrophages[23]. However, the viral infection results in TNF-α and IL-12 dependent production of IFN-γ by T cells and endogenous IFN-γ production by APCs. It is then IFN-γ that down regulate the scavenger receptor express by alveolar macrophages, termed as Macrophage receptor with Collagenous structure (MARCO), which has a role in bacterial phagocytosis. However, owing to the viral infection, this phagocytosis also gets inhibited. Prolonged desensitization of alveolar macrophages to bacterial TLR ligands such as lipoteichoic acid (TLR2), lipopolysaccharide (TLR4) and flagellin (TLR5) also occurs due to IAV infection [13] and lasts for several months owing to the longer life span of macrophages that reside in airway epithelium. Hence, the desensitized macrophages produce decreased number of chemokine. Decreased NFκB activation and consequently reduced recruitment of neutrophils to the site of infection also takes place. Eventually, this antiviral mechanism and altered macrophage function leads to an increased risk of bacterial colonization and enhanced bacterial invasion upon secondary infection with *S. pneumoniae* in case of mice [13], [18].

Table 1. Host factors involved in Combined Viral-Bacterial Pneumonia and in Post-influenza Pneumonia. MARCO, TLR 2, 4 and 5 are identified as the PRRs that play a critical role in IAV-SP co-infection and further affect the signaling of cytokine and chemokine such as IFN-γ, IFN-α/β and IL-10. Abbreviation: MARCO, Macrophage receptor with Collagenous structure; TLR, Toll-like receptor; PRR, Pattern recognition receptor; IFN, Interferon; IL, Interleukin.

Host factors	Combined Viral-Bacterial Pneumonia	Post-influenza Pneumonia
Pattern recognition receptors	MARCO[a]	TLR2[b] TLR4[b] TLR 5 [b]
Cytokine/Chemokine	IFN-γ[a] IFN-α/β[c]	IL-10[d]
Immune cells	Neutrophil function[e, f, g, h, i] Neutrophil recruitment[j, k, l] Neutrophil apoptosis[m, n] Macrophages[i, o] Monocytes[i]	Neutrophil function[d] Neutrophil recruitment[b]
Mechanical factors	Epithelial injury[p] Mucociliary velocity[q]	Unknown

[a]Sun & Metzger, 2008; [b]Didierlaurent et al., 2008; [c]Shahangian et al., 2009; [d]van der Sluijs et al., 2004; [e]LeVine et al., 2001; [f]McNamee & Harmsen, 2006; [g]Abramson & Hudnor, 1994; [h]Verhoef, Mills, Debets-Ossenkopp, & Verbrugh, 1982; [i]Abramson, Mills, Giebink, & Quie, 1982; [j]Larson, Parry, & Tyrrell, 1980; [k]Shahangian et al., 2009; [l]Ruutu, Vaheri, & Kosunen, 1977; [m]Engelich, White, & Hartshorn, 2001; [n]Colamussi, White, Crouch, & Hartshorn, 1999; [o]Debets-Ossenkopp, Mills, Van Dijk, Verbrugh, & Verhoef, 1982; [p]Plotkowski et al., 1986; [q]Pittet et al., 2010.

The heightened pathology and increased morbidity and mortality associated with IAV-SP co-infection is a serious concern and currently no vaccine exists that can concurrently target the co-infection of IAV and *S. pneumoniae*. The periodic administration of more than one vaccine or even administration of same vaccine to boost immune response to target co-infection is not only time-consuming but cost ineffective as well. It is therefore necessary to propose a vaccine construct having the ability to target co-infection with single administration without compromising on the immune response generated. In this regard multi-epitope peptide vaccine construct can prove to be potential candidates because of their ability to incorporate the epitope of each pathogen, which eventually broadens the scope of immune responses generated in the human body. The study aims to initially analyze the immune response generated by each targeted receptor of human body involved in IAV-SP co-infection. Based on this analysis the study aims to propose the best

combination contained within the construct that can be incorporated in the vaccine formulation. If the multi-epitope peptide based vaccine construct proves to induce an efficient immune response similarly during biological validation, then it will help prevent the detrimental effects of IAV-SP co-infection and will also help to design vaccine with different combinations for other types of co-infection as well.

2. MATERIALS AND METHODS

The steps of general methodology applied to the study have been enlisted in Fig 1.

Since the study involved strains of two different pathogens, therefore, the tools for target prediction utilized at each step also varied as shown in Fig 2.

2.1 Strain Selection

Influenza A Virus strain A/New York/392/2004 (H3N2) was selected from influenza viruses and *S. pneumoniae* R6 strain was selected from *S. pneumoniae* species.

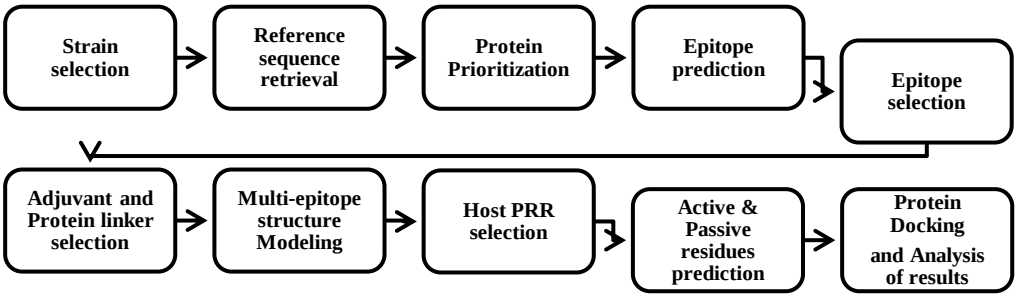

Figure 1.General Methodology applied to the study. The study focused on applying in-silico approaches to predict the vaccine targets for multi-epitope peptide construct. Protein prioritization followed by epitope prediction led to finalization of probable vaccine candidates which were then modeled to analyze the protein interactions.

