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Computers in Biology and Medicine 143 (2022) 105272

Contents lists available at ScienceDirect

Computers in Biology and Medicine

journal homepage: www.elsevier.com/locate/compbiomed

High-specificity targets in SARS-CoV-2 N protein for serological detection and distinction from SARS-CoV

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ARTICLE INFO

Keywords:
Novel coronavirus (SARS-CoV-2)
Serological detection
N protein
Cross-reactivity
Epitope

ABSTRACT

Numerous serological detection kits are being rapidly developed and approved for screening and diagnosing suspected coronavirus disease 2019 (COVID-19) cases. However, cross-reactivity between pre-existing antibodies against other coronaviruses and the captured antigens in these kits can affect detection accuracy, emphasizing the necessity for identifying highly specific antigen fragments for antibody detection. Thus, we performed a conservation and specificity analysis of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid (N) protein. We also integrated various B-cell epitope prediction methods to obtain possible dominant epitope regions for the N protein, analyzed the differences in serological antibody levels for different epitopes using ELISA, and identified N protein epitopes for IgG and IgM with high-specificity. The SARS-CoV-2 N protein showed low mutation rates and shared the highest amino acid similarity with SARS-CoV; however, it differed substantially from other coronaviruses. Tests targeting the SARS-CoV-2 N protein produce strong positive results in patients recovering from SARS-CoV. The N18–39 and N183-197 epitopes for IgG and IgM detection, respectively, can effectively overcome cross-reactivity, and even exhibit good specificity between SARS-CoV-2 and SARS-CoV. The antibody levels detected with these were consistent with those detected using the complete N protein. These findings provide a basis for serological diagnosis and determining the kinetics of SARS-CoV-2 antibody detection in patients.

1. Introduction

An acute infectious pneumonia, coronavirus disease 2019 (COVID-19), recently erupted worldwide. Patients commonly present with pneumonia and chest CT abnormalities as primary symptoms, followed by acute cardiac injury and secondary infection as complications, and even death in serious cases [1,2]. The pathogen is a novel coronavirus, namely, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the seventh known coronavirus to have infected humans [3]. Although the transmission rate of SARS-CoV-2 is yet to be confirmed, it has rampaged across the globe at an alarming rate. On January 30, 2020, the World Health Organization (WHO) declared COVID-19 as an international public health emergency. As of April 8, 2021, this disease has spread to 223 countries and regions, with nearly 132.73 million reported cases and more than 2.88 million deaths [4,5].

Nasopharyngeal swab nucleic acid tests are recommended for the clinical diagnosis of SARS-CoV-2 and are currently the gold standard for confirming SARS-CoV-2 infection [6–8]. However, the reported negative results obtained using this platform necessitate repeated testing, thereby limiting the use of nucleic acid testing in epidemic control and clinical

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https://doi.org/10.1016/j.compbiomed.2022.105272
Received 25 October 2021; Received in revised form 14 January 2022; Accepted 24 January 2022
Available online 29 January 2022

diagnosis. In fact, some cases have only been confirmed by alveolar lavage fluid assessment, while antibody testing can also be employed for supplementary diagnosis, particularly for suspected patients with consistent negative nucleic acid results [9,10]. In addition, antibody detection can help determine a patient's infection stage to guide clinical treatment [11], or can be used for serological surveys and past exposure surveys in high-risk population groups to develop prevention and control strategies [12].

The nucleocapsid (N) protein of coronaviruses has strong antigenicity and plays an important role in inducing the host immune responses during SARS-CoV-2 infection [13]. Moreover, it has been widely applied as the main target in diagnosing SARS-CoV infection [14–17]. Until February 10, 2021, 27 SARS-CoV-2 antibody detection kits have been approved by the National Medical Products Administration of China (NMPA), and these primarily use SARS-CoV-2 N or S, or specific N or S protein fragments as capture antigens [18]. However, most clinical trials conducted with these kits have only assessed patients with COVID-19, whereas samples infected with other coronaviruses are not included; in particular, SARS-CoV, has been widely confirmed to be highly similar to SARS-CoV-2. Recent studies have reported that 23% and 16% of the known SARS-CoV T-cell and B-cell epitopes, respectively, map identical to those of SARS-CoV-2, thus increasing the false positives caused by cross-reactivity [19,20]. However, it remains unclear whether the currently approved SARS-CoV-2 antibody detection kits can avoid cross-reaction with pre-existing antibodies against other coronaviruses.

Several unaddressed issues have restricted the application of diagnostic ELISA kits in clinical practice, including (1) whether pre-existing N protein antibodies in persons infected with other coronaviruses, especially SARS-CoV, can cross-react with the SARS-CoV-2 N protein, thus affecting the accuracy of diagnostic results; (2) whether the detection level of the N protein high-specificity region is consistent with that of the complete N protein; and (3) which epitope represents the optimal N protein high-specificity region. It is, therefore, necessary to clarify the specificity of SARS-CoV-2 N protein, as the main antibody detection target, and to determine a high-specificity region that can accurately quantify antibody levels.

In this study, the SARS-CoV-2 N protein coding genome was downloaded from a public database to analyze its conservation, and its specificity was analyzed by incorporating other coronaviruses including Bat-CoV, SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-OC43. B-cell epitope prediction software and online servers were integrated to obtain possible high-specificity epitope regions for the SARS-CoV-2 N protein. Finally, clinical serological testing was performed using ELISA to identify the epitope regions suitable for diagnosing IgM and IgG levels. Our findings could help elucidate the target of the SARS-CoV-2 N protein involved in the host immune response, and provide a theoretical basis for antibody detection methods that can avoid cross-reactivity.

2. Materials and methods

2.1. Collection of samples and epidemiological information

In total, 22 serum samples were collected from patients with COVID-19; of these, 19 were collected during hospitalization, whereas the remaining three were from patients who were discharged following qPCR testing of throat swabs, turning from positive to negative and subsequently reverting to positive during follow-up. Seven patients were sampled upon admission to the Second People's Hospital of Guangdong Province. Twelve patients were sampled when admitted to Yangjiang People's Hospital. Eight serum samples of SARS-CoV-infected patients were provided by Zhujiang Hospital, Southern Medical University. These samples were collected during the recovery period of patients infected with SARS-CoV in 2003. The epidemiological data for all patients and samples, including age, gender, time of admission, patient symptoms, disease classification, and sampling time were obtained and collated by the collection unit and subsequently subjected to statistical analysis at our laboratory.

