ORIGINAL ARTICLE

Establishment of an indirect ELISA-based method involving the use of a multiepitope recombinant S protein to detect antibodies against canine coronavirus

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Abstract

Here, we report the development of an indirect enzyme-linked immunosorbent assay (ELISA) method that involves using multiepitope recombinant S protein (rSP) as the coating antigen to detect antibodies against canine coronavirus (CCoV). rSP was designed by arranging its four S fragments (91–135 aa, S1 gene; 377–434 aa, S2 gene; 647–671 aa, S3 gene; 951–971 aa, S4 gene; 207–227 aa) and two T-cell epitopes in tandem: T–E1–E2–E3–E4–T. This multiepitope antigen, which has a molecular weight of approximately 25 kDa and contains a His-tag, was recognized by a CCoV-positive serum in a Western blot assay. The optimal concentration of rSP as a coating antigen in the ELISA was 2 µg/mL, and the optimal dilution of enzyme-labeled secondary antibody was 1:10,000. The cutoff OD_{450} value was established at 0.2395. No reactivity was observed with antisera against canine distemper virus, canine parvovirus, or feline calicivirus, indicating that this assay is highly specific. We also tested 64 clinical serum samples using our newly established method, and the positive rate was found to be 82.8%. In conclusion, our assay was found to be highly sensitive and specific for the detection of antibodies against CCoV, and it can therefore serve as a new, efficient diagnostic method.

Introduction

Canine coronavirus (CCoV), an enteric virus that mainly causes diarrhea with varying severity, has been described since 1971, and it is associated with high morbidity and low mortality [1–3]. In cases of coinfection with other pathogens such as canine adenovirus or canine parvovirus (CPV), CCoV infection can lead to severe clinical signs and can be fatal [4]. In recent years, the number of cases of highly pathogenic CCoV infection has increased considerably [5]; moreover, the antibody-positive rate has also increased each year.

CCoV is an enveloped virus with a single-strand positive-sense RNA genome of approximately 26–32 kilobases [6]. Like other coronaviruses, CCoV contains four main structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [7]. The S protein (SP) is a major antigenic determinant, and it induces the production of neutralizing antibodies in the host, mediates host-cell receptor binding and viral entry, and triggers fusion between the viral and host-cell membranes. In addition, it is an important determinant of cell tropism and pathogenicity [8, 9]. CCoV detection primarily relies on serological, etiological, and molecular approaches. Three assays – serum neutralization, indirect immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) – are widely used to detect CCoV-specific antibodies. Serum neutralization is the classical serological detection method, but it is cumbersome and time-consuming. Furthermore, this method needs to be performed in a laboratory with a high biosafety level, and for these reasons, serum neutralization is rarely used for rapid clinical

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Handling Editor: Sheela Ramamoorthy.

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detection [10–12]. As reported previously [13], the sensitivity of ELISA for detection of CCoV-specific antibodies is significantly higher than that of the serum neutralization test and can be used as an alternative. Indirect immunofluorescence is another method for detecting CCoV-specific antibodies, but the equipment required is expensive [14]. Thus, at present, ELISA is widely used for serological profiling of CCoV, with most established methods using either the M or N protein as the antigen, as these proteins are highly conserved and immunogenic [15–17]. SP reportedly plays a key role in inducing neutralizing antibodies and conferring protection against CCoV [18–21]. Hence, the use of SP as the antigen in ELISA appears to be especially suitable for CCoV detection. SP is a large protein with considerable hydrophobicity, and it is highly glycosylated, which makes the heterologous expression of full-length SP challenging. We therefore constructed a multiepitope antigen of SP to establish an ELISA-based method to detect antibodies against CCoV. We also tested the sensitivity and specificity of our method.

Materials and methods

Predicting and screening linear B-cell epitopes

Based on the sequence of the gene encoding the SP of CCoV (Table 1), as described previously by Miller et al. and Qiao et al. [22, 23], we predicted and screened linear B-cell epitopes using DNAStar software (protean) and the IEDB website (http://www.iedb.org/). We screened areas where the antigenic index, surface probability, and hydrophilicity values are positive, and where the beta turn area overlaps these areas best. A three-dimensional structural model of the protein was built using SWISS-MODEL (PyMOL).

Expression, purification, and identification of recombinant SP (rSP)

To obtain the recombinant plasmid pET28α-S1, codon optimization was first performed, and the sequences encoding the selected portions of rS1 were synthesized and cloned into the *Bam*HI–*Xho*I restriction sites of pET28α (+) by BGI (Beijing, China). The recombinant plasmid was then introduced into competent *Escherichia coli* BL21 (DE3) cells by transformation. The fusion protein was expressed in *E. coli* BL21 (DE3) cells, positive colonies were selected, and protein expression was induced using 1 mM IPTG. The expression temperatures were optimized (37°C and 16°C), and samples were collected at 8, 12, 16, and 20 h after IPTG induction for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%). Protein purification was carried out using a His-Tagged Protein Purification Kit (Kangwei Century, Beijing, China) according to the manufacturer's instructions.