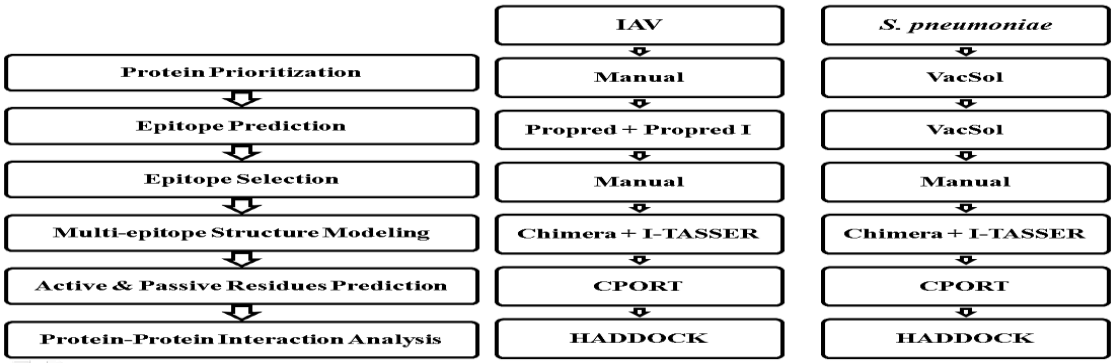

Figure 2.Tools utilized for target prediction. The tools were first identified based on the studies reported over the years. Some steps were performed manually to facilitate the performance of sub-steps involved.

2.2 Reference Sequence Retrieval

The complete genomes Protein sequences (Reference sequence) of each pathogen was retrieved from National Center for Biotechnology Information (NCBI) with accession numbers PRJNA15622 and PRJNA278 for IAV and *S. pneumoniae* respectively. Reference sequences were selected as they are updated to reflect current knowledge of sequence data and biology. Some other features of Reference Sequence collection include non-redundancy, explicitly linked protein and nucleotide sequences along with format consistency and data validation.

2.3 Protein Prioritization and Prediction of T cell epitopes

Protein prioritization was manually performed for IAV, whereas, VacSol, an in house pipeline designed at Integrative Biology Laboratory, ASAB, NUST was used to prioritize proteins in case of *S. pneumoniae*. VacSol, a high throughput *in silico* pipeline uses subtractive reverse vaccinology to predict potential therapeutic targets in prokaryotic pathogens [35]. This pipeline also helped to predict both B and T cell epitopes of the prioritized proteins of *S. pneumoniae*. The determination of the T cell epitopes required both HLA I and HLA II binding peptide sequences. HLA class I binding promiscuous epitopes in the reference sequence of IAV were predicted by the help of ProPred I (www.imtech.res.in/raghava/ProPred1/) [36]. 4% default threshold value was opted with proteasome and immunoproteasome filters enabled at 5% threshold value to maximize the efficiency of determining T cell epitopes. The epitopes determined by ProPred I have the ability to bind to 47 HLA class I alleles. However, ProPred [37] was used at a cut off value of 3% threshold to predict epitopes for HLA class II alleles. ProPred predicts antigenic epitopes that have the ability to bind to 51 HLA class II alleles.

2.4 Epitope Selection

2.4.1 Antigenic Prediction

Analysis of the antigenic properties of the predicted epitopes was performed using VaxiJen version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) [38]. To obtain antigenic sequences a threshold value of 0.5 antigenic score was maintained, which gives 87% accurate results for viruses. Alignment-independent prediction of protective antigens is performed by Vaxijen server on the basis of the physiochemical properties of the antigens [39].

2.4.2 Class I Immunogenicity Prediction

All the HLA-I binding antigenic epitopes of IAV were scanned for MHC-I immunogenicity using Immune Epitope Database (IEDB) Analysis tool

(http://www.iedb.org/) [40]. Default parameters were selected to perform the immunogenicity prediction, which uses amino acid position within the peptide and their properties [39].

2.4.3 Validation of Predicted Epitopes

To comment on the novelty of the predicted epitopes IEDB database was utilized as it contains experimentally confirmed data characterizing antibody and T cell epitopes studied in NHPs, homo sapiens and other animal species [39].

2.5 Adjuvant and Protein Linker Selection

Cholera Toxin B (CTB) was investigated as a classical mucosal adjuvant that has the ability to enhance vaccine immunogenicity [41]. Hence, CTB was used as an adjuvant for the multi-epitope construct because of its efficient generation of immune response during the infection of IAV and *S. pneumoniae*.

$(Gly_4Ser)_3$, a flexible protein linker commonly used for protein engineering and design [42], [43], was selected as the protein linker for the multi-epitope vaccine construct against IAV-SP co-infection.

2.6 Multi-epitope Structure Designing and Modeling

UCSF Chimera 1.11.2 was used for multi-epitope structures designing that involved linkage between the adjuvant, IAV epitope and *S. pneumoniae* epitope through the flexible protein linker $(Gly_4Ser)_3$[44]. The different combination of constructs were later modeled using I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER), which is a server for protein structure and function prediction [45].

2.7 Host Pattern Recognition Receptors Selection

Macrophage receptor with collagenous structure (MARCO) and Toll-like receptors (TLRs) were selected as the Pattern Recognition Receptors (PRRs) of the host because of their immune response generated during IAV-SP co-infection. The TLRs included TLR 2, TLR 4 and TLR 5. The Protein Data Bank (PDB) file of each receptor was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) (http://www.rcsb.org/pdb/home/home.do) [46].

2.8 Determination of Protein-Protein Interactions

CPORT (Consensus Prediction Of Interface Residues in Transient complexes)(http://haddock.science.uu.nl/services/CPORT/) was used for the prediction of active and passive residues involved in the interaction of each multi-epitope and the selected PRRs [47]. A combination of six methods is utilized by CPORT to accomplish the task [48]–[53].

The structures of each combination of multi-epitope construct against each PRR were fed into guru level interface HADDOCK (High Ambiguity Driven protein-protein DOCKing) (http://milou.science.uu.nl/services/HADDOCK2.2/haddock.php) server using default settings [54], [55]. Guru level interface being an advanced HADDOCK interface allows the identification of flexible regions from the simulation perspective, unlike easy level interface. The top clusters in each case were refined for better orientation, which led to improved HADDOCK scores. To analyze intermolecular and intramolecular interactions PDBsum was utilized [56].

3. RESULTS AND DISCUSSION

3.1 Prioritized Proteins and Predicted Epitopes

Based on the requirement and methodology of the study, the epitopes were predicted using the prioritized protein of each pathogen. Hemagglutinin (HA) was the prioritized protein of IAV, whereas, Probable Thiol Peroxidase (Tpx) was prioritized in case of *S. pneumoniae*.