2.2. Genome alignment and phylogenetic tree analysis of N protein

To determine the conservation of SARS-CoV-2 N protein, all M, N, E, and S protein sequences of SARS-CoV-2 were downloaded from GASID database (https://www.gisaid.org/). Following removal of sequences with unknown amino acids or incomplete full-length sequences (419 amino acid for M protein, 222 for N protein, 75 for E protein, and 1273 for S protein, respectively). The human SARS-CoV-2 M, N, E, and S protein sequences from Wuhan-Hu-1, China (GenBank number: MN908947.3) were used as the reference sequence to analyze the mutations. All M, N, E, and S proteins were aligned to the reference using DIAMOND [21]. A mutation rate was obtained after each protein was aligned with the reference sequence and all mutation rates were calculated using the R environment (v4.0.2).

For the specificity of SARS-CoV-2 N protein, the N protein genomes of the other coronaviruses, including Bat-CoV, SARS-CoV, MERS-CoV, HCoV-229E, and HcoV-OC43, were obtained from GenBank database (https://www.ncbi.nlm.nih.gov/). Sequence alignment was conducted in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL) using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was performed using MEGA6.0 (http://www.megasoftware.net/), and the maximum likelihood method was used to construct the phylogenetic tree using the Tamura-Nei model and Gamma distribution with 1000 iterations [22].

2.3. B-cell epitope prediction for SARS-CoV-2 N protein

B-cell epitope prediction software and online servers were integrated to obtain the dominant epitope regions shared by all three methods. First, ABCpred (http://crdd.osdd.net/raghava/abcpred/ABC_submission.html) and BCEpred (http://crdd.osdd.net/raghava/bcepred/bcepred_submission.html), which have scoring systems and present results in a table format, as well as the authoritative database IEDB (http://tools.iedb.org/bcell/), were used to perform B-cell epitope prediction of the SARS-CoV-2 N protein. SOMPA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), DNAstar's Protean (https://www.dnastar.com/), and Discovery Studio 2.5 (DS2.5) (http://accelrys.com/products/collaborative-science/biovia-discovery-studio/) were used for stepwise verification of the secondary structure and three-dimensional spatial conformation of the dominant regions. The three-dimensional structure of SARS-CoV-2 N protein (GenBank accession number MN908947.3), was resolved by the Zhangyang Lab and is available for copyrighted free use (https://zhanglab.ccmb.med.umich.edu/COVID-19/). The three-dimensional structure of residue 1–249 of SARS-CoV N protein (GenBank accession number NC004718.3) was predicted using the C–I-TASSER On-line Server of Zhangyang Lab (https://zhanglab.dcmb.med.umich.edu/C-I-TASSER/) with the submission number CIT1856 (https://zhanglab.dcmb.med.umich.edu/C-I-TASSER/output/CIT1856/). Finally, the potential B-cell epitopes were identified.

2.4. ELISA testing

SARS-CoV-2 N protein expressed by prokaryotic vectors (provided by Nanjing Vazyme Medical Technology Co., Ltd) or polypeptide fragments of epitope regions (Synthesized by the GL Biochem (Shanghai) Ltd) at 5 μg/ml in 100 μl, were absorbed overnight at 4 °C to ELISA plates (JET BIOFIL, China) and blocked with 300 μl of 5% skimmed milk powder at 37 °C for 2 h. The ELISA plates were then incubated for 1 h at 37 °C with 100 μl of primary antibody solution consisting of patients' serum samples diluted 100-fold and subjected to a two-fold gradient dilution, followed by binding with 100 μl HRP-labeled mouse anti-human IgG and IgM secondary antibodies, respectively. After staining with 100 μl TMB

solution for 5 min and reaction termination with 50 μl of 2 M sulfuric acid stop solution, the OD values were read at 450 nm. (Sample OD-negative OD)/(Negative OD-blank OD) $\geq$ 2.1 served as the criterion for determining a positive reaction. The highest dilution for a positive test was regarded as the patient's serum antibody titer.

2.5. Colloidal gold strip testing

Two colloidal gold kits for the emergency detection of SARS-CoV-2 antibodies, approved by the Food and Drug Administration of China, were used and purchased from Nanjing Vazyme Medical Technology Co., Ltd and Wondfo Biotech Co., Ltd. The former can simultaneously detect IgG and IgM, whereas the latter can only detect IgG. The test cards were placed horizontally, according to the manufacturer's instructions, and serum was added to the sample well. Then, 20 μl of diluted samples were added and the results were read after 10–15 min.

2.6. Statistical analysis

ELISA data were collated and statistically analyzed using SPSS 20.0. A paired samples *t*-test was performed to analyze the serum antibody titer of clinical patients using SARS-CoV-2 N protein and nine epitope polypeptides.

3. Results

3.1. Conservation and specificity of SARS-CoV-2 N protein

All M, N, E, and S protein amino acid sequences were downloaded from GASID on December 16, 2021. Finally, 5,436,842 M protein, 5,167,111 N protein, 5,590,251 E protein, and 3,753,058 S protein sequences were retained. The viral structural proteins showed low mutation rates in their amino acid sequences during the transmission process and were highly conserved. Among them, the mutation rates of S and N proteins, which are closely associated with virus-host interactions, are 0.572% and 0.677%, respectively (Table 1).

