FOUR POTENTIAL BROAD-SPECTRUM NEUTRALIZING EPITOPES IN THE SPIKE PROTEIN OF SARS-COV-2 PREDICTED BY BIOINFORMATICS

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ABSTRACT

Introduction*: To date, seven human coronaviruses (HCoVs) have been discovered. SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is a newly emerged HCoV, which can induce fatal pneumonia infection. Coronavirus is a single-stranded RNA virus that is susceptible to mutation, making it difficult to develop vaccines. Broad-spectrum vaccines based on conserved neutralizing epitopes can not only prevent epidemic strains, but also has broad-spectrum antiviral property. In this study, bioinformatics was used to predict broad-spectrum neutralizing epitopes of HCoVs.*

Methods*: The spike (S) protein amino acid sequence of SARS-CoV-2 Wuhan-Hu-1 strain was used as a template for epitope prediction. The linear B cell neutralization epitopes were primarily predicted by four online prediction servers, including ABCpred2006, BepiPred2.0, COBEpro and SVMTriP. The peptide fragments were selected according to their common prediction results, and were further screened using the server VaxiJen2.0. The conservation of high-score candidate epitopes was analyzed by Clustal X2 software, and the spatial position of candidate epitopes in S protein were analyzed by the PyMOL software.*

Results*: According to the analysis above, four potential broad-spectrum linear B cell neutralizing epitopes were screened, which include the epitopes P6 (aa496-501) and P14 (aa1163-1168) conserved between SARS-CoV-2 and SARS-CoV; P15 (aa1196-1201) conserved among SARS-CoV-2, SARS-CoV and MERS-CoV; P12 (aa811-816) conserved among SARS-CoV-2, SARS-CoV and HCoV-OC43.*

Conclusions*: Four potential broad-spectrum linear B cell neutralizing epitopes in the S protein of SARS-CoV-2 were predicted, which lay a foundation for the development of HCoVs broad-spectrum vaccines.*

Keywords: *coronavirus, SARS-CoV-2, broad-spectrum neutralizing epitope, bioinformatics, vaccine.*

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Introduction

Human coronavirus (HCoV) is an enveloped single-stranded positive-sense RNA virus. Seven HCoVs have been identified so far, namely, SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-HKU1, HCoV-299E HCoV-OC43 and HCoV-NL63[(1)]. SARS-CoV-2 is a novel HCoV first discovered in January 2020. Like SARS-CoV and MERS-CoV that have been discovered previously, SARS-CoV-2 also causes deadly pneumonia[(2, 3)]. However, SARS-CoV-2 is different since it exhibits extremely strong infectivity and spreads throughout the world at an astonishing speed. SARS-CoV-2 has already caused a global pandemic and posed a serious threat to human health and life. In contrast, HCoV-HKU1, HCoV-299E, HCoV-OC43 and HCoV-NL63 are less hazardous, causing only common cold and respiratory tract symptoms. Vaccination is an effective way to prevent infectious diseases. However, no HCoVs vaccines have been approved for marketing to date. It has been shown that RNA viruses are highly mutable, which is one of the major reasons for the failure of some vaccines against the existing epidemic strains. This also adds to the difficulty in developing and updating effective vaccines[(4, 5)]. Therefore,

to develop broad-spectrum vaccines on conserved neutralizing epitopes in HCoVs is of great value in to preventing such infectious diseases.

The HCoVs genome mainly encodes four structural proteins, namely, spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N) Fig. 1). S protein is a key protein mediating the entry of HCoVs into the target cells of the host. The S protein consists of two subunits, S1 and S2. HCoVs binds to the target cells via the receptor-binding domain (RBD) within the S1 subunit[6, 7]. Then the proteases cleave the S protein at the S1/S2 and S2' sites, driving the conformational change of the two heptad repeats (HR1 and HR2) within the S2 subunit to form a six-helix bundle (6-HB). In this way, the fusion of the virus and the target cell membrane triggers viral infection[8]. S protein is also an important antigen protein. Some neutralizing epitopes have been identified in the S protein of SARS-CoV and MERS-CoV[9-14]. In previous studies, it was found that the antibodies targeting the S protein of SARS-CoV can also neutralize SARS-CoV-2[15, 16], indicating that the S protein contains potential broad-spectrum neutralizing epitope and could serve as the essential target for the development of broad-spectrum HCoVs vaccines.