3.1.1 Influenza A Virus Prioritized Proteins

The proteins of IAV were prioritized on the basis of three factors as summarized in Table 2. Proteins having greatest % identity with humans and annotation were preferred. Besides, Host Apical and Virion membranes were the preferred subcellular locations for the proteins that were needed to be prioritized. Although both Hemaglutinnin and Neuraminidase were obtained as the prioritized proteins but based on the requirements of selecting one best protein, only Hemaglutinnin was considered for further predictions.

Table 2. Prioritized proteins of IAV. The proteins of IAV were prioritized on the basis of their homology with humans, subcellular location and annotation. Analysis of each factor utilized respect tool mentioned within the bracket.

Protein	% Identity with Humans (UniprotKB: Blastp)	Subcellular Location (UniprotKB and Virus-mPLoc)	Annotation (UniprotKB: 5-point-system)
PA-X protein	22.2 – 30.4	• Host Cytoplasm	2/5
Hemagglutinin	20.2 – 34.1	• Host Apical Cell Membrane • Virion Membrane	3/5
Matrix protein 2	31.9	• Host Apical Cell Membrance	2/5
Matrix protein 1	25.3 – 36.4	• Host nucleus • Peripheral virion membrane protein	2/5

		(cytoplasmic side) • Host nucleus	
Neuraminidase	20.9 – 27.7	• Host Apical Cell Membrance	3/5
Nucleocapsid protein	22.9 – 36.1	• Host Nucleus	2/5
Non-structural protein 2	21.3 – 39.5	• Host Nucleus • Virion	2/5
Non-structural protein 1	34.4 – 26.5	• Host cytoplasm • Host nucleus	3/5
Polymerase acidic protein	18.5 – 30.1	• Host cytoplasm • Host nucleus	2/5
Polymerase basic protein 1	27.4 – 33.3	• Host cytoplasm	2/5
PB1-F2 protein	33.3 – 52.9	• Host cytosol • Host mitochondrion inner membrane • Host nucleus	2/5
Polymerase basic protein 2	-	• Host nucleus	2/5

3.1.2 Hemaglutinnin Predicted Epitopes

The epitope prediction of Hemaglutinnin resulted in two T-cell epitopes demonstrated in Table 3.

3.1.3 *S. pneumoniae* Prioritized Proteins and Predicted Epitopes

The use of VacSol helped in prioritizing two proteins of *S. pneumoniae* based on the factors summarized in Table 4. However, based on the requirement of prioritizing one best protein, Probable thiol peroxidase was selected for further predictions. Each prioritized protein of *S. pneumoniae* was further used to predict T-cell epitopes based on the factors enlisted in Table 5.

Table 3. Predicted epitopes of Hemaglutinnin. The epitope prediction of Hemaglutinnin, based on MHC-I and II allele count, immunogenicity and antigenicity, gave two T-cell epitopes. VaxiJen was used for antigenicity check, whereas, Class I Immunogenicity IEDB tool was used for immunogenicity. Abbreviation: MHC, Major Histocompatibilty Complex; IEDB, Immune Epitope Database.

T Cell Epitope	MHC-I Allele Count	MHC-II Allele Count	Location	Immunogenicity (IEDB)	VaxiJen (Threshold = 0.5)	
					Score	**Antigenicity**
IEVTNATEL	12	2	50	0.17425	0.8869	Probable Antigen
LWSYNAELL	8	0	437	0.05113	0.6384	Probable Antigen

Table 4. Prioritized proteins of *S. pneumoniae*. Six factors were taken in to account by VacSol to prioritize the proteins of *S. pneumoniae*.

Protein	Non-Homologous	Localization	Essential	Virulent	Helices < 2	Annotated
Zinc-binding lipoprotein AdcA	✓	✓	✓	✓	✓	✓
	0	Periplasmic	1	3	IN: 1	40.42 %
Probable thiol peroxidase	✓	✓	✓	✓	✓	✓
	0	Periplasmic	1	4	OUT: 0	100 %

Table 5. Predicted epitopes of *S. pneumoniae*. VacSol predicted a total of five T-cell epitopes based on MHC-I and MHC II allele count. VaxiJen score for antigenicity was also taken in to consideration. Abbreviation: MHC, Major Histocompatibilty Complex.

Protein	T Cell Epitope	Location	MHC-I Allele Count	MHC-II Allele Count	VaxiJen (Threshold = 0.5)	
					Score	**Antigenicity**
Zinc-binding lipoprotein AdcA	FLLCLGACG	21	6	6	0.4059	Probable Antigen
	LESDPQNDK	286	3	5	0.9905	Probable Antigen
	MVKEVSGDL	51	40	6	0.806	Probable Antigen
Probable thiol peroxidase	LAGLDNTVV	70	6	4	1.2005	Probable Antigen
	FTGKQLQVG	12	2	2	1.4001	Probable Antigen

3.2 Multi-epitope Peptide Vaccine Construct Design

The study aimed to propose a multi-epitope peptide vaccine construct, hence the best epitope of each pathogen, an adjuvant and a linker were incorporated in to the construct as demonstrated in Fig 3.

3.3 Multi-epitope Peptide Vaccine Construct Combinations

A total of four combinations were proposed using one best epitope from each pathogen. The combinations have been represented in Table 6.

Figure 3. Proposed design of Multi-epitope peptide vaccine constructs. LWSYNAELL and FTGKQLQVG were incorporated as IAV and *S. pneumoniae* epitopes, respectively.

TABLE 6. Combinations for multi-epitope peptide vaccine construct. The table represents the possible combinations involving Hemaglutinnin and Probable Thiol Peroxidase T-Cell Epitopes.

Multi-epitope	Hemaglutinnin T- Cell Epitope	Probable Thiol Peroxidase T-Cell Epitope
1	IEVTNATEL	LAGLDNTVV
2	LWSYNAELL	LAGLDNTVV
3	IEVTNATEL	FTGKQLQVG
4	LWSYNAELL	FTGKQLQVG

3.4 Protein-Protein Interaction

HADDOCK results summarized in Table 7 helped to identify the best multi-epitope combination for the vaccine construct. The visual representation of the interaction of multi-epitope 4 with MARCO, TLR 2, TLR 4 and TLR 5 has been indicated in Fig 4, 5, 6 and 7, respectively.

TABLE 7. Protein-Protein interaction results. Based on the lowest HADDOCK and RMSD score, multi-epitope 4 was proposed as the best combination.