The N protein sequences of the other coronaviruses were incorporated in constructing the phylogenetic tree. Coronaviruses of the same lineage with the capacity for human-to-human transmission were clustered on the same branch, on which SARS-CoV and SARS-CoV-2 showed the closest genetic relationship. Bat-CoV, isolated at different times from different bat species, was clustered with SARS-CoV and SARS-CoV-2 (Fig. 1a). Five sequences for HCoV-229E, 26 for HCoV-OC43, 21 for SARS-CoV, and 23 for MERS-CoV, from strains isolated in different years from regions, were aligned with the amino acid sequences of the N protein of MN908947.3. The results showed that the SARS-CoV-2 N protein differed significantly from the weakly pathogenic HCoV-229E and HCoV-OC43, which are commonly found in the population, as well as MERS-CoV, which can cause severe respiratory syndrome, thus showing strong specificity. However, compared with SARS-CoV, which has several long and highly conserved regions, the SARS-CoV-2 N protein showed weak conservation (Table 2 and Fig. 1b).

Table 1
Mutation rates of amino acid sequences for structural proteins during SARS-CoV-2 transmission.

Protein	Number	Mean ± SD (mutation rates %)	Functions in SARS-CoV
S	3,753,058	0.572 ± 0.234	Induction of neutralizing antibodies, research target of drugs and vaccines [23]
E	5,590,251	0.126 ± 1.61	Related to envelope formation [24]
M	5,436,842	0.277 ± 0.244	Related to envelope formation and membrane transport [25]
N	5,167,111	0.677 ± 0.369	Strong antigenicity, induction of cellular immune response; main target for the establishment of serological diagnostic methods [14–17,26]

3.2. Feasibility analysis of SARS-CoV-2 N protein as a target for clinical serological antibody detection

The ELISA results, with N protein as the target, were highly consistent with the qualitative IgG detection results using colloidal gold test strips. The ELISA method showed greater sensitivity and could detect antibodies in serum earlier. Alternatively, the IgM test results varied greatly with only four tests proving positive, despite poor regularity of symptom onset distribution. ELISA detected positive results as early as six days after symptom onset. IgM could be detected in mild cases for approximately 17 days and in severe cases (15 and 17) after more than 20 days of infection (Table 3).

The mean age of the 19 included patients was 46.2 years, with 12 men (63.2%) and 7 women. More patients presented with cough (73.7%) on admission compared to fever (47.4%); even among the three severe cases, only one was febrile (Fig. 2).

Moreover, IgM appeared earlier than IgG, within the first week of symptom onset. Within the first 6 days of symptom onset, a seropositive reaction could be detected even after 800-fold dilution of the patient's serum. The levels of both antibodies increased in the second week, with IgG showing a more significant increase. Thereafter, serum IgG maintained a high antibody titer until the recovery period (after the third week of symptom onset), whereas IgM began to decrease gradually. In the serum of nine patients tested after 19 days, IgM was only detected in serum collected from two severe cases at 20 and 21 days, whereas that of the remaining patients, subjected to a 100-fold dilution, tested negative (Fig. 2a and b). Further, there were no significant differences in the serum IgG and IgM levels between the nine mild cases and three severe cases during the recovery period (Fig. 2c). As for the three mild cases with "re-detectable positives", the serum IgG levels did not differ significantly between the recovery period during hospitalization and after discharge. Meanwhile, IgM became positive in all cases after discharge, with a titer significantly higher than that of the serum levels during recovery (Fig. 2d).

The serum colloidal gold test strip results for eight patients infected with SARS-CoV in the recovery stage were all strongly positive for IgG and negative for IgM (Fig. 2e). The use of the SARS-CoV-2 N protein for ELISA detection produced results that were was highly consistent with the test strip results, even when the antibody titer was as high as 10^4, suggesting that use of the SARS-CoV-2 N protein as the detection target will show strong cross-reactivity with the serum of patients infected with SARS-CoV, resulting in erroneous results. (Fig. 2f).

3.3. Epitope prediction for SARS-CoV-2 N protein

ABCpred, BCEpred, and IEDB were used to perform epitope prediction for the N protein of SARS-CoV-2, and the results of the three software were collated and compared. Five dominant epitope regions shared among the three were obtained, namely, N18–39, N183–197, N249–266, N276-299, and N365-391 (Fig. 3a and Table 4).

In general, the secondary structure of a good epitope should be flexible, hydrophilic (>0), surface accessible (>0) and antigenic (>0). At the same time, the tertiary structure should contain β-turns or coils, which are easily displayed on the surface as antigenic epitopes to facilitate binding of the antibody because of their loose structure, rather than α-helices and β-sheets, which are difficult for antibody binding. These regions in our study showed good flexibility (Fig. 3b), hydrophilicity (Fig. 3c), antigenicity (Fig. 3d), and surface accessibility (Fig. 3e). Simulation of the N protein three-dimensional structure showed that the five regions were located on the protein surface. N18–39 and N183-197 were free random-coil loop structures (Fig. 4a), whereas N249–266, N276-299, and N365-391 showed similar random coil + α helix