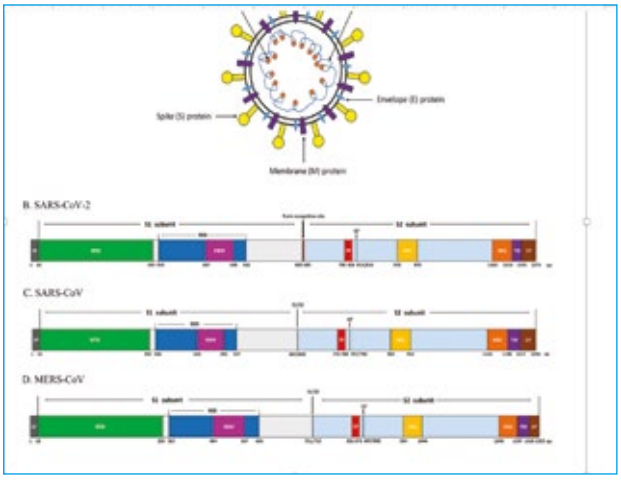


Fig. 1: The HCoVs pattern and the functional, structural domain patterns of the S protein of SARS-CoV-2, SARS-CoV, and MERS-CoV. A, The HCoVs structural proteins include S, M, E and N proteins.. B-D, The S protein structural schematics of SARS-CoV-2 (Uniprot: P0DTC2), SARS-CoV (Uniprot: P59594) and MERS-CoV (Uniprot: K9N5Q8). The S protein contains S1 and S2 subunits. SP, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; RBM, receptor-binding motif; FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, transmembrane; CP, cytoplasmic tail.

Identification of neutralizing epitopes by using conventional experimental methods is time-consuming and laborious. Bioinformatics prediction combined with experimental verification can save time and effort and speed up the identification of epitopes. In this study, multiple bioinformatics were employed to predict linear B-cell epitopes of SARS-CoV-2. The predicted epitopes were then aligned with the other six HCoVs to obtain the potential broad-spectrum neutralizing epitopes of HCoVs. These predicted epitopes could serve as the candidate targets for the development of broad-spectrum vaccines against HCoVs.

Methods

Data acquisition

Sequences of S protein were downloaded from the GenBank database of NCBI, including the reference amino acid sequences of SARS-CoV-2 (YP_009724390.1), SARS-CoV (NP_828851.1), MERS-CoV (YP_007188579.1), HCoV-OC43 (YP_009555241.1), HCoV-HKU1 (YP_173238.1), HCoV-NL63 (YP_003767.1), HCoV-229E (NP_073551.1), and the full-length amino acid sequences from all over the world were obtained, including 180 for SARS-CoV-2, 209 for SARS-CoV, 472 for MERS-CoV and 288 for HCoV-OC43.

Crystal structure models of the S protein were downloaded from the PDB database, including the SARS-CoV-2 S protein (PDB: 6VYB, 6LXT) and the SARS-CoV S protein (PDB: 5X5B).

Epitope prediction

In this study, the amino acid sequence of the SARS-CoV-2 strain Wuhan-Hu-1 (YP_009724390.1) was used as the template. Four linear B-cell epitope prediction online servers with different algorithms, namely, ABCpred2006 (threshold: 0.51), BepiPred2.0 (threshold: 0.5), COBEpro (threshold: 0.5) and SVMTriP (default threshold), were employed to preliminarily predict the linear B-cell neutralizing epitopes. To increase prediction accuracy, VaxiJen2.0 (threshold: 1.0), the online server with another different algorithm and a higher prediction accuracy rate (70%~89%), was used for further screening, shortening the scope by deleting the amino acid one by one. The top three highest-scoring peptides in each scope were chosen as the candidate neutralizing epitopes. If the top three peptides had similar positions, only the peptide with the highest scoring was chosen.