	IAV EPITOPE	SP EPITOPE	RECEPTOR	HADDOCK SCORE	RMSD SCORE
MULTIEPITOPE 1	IEVTNATEL	LAGLDNTVV	MARCO	-143.4 +/- 0.6	0.3 +/- 0.2
			TLR2	-119.3 +/- 1.5	0.3 +/- 0.2
			TLR4	-138.0 +/- 3.3	0.2 +/- 0.1

			TLR5	-129.8 +/- 7.6	0.3 +/- 0.2
MULTIEPITOPE 2	LWSYNAELL	LAGLDNTVV	MARCO	-138.4 +/- 1.6	0.3 +/- 0.2
			TLR2	-132.4 +/- 4.1	0.3 +/- 0.2
			TLR4	-124.8 +/- 1.2	0.3 +/- 0.1
			TLR5	-212.2 +/- 5.3	0.3 +/- 0.2
MULTIEPITOPE 3	IEVTNATEL	FTGKQLQVG	MARCO	-141.6 +/- 6.5	0.3 +/- 0.2
			TLR2	-107.2 +/- 1.0	0.3 +/- 0.2
			TLR4	-152.3 +/- 4.2	0.3 +/- 0.1
			TLR5	-262.0 +/- 6.4	0.3 +/- 0.2
MULTIEPITOPE 4	LWSYNAELL	FTGKQLQVG	MARCO	-143.9 +/- 3.3	0.3 +/- 0.2
			TLR2	-131.8 +/- 6.0	0.3 +/- 0.1
			TLR4	-171.4 +/- 4.1	0.3 +/- 0.2
			TLR5	-262.0 +/- 6.4	0.3 +/- 0.2

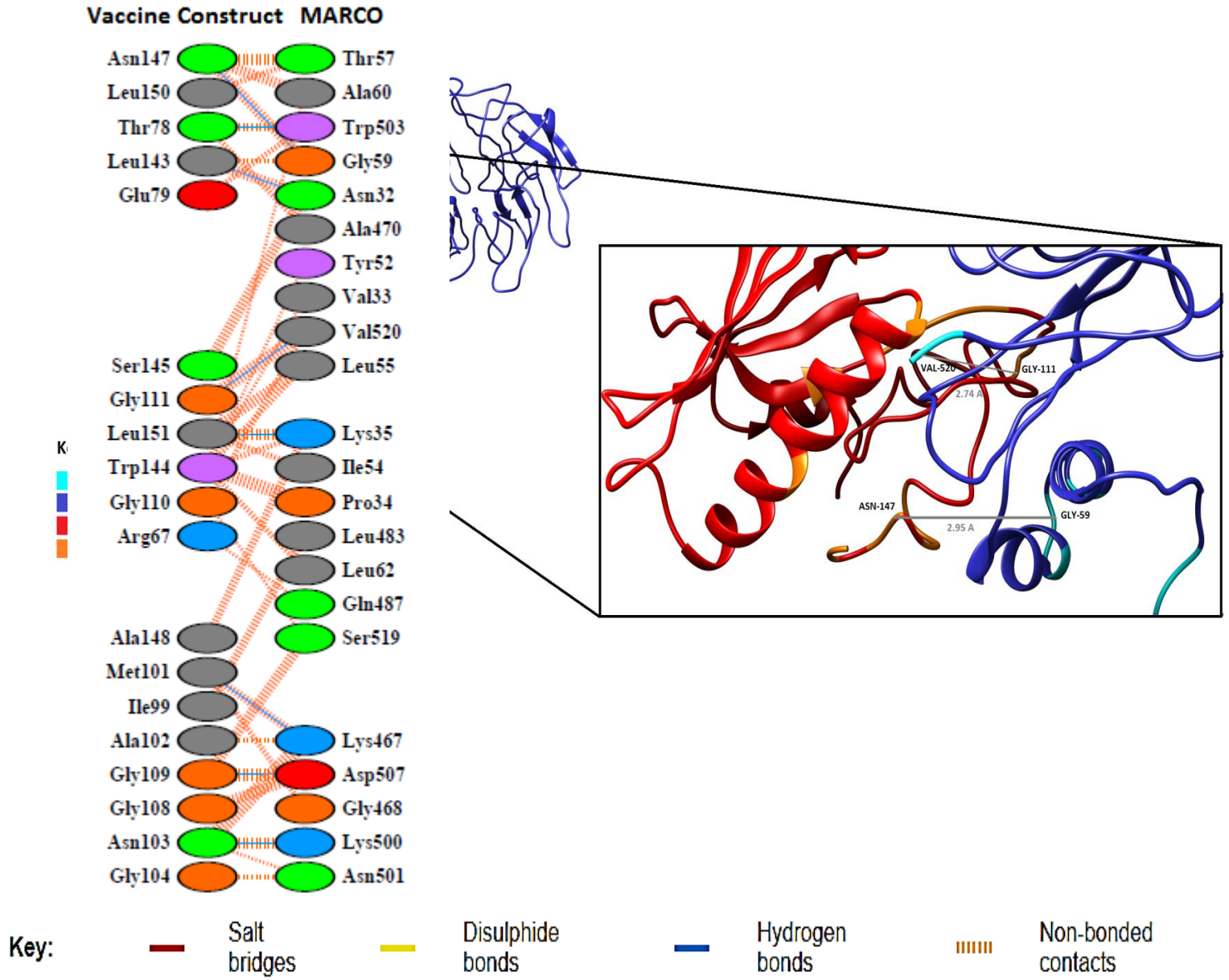

Figure 4. Interaction between Vaccine Construct (Multi-epitope 4) and MARCO. The figure indicates the interacting residues an construct and MARCO. Abbreviation: MARCO, Macrophage receptor with collagenous structure.