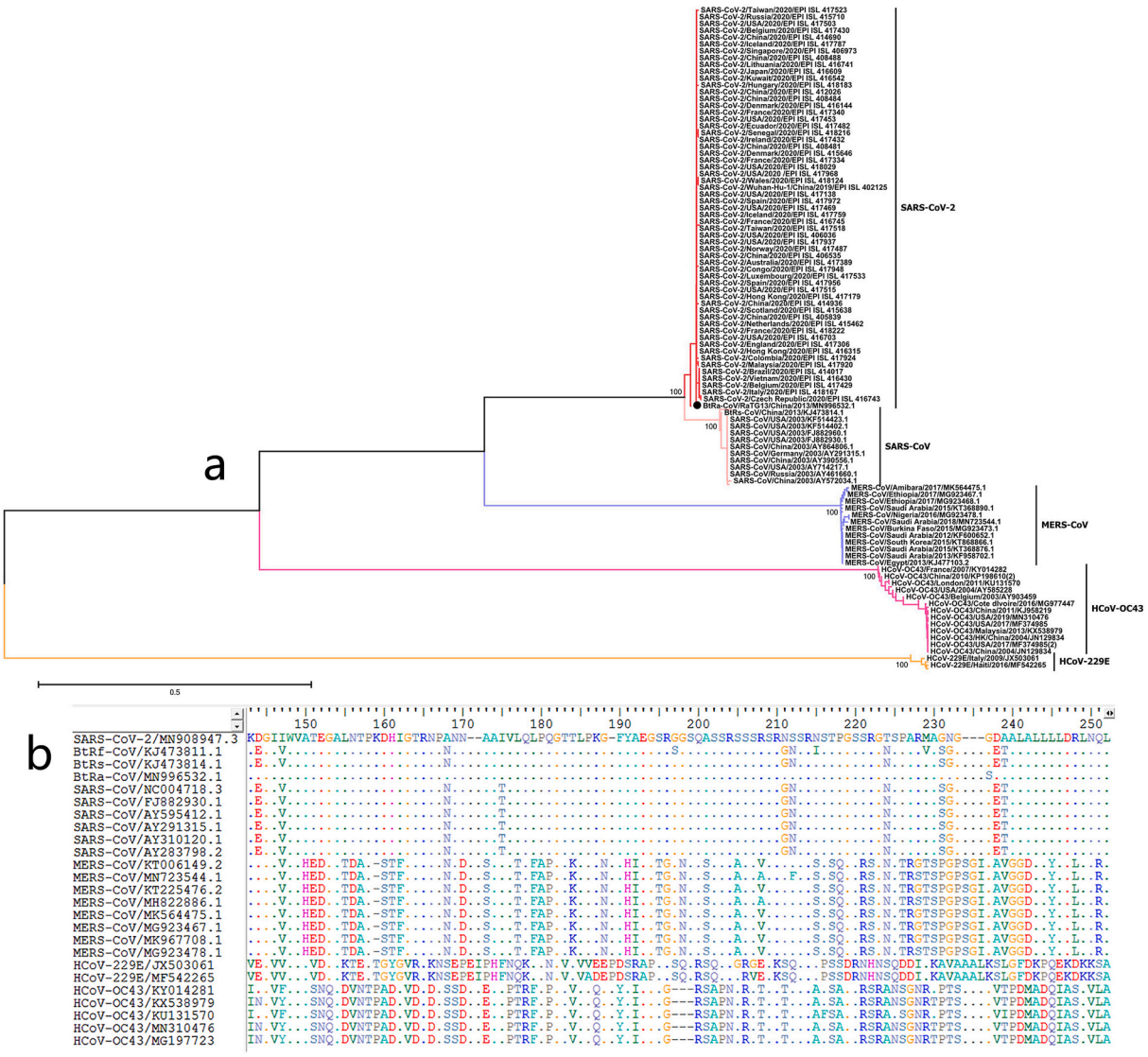

Fig. 1. Specificity analysis of the SARS-CoV-2 N protein. (a) Phylogenetic tree of the N protein from SARS-CoV-2, Bat-CoV, SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-OC43 from outbreaks in different epidemic years in different countries and regions. (b) N protein sequence alignment of SARS-CoV-2, Bat-CoV, SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-OC43.

Table 2
Conservative rates of amino acid sequences for N proteins between SARS-CoV-2 and other coronaviruses.

Coronaviruses	conserved sites/Length	Conservative rate (%)	Containing long highly conserved regions (More than 15 amino acids)
SARS-CoV	381/422	90.28	YES
MERS-CoV	198/413	47.94	NO
HCoV-229E	94/389	24.16	NO
HCoV-OC43	125/448	27.9	NO

structural characteristics (Fig. 4b and c); all of them showed good antigenicity.

The N proteins of SARS-CoV-2 and SARS-CoV showed many long and conserved regions. To analyze the effects of pre-existing antibodies in the SARS-CoV-infected population on the detection of SARS-CoV-2 when using the five epitope regions for serological diagnosis, sequence alignment was performed among the five epitope regions of the SARS-CoV-2 and the SARS-CoV N proteins. N18–39 and N365-391 differed significantly between SARS-CoV-2 and SARS-CoV and showed strong specificity. N183–197 and N276-299 showed two and one differential sites, respectively, but had > ten consecutive fully conserved amino acid residues. The N249-266 region was fully conserved in both (Fig. 4d). Therefore, nine polypeptides corresponding to the five epitope regions of the SARS-CoV-2 and SARS-CoV N proteins were synthesized and used for verification in subsequent ELISA testing (Table 4).

3.4. Feasibility analysis of using five epitopes for clinical serological ELISA testing

Based on the positive ELISA results for IgG and IgM in the serum samples of the 19 included patients, IgM antibody tests for N18–39 and N365-391 were negative. The other three polypeptides showed positive reactions for IgG and IgM. (Fig. 5a and b).

For IgG detection, the five-polypeptide epitope of the SARS-CoV-2 N protein showed specific positive reactions for IgG in serum. However, the N18-39 polypeptide alone did not exhibit significant differences for N protein following the detection of serum IgG levels in the 19 patients. The detection levels of the other four polypeptides were lower than those of the N protein (Fig. 5a). In addition, the detection level of the N18-39 polypeptide in the corresponding region of the SARS-CoV N

Table 3
Epidemiological information and serological antibody test results for COVID-19 patients.

Patient	Age	Sex	City	Date	Fever	Cough	Disease	IgG			IgM	
								Vazyme	Wondfo	ELISA	Vazyme	ELISA
1	41	♀	GZ	6	✓	×	Mild	–	–	–	+	–
2	24	♂	YJ	6	×	✓	Mild	–	–	–	–	–
3	27	♀	YJ	8	×	✓	Mild	+	+	+	+	–
4	58	♂	GZ	8	×	✓	Mild	+	–	–	+	–
5	35	♂	GZ	13	×	✓	Mild	+	–	–	+	–
6	49	♀	GZ	13	×	✓	Mild	+	+	+	+	+
7	57	♂	GZ	14	✓	×	Mild	+	–	+	+	–
8	42	♂	GZ	15	✓	✓	Mild	+	+	+	+	+
9[a]	28	♂	YJ	16	✓	×	Mild	+	+	+	+	–
10	61	♂	YJ	17	✓	×	Mild	+	+	+	–	–
11	56	♂	YJ	17	×	✓	Mild	+	+	+	+	–
12[a]	60	♀	YJ	17	×	✓	Mild	+	+	+	+	–
13	23	♀	YJ	19	✓	✓	Mild	+	+	–	–	–
14	35	♂	YJ	19	×	✓	Severe	+	+	+	–	–
15	56	♀	YJ	20	✓	✓	Severe	+	+	+	+	+
16	56	♀	GZ	21	✓	×	Mild	+	+	+	–	–
17	77	♂	YJ	21	×	✓	Severe	+	+	+	+	–
18	24	♂	YJ	21	×	✓	Mild	+	+	+	–	–
19[a]	69	♂	YJ	24	✓	✓	Mild	+	+	+	–	+

[a] Represents patients who tested positive using QPCR and ELISA detection after discharge. ♀: Male; ♂: Female; GZ: Guangzhou; YJ: Yangjiang.