Spatial configuration analysis

PyMOL software was used to map the candidate epitopes on the SARS-CoV-2 S protein, and analyze the RBD structures of SARS-CoV-2 and SARS-CoV.

Conservation and identity analysis of amino acid sequences

The conservation of the candidate epitopes was analyzed with Clustal X2. The identity of the S protein amino acid sequences was determined by running Clustal W alignments with the MegAlign software of DNAstar2.0.

Results

The candidate linear B-cell epitopes in the SARS-CoV-2 S protein

The peptides S1 to S12 were predicted by ABCpred2006, BepiPred2.0, COBEpro, and SVMTriP, as shown in Supplementary Table 1. After a further screening with VaxiJen2.0, 17 candidate linear B-cell epitopes were obtained, as shown in Table 1. Ten candidate epitopes were located in major functional domains of the S protein, among which P2-P8 were located in RBD, P12 was near the S2' cleavage site, P14 and P15 were in the HR2 domain. Previous studies on HCoVs have already identified some neutralizing epitopes in these three key functional domains[17-20]. Therefore, these candidates were further analyzed for screening broad-spectrum neutralizing epitopes.

Name	Location	Peptide	VaxiJen2.0 Score
P1	317-322	NFRVQP	2.6356
P2	331-336	NITNLC	2.1975
P3	412-417	PGQTGK	2.845
P4	419-424	ADYNYK	2.469
P5	458-463	KSNLKP	2.5009
P6	496-501	GFQPTN	2.4879
P7	529-534	KSTNLV	1.8156
P8	534-539	VKNKCV	1.4975
P9	649-654	CLIGAE	1.7692
P10	672-677	ASYQTQ	1.0927
P11	683-688	RARSVA	1.8446
P12	811-816	KPSKRS	1.7987
P13	1110-1115	YEPQII	1.2991
P14	1163-1168	DVDLGD	3.2358
P15	1196-1201	SLIDLQ	2.2716
P16	1250-1255	CGSCCK	2.0904
P17	1262-1267	EPVLKG	1.3338

Table 1: Candidate linear B-cell neutralizing epitopes in the SARS-CoV-2 S protein.

Position mapping of the candidate linear B-cell neutralizing epitopes on the SARS-CoV-2 S protein

The RBD structure of SARS-CoV-2 has two conformations, referred to as the "up" and the "down", and the "up" is the key conformation for binding to the target cells[21, 22]. Ten candidate epitopes of interest were mapped on the S protein of the "up" RBD, as shown in Fig. 2-A. The epitopes P2-P8 and P12 were all in the loop structure on the protein surface, which indicates that they might serve as the recognition sites for functional antibodies. Since this model could only construct the amino acids from positions 27 to 1147, epitopes P14 and P15 were not localized in this model. The S2 subunit model of the SARS-CoV-2 S protein was obtained in addition, and the epitopes P14 and P15 were mapped on it, as shown in Fig. 2-B. Likewise, the two candidate epitopes were also located in the loop structure.

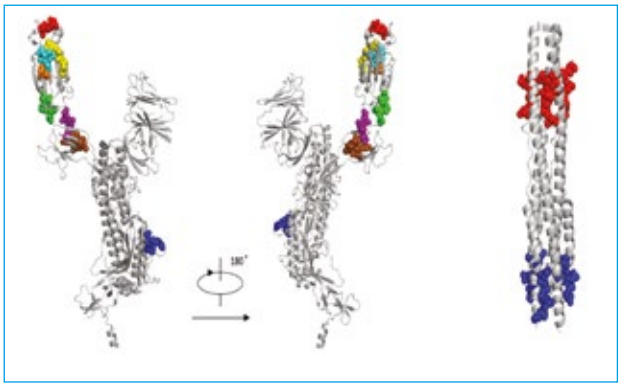


Fig. 2: Position mapping of the candidate linear B-cell epitopes on the SARS-CoV-2 S protein. The candidate epitopes were represented by using the Spheres model, with different epitopes in different colors. **A**, The S protein model (PDB: 6VYB, chain B), green for P2, yellow for P3, cyan for P4, orange for P5, red for P6, purple for P7, brown for P8, blue for P12. **B**, The S2 subunit model (PDB: 6LXT), red for P14, blue for P15.