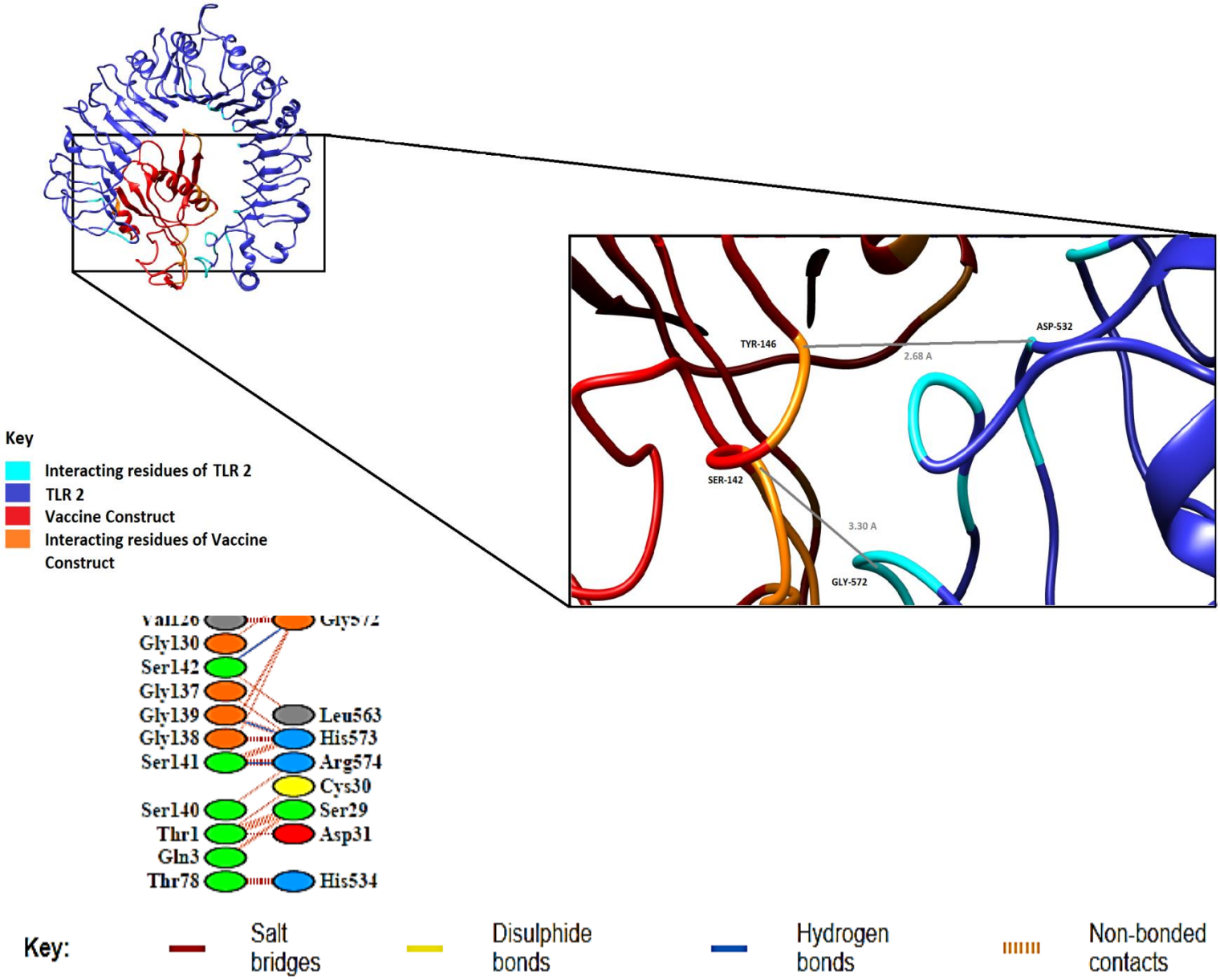

Figure 5. Interaction between Vaccine Construct (Multi-epitope 4) and TLR 2. The figure indicates the interacting residues vaccine construct and TLR 2. Abbreviation: TLR, Toll-like receptor.