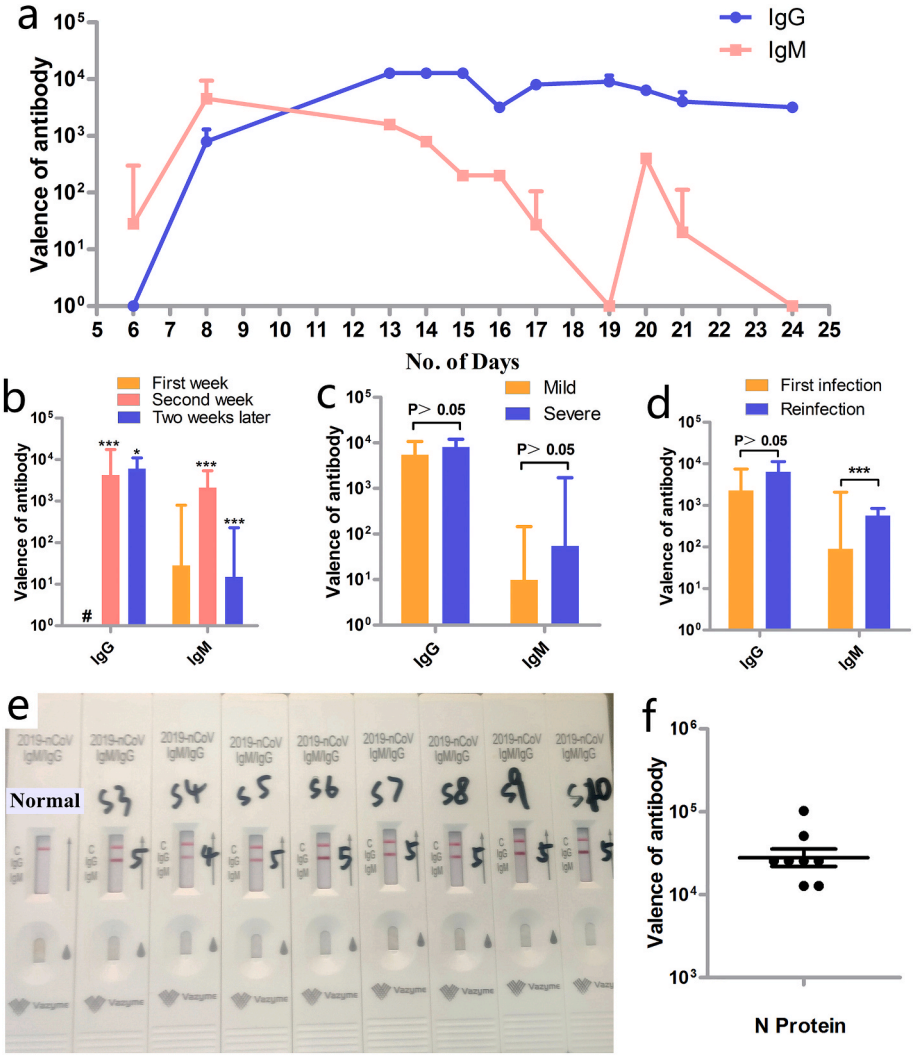

Fig. 2. Serum antibody titer of clinical patients for SARS-CoV-2 N protein detected using ELISA. (a) During disease progression, patients' serum IgG and IgM antibody titers showed dynamic changes. A detection value of 100 indicates that the ELISA result was negative for serum diluted 100-fold. (b) Differences in the serum IgG and IgM antibody titers of COVID-19 patients in the first, second, and two weeks after onset. #, tested negative for serum diluted 100-fold; *P < 0.05, ***P < 0.001 between the latter and former periods. (c) Differences in serum IgG and IgM antibody titers between mild and severe COVID-19 cases two weeks after onset. (d) Comparison of serum IgG and IgM antibody levels in patients with "re-detectable positives" during hospitalization and after discharge, ***P < 0.001. (e) SARS-CoV-2 antibody detection kit for testing the serum of SARS-CoV infected patients during the recovery period. (f) ELISA method targeting the SARS-CoV-2 N protein to test the serum of SARS-CoV infected patients during recovery.