Potential broad-spectrum neutralizing epitopes of HCoVs

The RBD determines the binding of the HCoVs to the host cells. Previous studies have shown that SARS-CoV-2 and SARS-CoV share the same cellular receptor angiotensin-converting enzyme 2 (ACE2). In the SARS-CoV-2 RBD, 18 amino acid residues were in direct contact with ACE2. In the SARS-CoV RBD, there are only 16. For both, the number of conserved amino acids interacting with ACE2 was 8[23, 24] (Fig. 3-B).. Our analysis showed that the amino acid sequence identity between SARS-CoV-2 RBD and SARS-CoV RBD was

73.4% (Supplementary Table 2). Ås to the structure, both RBD were conservative, with a root mean square deviation (RMSD) of 1.038 A over 149 Cα atoms (Fig. 3-A). The candidate epitopes P2-P8 were located within RBD. P6 contained four RBD/ACE2 binding sites, including Gly496, Asn498, Thr500 and Asn501. The Thr487 residue in SARS-CoV, corresponding to the Asn501 in SARS-CoV-2, was determined as the important binding site of SARS-CoV RBD for ACE2[25]. Moreover, these

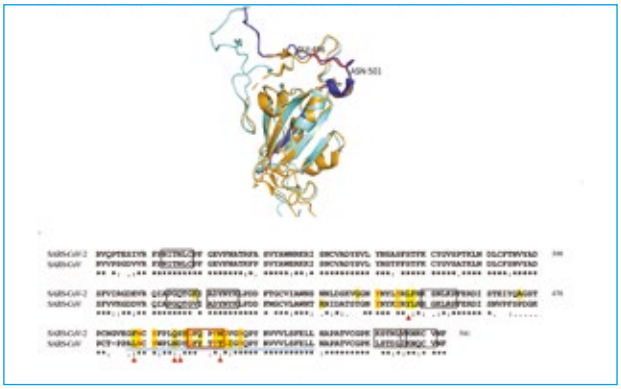


Fig. 3: A, Crystal structure alignment of RBD between SARS-CoV-2 and SARS-CoV. The orange color represents the crystal structure of SARS-CoV-2 RBD, cyan the crystal structure of SARS-CoV RBD, red the P6 peptide of SARS-CoV-2, and blue the linear neutralizing epitope of SARS-CoV that had been identified. **B**, Alignment between the SARS-CoV-2 RBD/ACE2 binding site and SARS-CoV RBD/ACE2. The yellow background represents the RBD/ACE2 binding site, and red font the same RBD/ACE2 binding site shared between SARS-CoV-2 and SARS-CoV. The red triangular symbol represents the important site for enhancing RBD/ACE2 binding in SARS-CoV. The blue underline represents the linear neutralizing epitope of SARS-CoV that has been previously identified. The box represents the localization of the candidate epitope P2-P8 in SARS-CoV-2, the red box the localization of P6.

two amino acid residues shared similar biochemical properties. Therefore, Asn501 might also be an important binding site for SARS-CoV-2 RBD/ACE2. The above results indicated that the antibodies for P6 peptide targeting these important sites might have a steric hindrance effect, thereby interfering or even blocking the binding of SARS-CoV-2 to the human ACE2 receptor. Besides, three amino acids in P6 were strictly conserved in SARS-CoV, and two were partially conserved. In Fig. 3-A, the alignment of the crystal structure of RBD indicates that the P6 was highly overlapped in SARS-CoV. Apparently, P6 was strongly conserved in SARS-CoV. And at the position corresponding to P6 in SARS-CoV, a neutralizing epitope (ALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFEL[17] has been identified, indicating that P6 might be a potential cross-neutralizing epitope against SARS-CoV-2 and SARS-CoV.