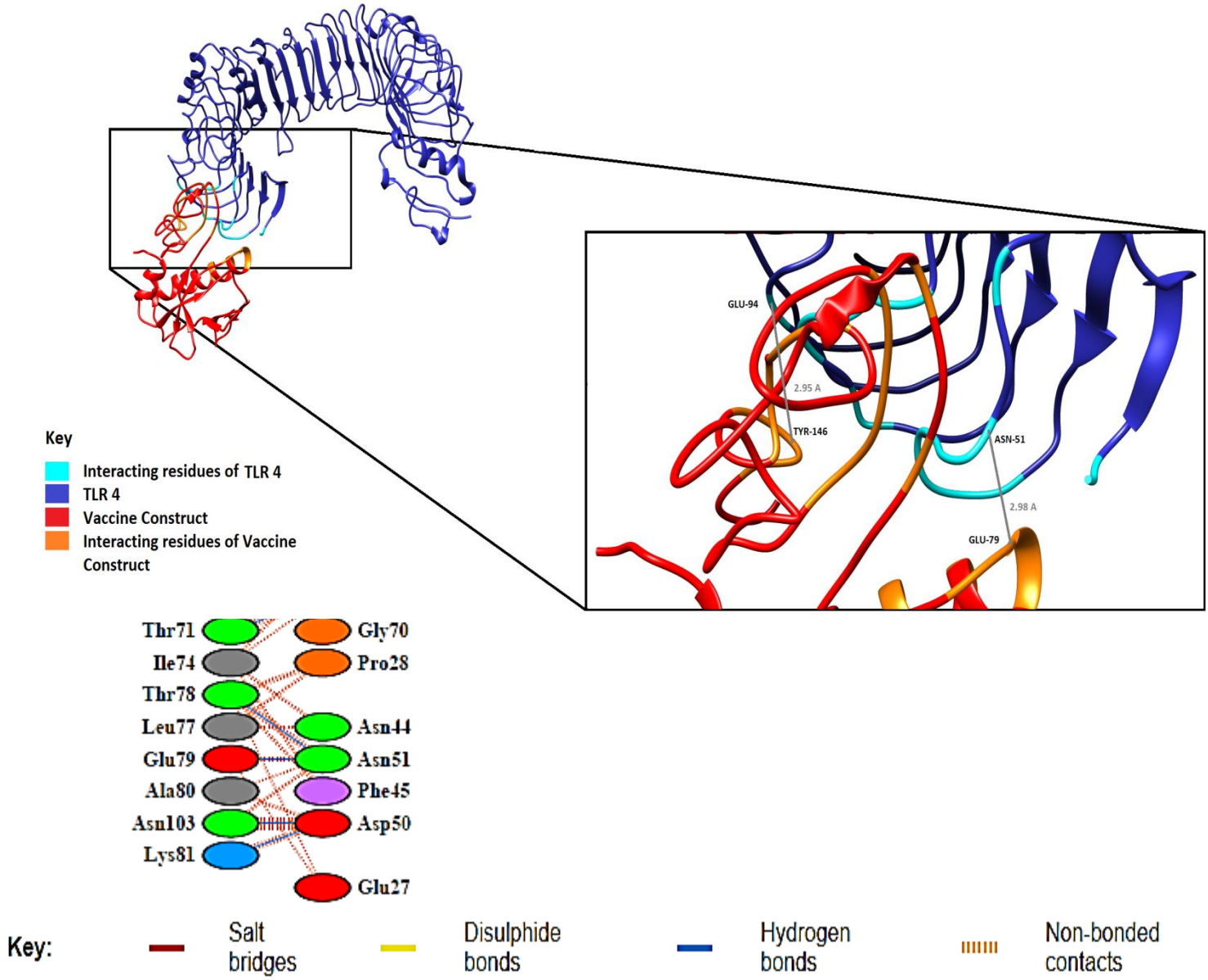

Figure 6. Interaction between Vaccine Construct (Multi-epitope 4) and TLR 4. The figure indicates the interacting residues a vaccine construct and TLR 4. Abbreviation: TLR, Toll-like receptor.

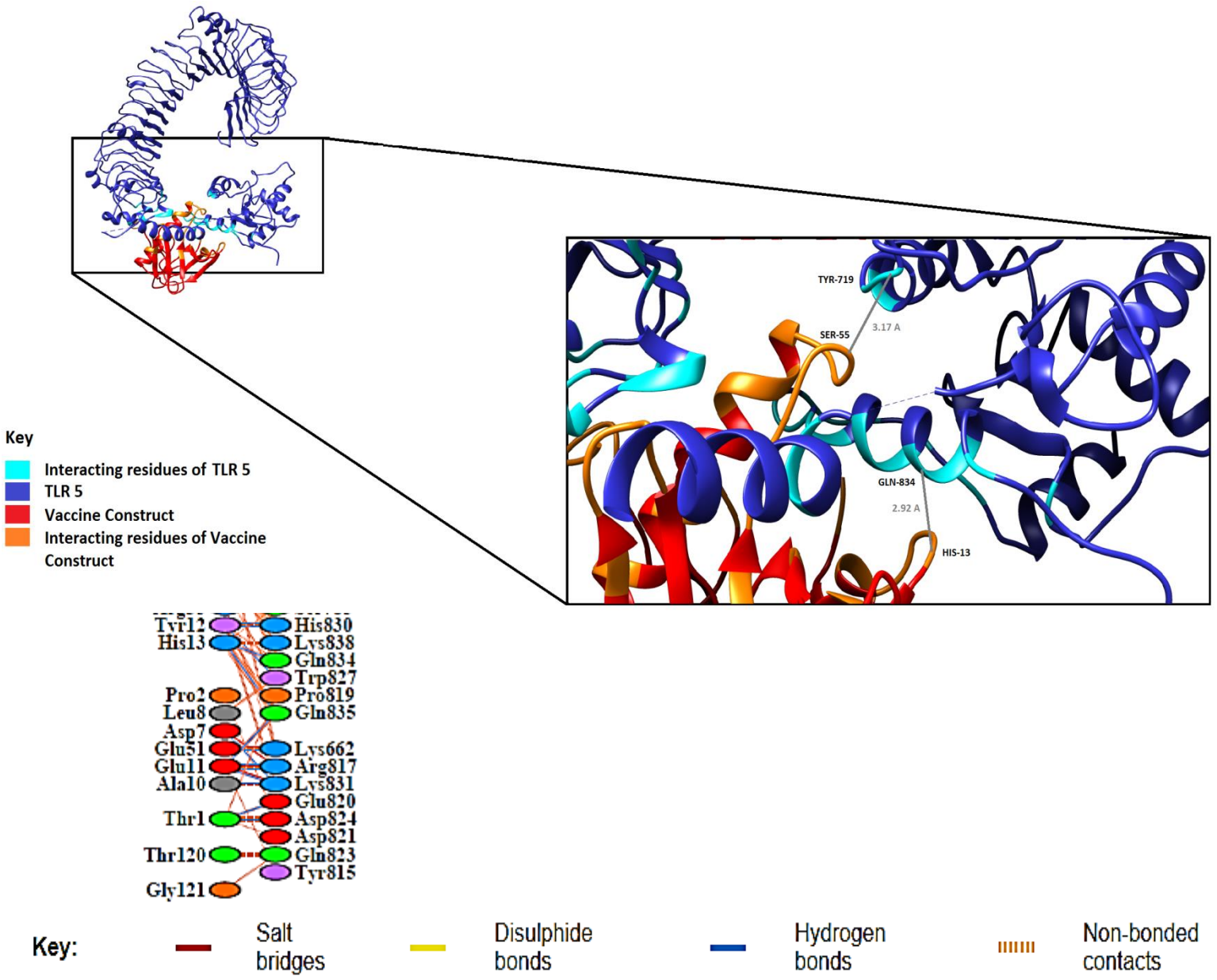

Figure 7. Interaction between Vaccine Construct (Multi-epitope 4) and TLR 5. The figure indicates the interacting residues and bo vaccine construct and TLR 5. Abbreviation: TLR, Toll-like receptor.

4. CONCLUSION

The results of this in-silico study helped in concluding that multi-epitope peptide based construct can be used as probable candidate against infectious diseases that involve co-infection of multiple pathogens. This hypothesis has been proven true particularly for the co-infection of IAV and *S. pneumoniae*. Significant Immune response was generated by each PRR particularly in case of the combination of multi-epitope 4 construct, which led to the conclusion that the very combination has the greatest potential to induce an effective immune response in human body if and when validated through animal model. The study considered PRRs as the only immune targets of human body; however, this approach can further be extended to Human Leukocyte Antigens (HLAs) in order to broaden the scope of the study in terms of immune response generated by the vaccine construct. Based on the promising results of this in-silico predication, the results can further be validated using animal model or the Lab-on-a-chip approach. Different adjuvants can also be made use of in order to boost the immune response generated. The scope of the study can also be broadened by including other strains of each pathogen. Besides, there exists an interesting link between microorganism infection and carcinogenesis, which has recently gained immense attention from researchers worldwide. Hence, the link between IAV-SP co-infection and Lung cancer can be studied in depth and by identifying the common signaling pathways a multi-epitope peptide vaccine construct or fusion proteins can also be proposed against this fatal combination.

Conflict of Interest

The author declares that there is no conflict of interest.