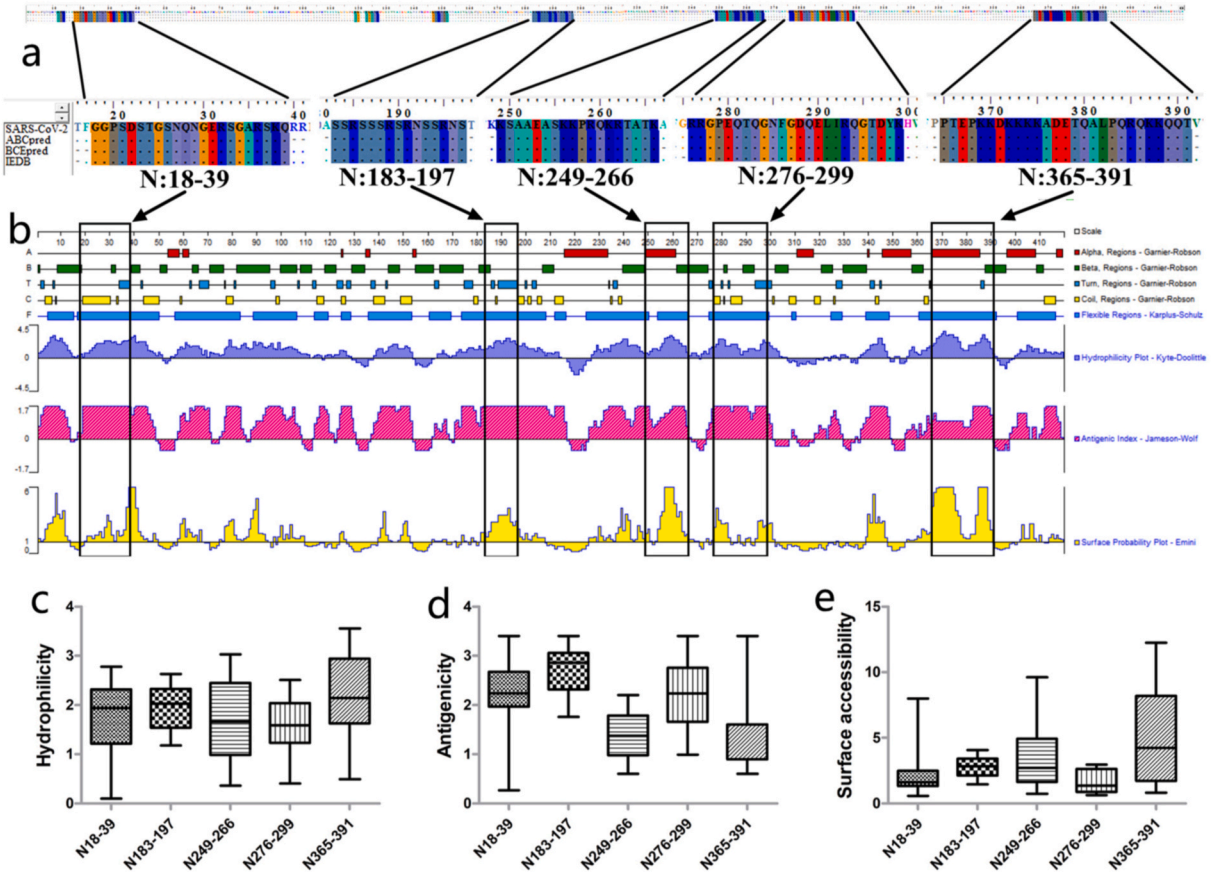

Fig. 3. Epitope prediction process for the SARS-CoV-2 N protein. (a) Alignment of epitope prediction results from ABCpred, BCEpred, and IEDB for SARS-CoV-2 N protein. (b) The secondary structure of the five epitope regions predicted by Protean of DNAstar. (c–e) Hydrophilicity, antigenicity and surface accessibility of the five epitope regions predicted by Protean of DNAstar.

Table 4
Synthetic polypeptides corresponding to the five epitope regions in SARS-CoV-2 and SARS-CoV N proteins.

Polypeptide	Region	Amino acid sequence	Length
SARS-CoV[a]			
P1	N19-40	GGPTDSTDNNQNGGRNGARPKQ	22
P2	N184-198	SSRSSSRSRGNSRNS	15
P3	N277-300	RRGPEQTQGNFGDQDLIRQGTDYK	24
P4	N366-392	PTEPKKDKKKKTDEAQPLPQRQKKQPT	27
SARS-CoV-2[b]			
P5	N18-39	GGPSDSTGSNQNGERSGARSKQ	22
P6	N183-197	SSRSSSRSRNSSRNS	15
P7	N249-266	KSAAEASKKPRQKRTATK	18
P8	N276-299	RRGPEQTQGNFGDQELIRQGTDYK	24
P9	N365-391	PTEPKKDKKKKADETQALPQRQKKQQT	26

[a] The GenBank accession number NC004718.3 was used for SARS-CoV.
[b] The GenBank accession number MN908947.3 was used for SARS-CoV-2.

protein (N19-40) was the lowest among all four corresponding polypeptides. Only 4 of 19 samples tested positive, and the antibody titer was as low as 100-fold (Fig. 5a). Meanwhile, similar results were obtained for SARS-CoV-infected patients, showing high antibody levels during the recovery period. Compared with the high IgG antibody titer detected with the N19-40 peptide of SARS-CoV, the N protein of SARS-CoV-2 showed a higher level of antibody titer detection, suggesting that there may be a large number of N proteins targeting different sites of cross-reactive antibodies that can interfere with the ELISA test results. Among the four peptides with different amino acid sequences between the two viruses, detection of the N18-39 polypeptide in the serum of eight patients was positive, with the most stable difference between samples and lowest titers, and only a 100–800-fold dilution produced positive results (Fig. 5c). This suggests that the N18-39 polypeptide has good specificity and accuracy when used to detect the IgG antibody levels in serum samples from patients with COVID-19.

Although a positive IgM reaction could be detected using the N278-299 polypeptide, the antibody level detected was significantly lower than that with the N protein. The detection levels of N183–197 and N249-266 showed good agreement with that of the N protein, with no statistically significant differences (Fig. 5b). There are two differential sites in the amino acid sequence of the SARS-CoV-2 N183-197 region and the corresponding N184-198 region in SARS-CoV, which caused N184-198 to produce negative results for the IgM of patients with COVID-19. Hence, the SARS-CoV-2 N183-197 polypeptide has good specificity. In contrast, the N249-266 polypeptide sequence is identical between viruses, and the presence of IgM antibodies can be detected in the sera of patients infected with either virus (Fig. 5b).

To analyze why SARS-CoV-2 N18–39 and N183-197 can be distinguished from SARS-CoV, we aligned the three-dimensional structure of the N protein of the two viruses through DS2.5. The results show that SARS-CoV-2 and SARS-CoV have similar N-terminal RNA-binding domains, whereas N18–39 and N183-197, containing differences between the two viruses, are located on both sides of the RNA-binding domain, respectively. The N21T→S, N25D→G, and N26 N→S mutations of the N18-39 region changed its three-dimensional structure, forming a free random-coil loop structure, while the N192G→N and N193 N→S mutations of N183-197 turned the region into a complex continuous loop structure (Fig. 5d).These changes may change the docking interface formed by the antigen-antibody complex, resulting in undetectable results in ELISA.