Among the predicted candidate epitopes, P12, P14 and P15 were all localized in the S2 subunit. Notably, the P12 (811-816aa) contained the S2' cleavage site (815/816aa), the second cleavage site of the S protein. The processing of the S2' cleavage site was a key event for activation of S protein. Furthermore, a neutralizing epitope (ILPDPLKPTKRSFIEDLLFNKVT)[18] was identified at the S2' cleavage site of SARS-CoV, indicating that antibodies targeting this site might interfere with the subsequent fusion between the virus and the host cell membrane by influencing the S2' cleavage. As shown in Fig. 4-A, S2'(R↓S) was highly conserved in HCoVs, and P12 was highly conserved in SARS-CoV and HCoV-OC43. Five out of six amino acids in P12 were completely conserved in SARS-CoV,

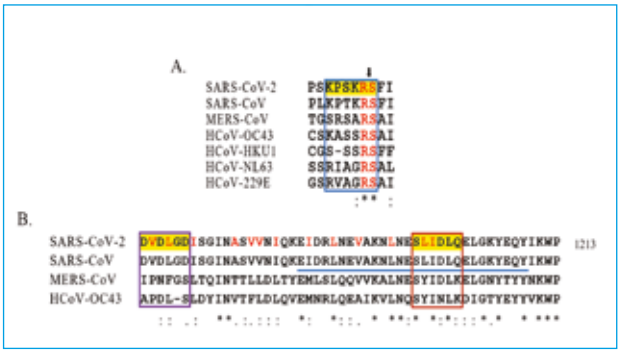


Fig. 4: A, Amino acid alignment of the S2' cleavage sites among seven HCoVs. The S2' cleavage site is represented by the arrowhead ↓. The red color represents the amino acids flanking the S2' cleavage site. The yellow background indicates the amino acid sequence of the candidate epitope P12 in SARS-CoV-2, and the blue box represents the location of P12. **B**, Amino acid alignment of HR2 in SARS-CoV-2 with that in SARS-CoV, MERS-CoV and HCoV-OC43. The red font represents the essential residue for the binding of HR2 to HR1; the yellow background indicates the amino acid sequence of the candidate epitopes P14 and P15 of SARS-CoV-2; the purple box represents the location of P14, the red box represents the location of P15, and the blue underlines the linear neutralizing epitope of SARS-CoV that had been previously identified.

except for that Ser813was substituted by Thr795with similar polarity. Besides, four amino acids of P12 in HCoV-OC43 were completely conserved. Substitution only occurred in two amino acids with similar polarity, i.e., Pro812→Ala900 and Lys814→Ser902 substitution. Therefore, P12 was considered to have similar antigenicity as the corresponding peptide in

SARS-CoV and HCoV-OC43, and that the P12-targeting antibodies possessed the potential to neutralize SARS-CoV-2, SARS-CoV and HCoV-OC43.

Conformational changes of HR2 and HR1 are the key events triggering viral membrane fusion. Recently, some researchers have successfully unraveled the core crystal structure of 6-HB in the S2 subunit of SARS-CoV-2 S protein, discovering the essential residues for the binding of HR2 to HR1[26]. P14 and P15 each contained two different essential residues for the binding of HR2 to HR1 (Fig. 4-B). Therefore, antibodies targeting these two sites may inhibit viral membrane fusion by influencing the binding of HR2 to HR1. Besides, according to the S protein amino acid alignment between SARS-CoV-2 and another six HCoVs (Supplementary Table 2), HR2 of SARS-CoV-2 had 100% identity with SARS-CoV. And more than 40% identity with MERS-CoV and HCoV-OC43. The alignment of the HR2 amino acid sequences of the four HCoVs was shown in Fig. 4-B. It was found that P14 was completely conserved in SARS-CoV and was the potential cross-neutralizing epitope against SARS-CoV-2 and SARS-CoV. P15 was completely conserved in SARS-CoV, and apart from this, it was highly conserved in MERS-CoV. Also, a neutralizing epitope (EIDRLNEVAKNLNESLIDLQELGKYEQY)[17] had been previously identified at the position of P15 in SARS-CoV, indicating that P15 might be a potential broad-spectrum neutralizing epitope among SARS-CoV-2, SARS-CoV and MERS-CoV. In MERS-CoV, two amino acid substitutions occurred in P15. The substitution of Gln1201by Lys1284with similar polarity, and the substitution of non-polar Leu1197) by polar Tyr1280. The L→Y substitution might influence the affinity of the P15-targeting antibodies to MERS-CoV.