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REFERENCES

M. P. Crotty *et al.*, "Epidemiology, co-infections, and outcomes of viral pneumonia in adults: an observational cohort study," *Medicine (Baltimore).*, vol. 94, no. 50, 2015.

L. S. Cauley and A. T. Vella, "NIH Public Access," vol. 19, no. 102, pp. 33–40, 2015.

W. P. Glezen and F. W. Denny, "Epidemiology of acute lower respiratory disease in children," *N. Engl. J. Med.*, vol. 288, no. 10, pp. 498–505, 1973.

T. F. Murphy, F. W. Henderson, W. A. Clyde, A. M. Collier, and F. W. Denny, "Pneumonia: an eleven-year study in a pediatric practice," *Am. J. Epidemiol.*, vol. 113, no. 1, pp. 12–21, 1981.

N. C. Oswald, R. A. Shooter, and M. P. Curwen, "Pneumonia complicating Asian influenza," *Br. Med. J.*, vol. 2, no. 5108, p. 1305, 1958.

D. A. J. Tyrrell, "The pulmonary complications of influenza as seen in Sheffield in 1949," *QJM*, vol. 21, no. 3, pp. 291–306, 1952.

D. B. Louria, H. L. Blumenfeld, J. T. Ellis, E. D. Kilbourne, and D. E. Rogers, "Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza," *J. Clin. Invest.*, vol. 38, no. 1 Pt 1-2, p. 213, 1959.

A. L. Bisno, J. P. Griffin, K. A. Van Epps, H. B. Niell, and M. W. Rytel, "Pneumonia and Hong Kong influenza: a prospective study of the 1968-1969 epidemic," *Am. J. Med. Sci.*, vol. 261, no. 5, pp. 251–263, 1971.

S. A. Madhi and K. P. Klugman, "A role for Streptococcus pneumoniae in virus-associated pneumonia," *Nat. Med.*, vol. 10, pp. 811–813, 2004.

W. T. Jones, J. H. Menna, and D. E. Wennerstrom, "Lethal synergism induced in mice by influenza type A virus and type Ia group B streptococci.," *Infect. Immun.*, vol. 41, no. 2, pp. 618–623, 1983.

G. J. Jakab, "Mechanisms of bacterial superinfections in viral pneumonias.," *Schweiz. Med. Wochenschr.*, vol. 115, no. 3, pp. 75–86, 1985.

K. F. van der Sluijs *et al.*, "IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection," *J. Immunol.*, vol. 172, no. 12, pp. 7603–7609, 2004.

A. Didierlaurent *et al.*, "Sustained desensitization to bacterial Toll-like receptor ligands after resolutionof respiratory influenza infection," *J. Exp. Med.*, vol. 205, no. 2, pp. 323–329, 2008.

L. A. Pittet, L. Hall-Stoodley, M. R. Rutkowski, and A. G. Harmsen, "Influenza virus infection decreases tracheal mucociliary velocity and clearance of Streptococcus pneumoniae," *Am. J. Respir. Cell Mol. Biol.*, vol. 42, no. 4, pp. 450–460, 2010.

A. M. LeVine, V. Koeningsknecht, and J. M. Stark, "Decreased pulmonary clearance of S. pneumoniae following influenza A infection in mice," *J. Virol. Methods*, vol. 94, no. 1, pp. 173–186, 2001.

S. Uematsu and S. Akira, "Toll-Like receptors (TLRs) and their ligands.,"

Handb. Exp. Pharmacol., no. 183, pp. 1–20, 2008.

K. F. van der Sluijs, T. van der Poll, R. Lutter, N. P. Juffermans, and M. J. Schultz, "Bench-to-bedside review: bacterial pneumonia with influenza - pathogenesis and clinical implications.," *Crit. Care*, vol. 14, no. 2, p. 219, 2010.

K. R. Short, M. N. Habets, P. W. M. Hermans, and D. A. Diavatopoulos, "Interactions between Streptococcus pneumoniae and influenza virus: a mutually beneficial relationship?," *Future Microbiol.*, vol. 7, no. 5, pp. 609–24, 2012.

M. Boyd, K. Clezy, R. Lindley, and R. Pearce, "Pandemic influenza: clinical issues," *Med. J. Aust.*, vol. 185, no. 10, p. S44, 2006.

J. A. McCullers and J. E. Rehg, "Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor," *J. Infect. Dis.*, vol. 186, no. 3, pp. 341–350, 2002.

M.-C. Plotkowski, E. Puchelle, G. Beck, J. Jacquot, and C. Hannoun, "Adherence of Type I Streptococcus pneumoniae to Tracheal Epithelium of Mice Infected with Influenza A/PR8 Virus 1--3," *Am. Rev. Respir. Dis.*, vol. 134, no. 5, pp. 1040–1044, 1986.

E. W. A. Brydon, H. Smith, and C. Sweet, "Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-H292) cells limits pro-inflammatory cytokine release," *J. Gen. Virol.*, vol. 84, no. 9, pp. 2389–2400, 2003.

I. Fujimoto, J. Pan, T. Takizawa, and Y. Nakanishi, "Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages," *J. Virol.*, vol. 74, no. 7, pp. 3399–3403, 2000.

K. Sun and D. W. Metzger, "Inhibition of pulmonary antibacterial defense by interferon-[gamma] during recovery from influenza infection," *Nat. Med.*, vol. 14, no. 5, p. 558, 2008.

A. Shahangian *et al.*, "Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice," *J. Clin. Invest.*, vol. 119, no. 7, pp. 1910–1920, 2009.

L. A. McNamee and A. G. Harmsen, "Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection," *Infect. Immun.*, vol. 74, no. 12, pp. 6707–6721, 2006.

J. S. Abramson and H. R. Hudnor, "Effect of priming polymorphonuclear leukocytes with cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and G-CSF) on the host resistance to Streptococcus pneumoniae in chinchillas infected with influenza A virus," *Blood*, vol. 83, no. 7, pp. 1929–1934, 1994.

J. Verhoef, E. L. Mills, Y. Debets-Ossenkopp, and H. A. Verbrugh, "The effect of influenza virus on oxygen-dependent metabolism of human neutrophils," in *Biochemistry and Function of Phagocytes*, Springer, 1982, pp. 647–654.

J. S. Abramson, E. L. Mills, G. S. Giebink,

and P. G. Quie, "Depression of monocyte and polymorphonuclear leukocyte oxidative metabolism and bactericidal capacity by influenza A virus.," *Infect. Immun.*, vol. 35, no. 1, pp. 350–355, 1982.