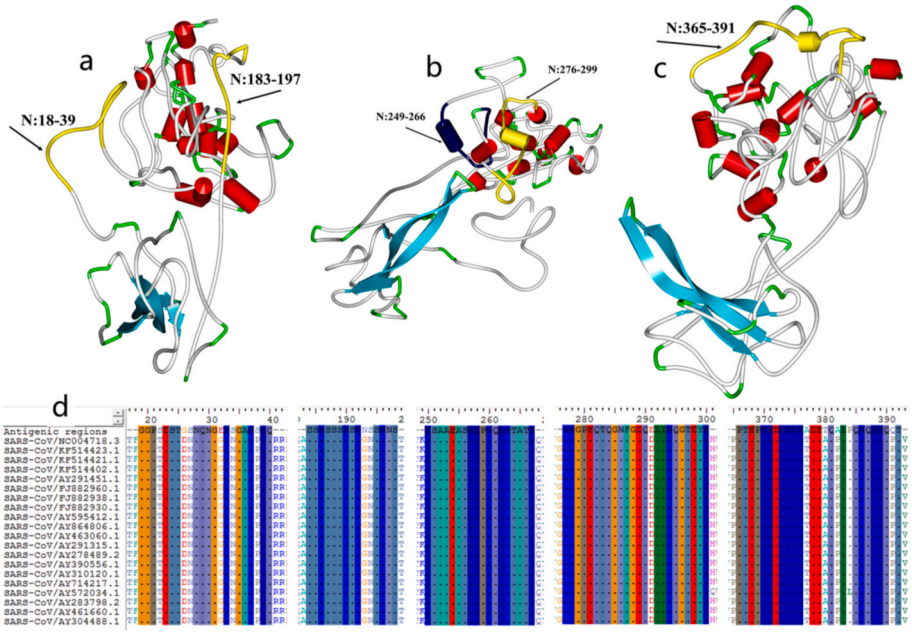

Fig. 4. Three-dimensional structure and specificity of the five epitope regions. (a–c) Three-dimensional structure of the five epitope regions. (d) Alignment of the SARS-CoV N protein sequences to analyze the specificity of the five epitope regions.

4. Discussion

The genome sequence data show that SARS-CoV-2 is a member of the betacoronavirus genus and belongs to the subgenus including SARS-CoV (Sarbecovirus), with approximately 79% similarity to SARS-CoV at the nucleotide level. The spike (S) protein, a key surface glycoprotein that interacts with the host cell receptor, and N protein have strong antigenicity and can induce cellular immune responses, are the main targets of serological diagnosis, and show a high degree of similarity between viruses [27,28]. This is consistent with the results of the conservation and specificity analysis conducted in this study. During SARS-CoV-2 evolution, the mutation rate of its N protein was relatively low and showed the closest genetic relationship with SARS-CoV, resulting in multiple continuous long conserved amino acid regions in the N protein, suggesting that the two may show cross-reactivity when using the N protein for antibody detection.

At present, experimental data on the expression patterns of SARS-CoV-2 antibodies are being obtained rapidly. For the detection of IgG and IgM antibodies against SARS-CoV N protein, the average time required for IgG to produce a positive result was one day less than that required for IgM, and could be detected as early as four days after symptom onset [29–31]. Our findings showed that in the serum samples of two patients collected six days after onset, the earliest collection time in this study, IgM was detected, whereas IgG was negative. However, as the disease progressed, both IgG and IgM were detectable, and the IgG titer increased until it superseded that of IgM. During the recovery period, IgG remained high, while IgM gradually disappeared. Kristi et al. found in a small-sample survey that in the first serum sample collected when patients were admitted to the hospital, virus-specific IgM and IgG titers were relatively low, or lower than the detection limit. However, by the fifth day, nearly all patients showed positive or elevated antibody levels [32]. In other studies, SARS-CoV-2 virus-specific IgG and IgM reached peak levels on 17–19 days and 20–22 days after symptom onset, respectively [33], while the IgG and IgM titers of patients with severe COVID-19 were higher than those of the non-severe group [34].

It is important to note that our research was conducted from March to June 2020, before the COVID-19 epidemic expanded globally, during the time when it was primarily concentrated in Wuhan, China, and its surrounding areas and the number of cases in Guangdong was relatively small. Thus, the 22 serum samples collected from COVID-19 patients in our study are representative of the spreading process at that point in the pandemic. As COVID-19 has now spread globally, its clinical symptoms, viral molecular characteristics, and antibody levels may have undergone significant changes. Nevertheless, a study that provides insights into the early stages of an epidemic involving a novel virus, allows detailed analysis of the virus evolution, disease progression, and symptomatic changes.

Studies have shown that SARS-CoV IgG antibody titers can remain high for an extended period, such that among 257 patients with SARS, antibody titers increased steadily for 4–6 months after symptom onset. Even in cases where the antibody titer decreased rapidly, ELISA tests yielded positive results for up to 48 months [35]. The latest research indicates that *anti*-SARS-CoV IgG can last up to 12 years, although IgG titers generally peaked in 2004 [36]. Additionally, in an enzyme immunoassay serological study, more IgG-positive patients were identified than IgM-positive patients, and a higher proportion of patients had earlier IgG than IgM seroconversion, suggesting that the pre-existing antibodies of other coronaviruses have cross-reactivity with the detection antigen, which likely affects the accuracy of serological COVID-19 diagnosis [37]. When SARS-CoV-2 antibody-approved colloidal gold test strips and the ELISA method targeting the SARS-CoV-2 N protein were used to test patients infected with SARS-CoV in their recovery period, strong positive results were revealed, suggesting that the N protein, as a target for antibody detection, has strong cross-reactivity in SARS-CoV-infected individuals with pre-existing antibodies, which will affect the accuracy of the results. In this study, of the five epitope regions obtained by epitope prediction, the use of two high-specificity epitope regions, N18–39 and N183-197, for IgG and IgM detection, respectively, effectively overcame the limitations of cross-reactivity. They showed good specificity even between SARS-CoV-2 and SARS-CoV, and the detected antibody levels were consistent with those detected using the complete N protein. Hence, not all highly conserved epitopes can be used alone in ELISA to detect antibody levels in the serum of patients. Notably, even the most specific N18-39 polypeptide, when testing the serum of SARS-CoV-infected patients with high IgG antibody levels, showed low levels of IgG. When testing populations with pre-existing immunity for SARS-CoV, high-sensitivity ELISA detection methods may produce false positives. However, after more than 15 years, the