Conservation analysis of potential broad-spectrum neutralizing epitopes

In order to expand the analysis of the conservation of the four potential broad-spectrum neutralizing epitopes, amino acid sequence alignments of full-length S proteins of HCoVs worldwide were performed by using the reference sequences as criteria (Supplementary Table 3). It was found that there is a high identity of 99.8% between SARS-CoV-2 S proteins. Although a total of 14 mutated sites were found as the evolution of SARS-CoV-2, none were located within the scope of the four potential broad-spectrum neutralizing epitopes. The amino acid sequences corresponding to the location of the four potential broad-spectrum neutralizing epitopes were also highly conserved among SARS-CoV, MERS-CoV and HCoV-OC43 by alignment, as shown in Supplementary Fig. 1.

Name	Location	Peptide	Length
S1	304-322	KSFTVEKGIYQTSNFRVQP	19
S2	329-363	FPNITNLCPFGEVFNATRFASVYAWNRKRISNCVA	35
S3	402-426	IRGDEVRQIAPGQTGKIADYNYKLP	25
S4	440-507	NLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIY-QAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQP	68
S5	516-540	ELLHAPATVCGPKKSTNLVKNKCVN	25
S6	648-666	GCLIGAEHVNNSYECDIPI	19
S7	672-690	ASYQTQTNSPRRARSVASQ	19
S8	803-818	SQILPDPSKPSKRSFI	16
S9	1107-1127	RNFYEPQIITTDNTFVSGNCD	21
S10	1129-1172	VIGIVNNTVYDPLQPELDSFKEELDKYFKNHT-SPDVDLGDISGI	44
S11	1180-1210	QKEIDRLNEVAKNLNESLIDLQELGKYEQYI	31
S12	1247-1267	CCSCGSCCKFDEDDSEPVLKG	21

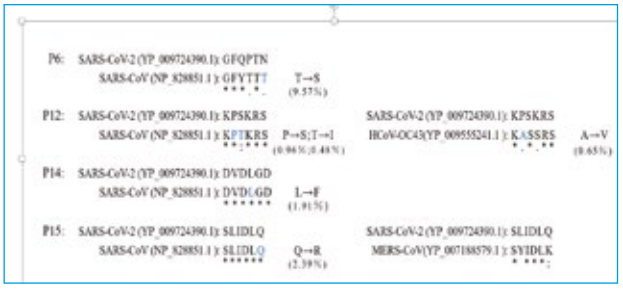
Supplementary Table 1: Peptides scope of the SARS-CoV-2 linear B cell neutralizing epitope co-predicted by the four servers.

	SARS-CoV-2 /SARS-CoV	SARS-CoV-2 /MERS-CoV	SARS-CoV-2 /HCoV-OC43	SARS-CoV-2 /HCoV-HKU1	SARS-CoV-2 /HCoV-NL63	SARS-CoV-2 /HCoV-229E
Spike	77.5%	28.7%	29.9%	28.2%	22.5%	28.1%
S1	66.0%	20.5%	21.4%	19.6%	12.7%	18.2%
S2	90.0%	43.7%	41.7%	39.1%	33.7%	35.4%
RBD	73.4%	19.5%	25.7%	21.7%	21.4%	26.5%
HR2	100.0%	43.1%	44.0%	37.3%	25.5%	31.4%

Supplementary Table 2: Identity analysis of amino acid reference sequences of S protein between SARS-CoV-2 and the other six HCoVs.

Subunit	Amino acid mutation site
S1:	28: Y→N, 49: H→Y, 50: S→L, 74: N→K, 157: F→L, 181: G→V, 221: S→W, 247: S→R, 408: R→I, 570: A→V, 614: D→G, 655: H→Y
S2:	797: F→C, 930: A →V

Supplementary Table 3: Amino acid mutation sites of the SARS-CoV-2 S protein.