H. E. Larson, R. P. Parry, and D. A. J. Tyrrell, "Impaired polymorphonuclear leucocyte chemotaxis after influenza virus infection," *Br. J. Dis. Chest*, vol. 74, pp. 56–62, 1980.

P. Ruutu, A. Vaheri, and T. U. Kosunen, "Depression of human neutrophil motility by influenza virus in vitro," *Scand. J. Immunol.*, vol. 6, no. 9, pp. 897–906, 1977.

G. Engelich, M. White, and K. L. Hartshorn, "Neutrophil survival is markedly reduced by incubation with influenza virus and Streptococcus pneumoniae: role of respiratory burst," *J. Leukoc. Biol.*, vol. 69, no. 1, pp. 50–56, 2001.

M. L. Colamussi, M. R. White, E. Crouch, and K. L. Hartshorn, "Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria," *Blood*, vol. 93, no. 7, pp. 2395–2403, 1999.

Y. Debets-Ossenkopp, E. L. Mills, W. C. Van Dijk, H. A. Verbrugh, and J. Verhoef, "Effect of influenza viras on phagocytic cells," *Eur. J. Clin. Microbiol.*, vol. 1, no. 3, pp. 171–177, 1982.

M. Rizwan *et al.*, "VacSol: a high throughput in silico pipeline to predict potential therapeutic targets in prokaryotic pathogens using subtractive reverse vaccinology," *BMC Bioinformatics*, vol. 18, no. 1, p. 106, 2017.

H. Singh and G. P. S. Raghava, "ProPred1: prediction of promiscuous MHC Class-I binding sites," *Bioinformatics*, vol. 19, no. 8, pp. 1009–1014, 2003.

H. Singh and G. P. S. Raghava, "ProPred: prediction of HLA-DR binding sites," *Bioinformatics*, vol. 17, no. 12, pp. 1236–1237, 2001.

I. A. Doytchinova and D. R. Flower, "VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines," *BMC Bioinformatics*, vol. 8, no. 1, p. 4, 2007.

H. Dar *et al.*, "Prediction of promiscuous T-cell epitopes in the Zika virus polyprotein: An in silico approach," *Asian Pac. J. Trop. Med.*, vol. 9, no. 9, pp. 844–850, 2016.

R. Vita *et al.*, "The immune epitope database (IEDB) 3.0," *Nucleic Acids Res.*, vol. 43, no. D1, pp. D405--D412, 2015.

J. Hou, Y. Liu, J. Hsi, H. Wang, R. Tao, and Y. Shao, "Cholera toxin B subunit acts as a potent systemic adjuvant for HIV-1 DNA vaccination intramuscularly in mice," *Hum. Vaccines Immunother.*, vol. 10, no. 5, pp. 1274–1283, 2014.

J. S. Klein, S. Jiang, R. P. Galimidi, J. R. Keeffe, P. J. Bjorkman, and L. Regan, "Design and characterization of structured protein linkers with differing flexibilities," *Protein Eng. Des. Sel.*, vol. 27, no. 10, pp. 325–330, 2014.

L. Song *et al.*, "An avian influenza A (H7N9) virus vaccine candidate based on the fusion protein of hemagglutinin globular head and Salmonella typhimurium flagellin.," *BMC Biotechnol.*,

vol. 15, p. 79, 2015.

R. Mukhopadhyay, S. Kundu, M. Debnath, and M. Biswas, "A comparative in silico 3D-docking study of reverse hydroxamate based MMP13 inhibitors and Quercetin 3-ObD-glucoside-a marine flavonoid glycoside."

A. Roy, A. Kucukural, and Y. Zhang, "I-TASSER: a unified platform for automated protein structure and function prediction," *Nat. Protoc.*, vol. 5, no. 4, pp. 725–738, 2010.

P. W. Rose *et al.*, "The RCSB Protein Data Bank: views of structural biology for basic and applied research and education," *Nucleic Acids Res.*, vol. 43, no. D1, pp. D345--D356, 2015.

S. J. de Vries and A. M. J. J. Bonvin, "CPORT: a consensus interface predictor and its performance in prediction-driven docking with HADDOCK," *PLoS One*, vol. 6, no. 3, p. e17695, 2011.

H. Chen and H.-X. Zhou, "Prediction of interface residues in protein--protein complexes by a consensus neural network method: test against NMR data," *Proteins Struct. Funct. Bioinforma.*, vol. 61, no. 1, pp. 21–35, 2005.

S. J. de Vries, A. D. J. van Dijk, and A. M. J. J. Bonvin, "WHISCY: What information does surface conservation yield? Application to data-driven docking," *Proteins Struct. Funct. Bioinforma.*, vol. 63, no. 3, pp. 479–489, 2006.

I. Kufareva, L. Budagyan, E. Raush, M. Totrov, and R. Abagyan, "PIER: protein interface recognition for structural proteomics," *Proteins Struct. Funct. Bioinforma.*, vol. 67, no. 2, pp. 400–417, 2007.

S. Liang, C. Zhang, S. Liu, and Y. Zhou, "Protein binding site prediction using an empirical scoring function," *Nucleic Acids Res.*, vol. 34, no. 13, pp. 3698–3707, 2006.

H. Neuvirth, R. Raz, and G. Schreiber, "ProMate: a structure based prediction program to identify the location of protein--protein binding sites," *J. Mol. Biol.*, vol. 338, no. 1, pp. 181–199, 2004.

A. Porollo and J. Meller, "Prediction-based fingerprints of protein--protein interactions," *Proteins Struct. Funct. Bioinforma.*, vol. 66, no. 3, pp. 630–645, 2007.

T. A. Wassenaar *et al.*, "WeNMR: structural biology on the grid," *J. Grid Comput.*, vol. 10, no. 4, pp. 743–767, 2012.

G. C. P. Van Zundert *et al.*, "The HADDOCK2. 2 web server: user-friendly integrative modeling of biomolecular complexes," *J. Mol. Biol.*, vol. 428, no. 4, pp. 720–725, 2016.

H. A. Dar, T. Zaheer, R. Z. Paracha, and A. Ali, "Structural analysis and insight into Zika virus NS5 mediated interferon inhibition," *Infect. Genet. Evol.*, vol. 51, pp. 143–152, 2017.