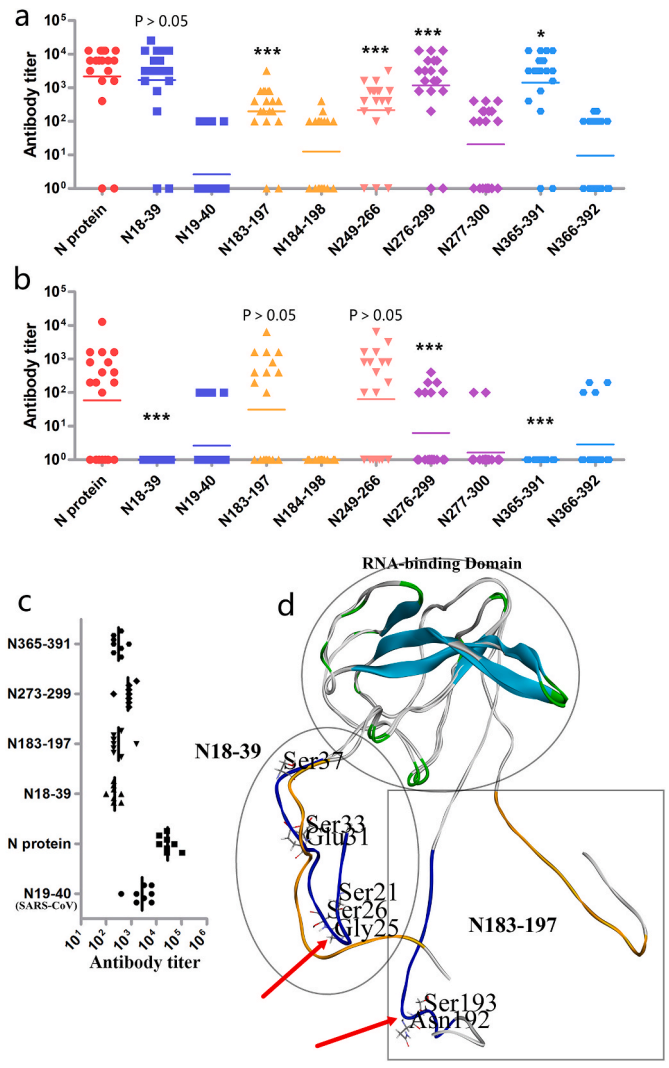

Fig. 5. The specificity and structural basis of using nine polypeptides in ELISA to detect serum IgG and IgM levels in patients. (a–b) Detection of IgG and IgM of COVID-19 patients by using nine polypeptides in ELISA, respectively. Detection value of 100 indicates that the ELISA results were negative for serum diluted 100-fold. The results of SARS-CoV-2 N18–39, N183–197, N249–266, N276-299, and N365-391 were analyzed with N protein by paired samples *t*-test. *P < 0.05 and ***P < 0.001. (c) Verification of serum cross-reactivity in the recovery stage of SARS-CoV infected patients. (d) Alignment of the three-dimensional structure of N protein residues 1–249 of two viruses. In the N18–39 and N183-197 regions, blue represents SARS-CoV-2 and orange represents SARS-CoV. The red arrow indicates that compared with SARS-CoV, the amino acid mutations at the corresponding position of SARS-CoV-2 enhances the loop structure.

levels of IgG-specific antibodies in people infected with SARS-CoV will be significantly reduced; however, whether they can be detected requires further exploration.

5. Conclusions

In this study, we found that patients infected with SARS-CoV produce strong positive test results in the recovery stage. However, specific regions of the N protein can be used for antibody detection, some of which showed good specificity between SARS-CoV-2 and SARS-CoV, and the detected antibody levels were consistent with those detected using the complete N protein. Our study provides new insights into antibody testing for COVID-19. Further, synthesizing these regions for mouse immunization can ensure that non-cross-reactive monoclonal antibodies can be screened, and can facilitate the construction of a double-antibody sandwich method to detect SARS-CoV-2 antigen.

Ethics approval and consent to participate

As this study involved collecting blood samples from patients, the purpose of the study was explained to all participating patients with COVID-19, and written informed consent was obtained. The study was approved by the ethics review committee of the Southern Medical University and performed in accordance with approved guidelines.

Author contributions

JY, ZQ, XL, and XH conceived the project, designed the experiments, undertook experiments, and wrote the manuscript; JY, XZ, KW, and NY provided the samples, performed the experiments and analyzed the data; QW, WX, LZ, and CW provided valuable structural insight and helped writing the manuscript. BZ participated in the construction and improvement of the research, and guided the experimental process and article writing. WZ participated in the entire research process and provided financial support.

Funding

This work was supported by the National Key R&D Program of China under grant number 2018YFC1602206; Yangjiang Science and Technology Program key projects under grant number 2019010; Guangzhou Science and Technology Program key projects under grant number 201803040006; Guangdong Science and Technology Program key projects under grant number 2021B1212030014; and the Basic Research Project of Key Laboratory of Guangzhou under grant number No.202102100001.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Peiwen Liu from Department of Pathogen Biology, Institute of Tropical Medicine, School of Public Health, Southern Medical University for providing guidance in the use of bioinformatics software such as sequence alignment and protein structure prediction; Editage (www.editage.cn) for English language editing; and Nanjing Vazyme Medical Technology Co., Ltd for providing two colloidal gold kits and SARS-CoV-2 N protein for emergency detection of SARS-CoV-2 antibodies.

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