Supplementary Fig. 1: Conservation analysis of the HCoVs sequences corresponding to the potential broad-spectrum neutralizing epitopes. The blue font represents the mutated amino acid residues. Enclosed in the brackets are the mutation rates of the corresponding amino acid residues.

Discussion

Rapid identification of neutralizing epitopes is crucial for vaccine development. In this study, bioinformatics methods were employed to predict

the epitopes using the S protein sequence of SARS-CoV-2 as a template. Then, through the alignment analysis with the reference sequence of HCoVs, the relatively conserved neutralizing epitopes of HCoVs were identified, providing reference for the development of broad-spectrum vaccines against HCoVs.

During the development of HCoVs vaccines, the S1 subunit is an important target for neutralizing antibodies, especially RBD. Previous studies have shown that neutralizing antibodies in the serum of the HCoVs-infected patients generally targeted to RBD, and these RBD-targeting specific monoclonal antibodies exhibited a strong neutralizing activity[27]. In this study, the predicted candidate epitope P6 was located within RBD and covered four essential sites for the binding to the ACE2 receptors on host cells. It was indicated that the P6-induced antibodies might have a steric hindrance effect, thereby interfering with or even blocking the binding of SARS-CoV-2 to the ACE2 receptor. Either in terms of the amino acid sequence or crystal structure, P6 is strictly conserved in SARS-CoV. Besides, a neutralizing epitope was identified at the position of P6 for SARS-CoV[17]. The above results indicated that P6 was a potential cross-neutralizing epitope between SARS-CoV-2 and SARS-CoV. However, from the perspective of evolution, RBD is located in the hypervariable region and prone to mutate. Our conservation analysis of the SARS-CoV-2 S protein also indicated amino acid mutation in the RBD of the SARS-CoV-2 S protein, with the substitution of 408 Arg by Ile[28]. Lost studies demonstrated that the epitopes targeting RBD are largely conformational epitopes[29-31] which are susceptible to change by a variety of factors. Therefore, RBD is not an ideal target for the development of broad-spectrum vaccines.

As compared with the S1 subunit, the amino acid sequence of the S2 subunit is more conserved and remains relatively conserved across different HCoVs. It was found in the previous studies on SARS, by Zhong [18] and Zhang[20] that serum antibodies in most of the recovered SARS patients targeted the S2 subunit and these S2-targeting antibodies could effectively neutralize SARS-CoV. Recently, Wu[32] also discovered high-titer neutralizing antibodies targeting S2 in the serum of the recovered patients infected with SARS-CoV-2. All of the above findings indicated that apart from the RBD, the S2 subunit is another important target for neutralizing antibodies against SARS-CoV-2. The potential of the S2 subunit to induce broadly neutralizing antibodies deserves our attention. In this study, the candidate epitopes, P12, P14 and P15, were all located in the S2 subunit. Among them, P12 contained the S2' cleavage site, the second cleavage site of the S protein. P14 and P15 were located within HR2, and both of them were related to the virus-host cell membrane fusion. Developing neutralizing antibodies that target such membrane fusion-related structural domains to block virus-host cell membrane fusion is another preventive strategy against viral infection. Lip[33, 34] demonstrated that the HR2 of SARS-CoV was able to induce the neutralizing polyclonal antibodies, that could inhibit cell membrane fusion. Another study also indicated that the peptides derived from HR2 of SARS-CoV and MERS-CoV could also inhibit infection effectively[35, 36]. In this study, our analysis showed that both P14 and P15 contained essential residues for the binding of HR2 to HR1. P14 was completely conserved in SARS-CoV. P15 was partially conserved in SARS-CoV, but highly conserved in MERS-CoV. All of the results above indicated good conservation of HR2, which may serve as a target for the broad-spectrum vaccine.

In summary, four potential cross-neutralizing epitopes were predicted by bioinformatics from the SARS-CoV-2 S protein, which could be served as the candidate targets for the development of broad-spectrum epitope vaccines against HCoVs.

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