Bacterial suspensions were lysed by ultrasonic disruption. The sample was centrifuged, and the pellet was resuspended in binding buffer. The supernatant was collected and passed through a nickel affinity chromatography column. The bound protein was eluted with elution buffer and collected in separate tubes with 0.5 ml per tube. Finally, the target protein was renatured in a gradient of 6 M, 4 M, and 2 M urea buffer solution at 4°C. The purified protein was collected and identified by Western blot and liquid chromatography–mass spectrometry.

The concentration of the recombinant protein was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). For mass spectrometry, 10 μg of protein was used. After using proteomics discovery software for mass spectrometry data extraction, we used MASCOT to search a local database using the amino acid sequence of the pET28α-S1 protein.

Table 1 Antigenic gene fragments

Name	Position	Amino acid sequence	Reference position	References
E1	91-135	AMENSTGNARGKPLLFHVHGEPVSVII-YISAYKNDVQQRPLLKHG	50-250 aa, 375-425 aa, 450-470 aa, 550-600 aa,	[22]
E2	377-434	CYNDTVSESSFYSYGEIPFGLTDGPRY-CYVLHNGTALKYLGTLPPSVKEI-AISKWGHF		
E3	647-671	ARTRTNEQVVRSLYVIYEEGDNIVG		
E4	951-971	ILPSHNSKRKYRSAIEDLLFD		
TE	207-227	FNNVTLLYSRSSTATWQHSAA	650-700 aa, 770-850 aa, 900-1025 aa, 1150-1225 aa, 1250-1452 aa, 537-539 aa, 557-559 aa, 566-568 aa, 14-16 aa, 94-96 aa	[23]

Establishment of indirect ELISA for detection of CCoV antibodies

Optimization of rSP and serum concentrations

Briefly, rSP diluted in an optimized coating buffer was coated onto 96-well plates. The optimal concentration of rSP was determined by testing various concentrations (0.5, 1, 2, and 4 μg/mL). After coating, the plates were washed three times with PBST (0.05% Tween 20 in PBS) and blocked using an optimized blocking buffer. CCoV-positive and negative sera (PBST was used to obtain 1:50, 1:100, 1:200, and 1:400 dilutions) was then added to rSP-coated wells. A matrix titration was used to determine the optimal concentration of rSP for coating onto the wells and the optimal serum dilution.

Selecting an optimal blocking buffer

We tested 1% BSA, 5% BSA, 5% non-fat milk, and 5% FBS to select an optimal blocking buffer based on the OD_{450} and P/N values.

Selecting the best secondary antibody incubation conditions

We diluted HRP-labeled mountain rabbit anti-canine IgG (Beijing Solarbio Biotechnology Co., Ltd.) in PBST (1:5000, 1:10,000, 1:15,000, and 1:20,000), and this was then added to each well, followed by incubation for 30, 45, 60, and 75 min. Subsequently, 3,3′,5,5′-tetramethylbenzidine (Beijing Solarbio Biotechnology Co., Ltd.) was added to each well, and the plate was incubated. Absorbance of each well was measured at OD_{450} using a spectrophotometric ELISA reader, and average values were calculated.

Determination of the cutoff value

Using our optimized conditions for rSP ELISA, 10 serum samples that were identified to be negative using a CCoV Antibody Fluorescence Detection Test Strip (Beijing Kwinbon Biotechnology Co.,Ltd, Beijing, China) were tested. The average S/P and standard deviation were calculated. $\bar{x} \pm 3S$ was used as the cutoff value of the rSP ELISA. S/P = (OD_{450} of samples to be tested - average OD_{450} of negative samples) / (average OD_{450} of positive samples - average OD_{450} of negative samples)

Analysis of the specificity of the rSP ELISA

The specificity of the rSP ELISA was determined using sera from animals that were positive for CCoV and from animals that were positive for canine distemper virus (CDV), CPV, and feline calicivirus (FCV), but negative for CCoV.

Analysis of the sensitivity of the rSP ELISA

For sensitivity analysis, five CCoV-positive serum samples with different antibody levels and a standard CCoV-positive serum sample were diluted (1:100–1:6400) and then subjected to rSP ELISA.

Comparison of rSP ELISA using clinical serum samples

Sixty-four clinical serum samples collected from the Beijing, Hebei, and Shandong regions were subjected to rSP ELISA. The results were compared to those obtained using a Canine Coronavirus Antibody Fluorescence Detection Test Strip (Beijing Kwinbon Biotechnology Co., Ltd, Beijing, China). In this assay, test strips are first coated with the N protein of CCoV, and then with sheep anti-canine IgG fluorescent microspheres. The serum for detection is diluted at 1:2000, and 80 μl of diluted serum is added to the sample well. After 5-10 min, test strips are inserted into a fluorescence immunoassay analyzer (Beijing Kwinbon Biotechnology Co.,Ltd, Beijing, China) for detection, and a fluorescence value greater than or equal to 0.5 is considered positive.

Results

Prediction and screening of B-cell epitopes

Based on the sequence of the gene encoding SP of CCoV, predicted linear B-cell epitopes of SP were chosen. The S domain in the CCoV strain is connected by a KK spacer and the sequence GGGGAS, and rSP consists of four fragments, namely E1 (91–135 aa, S1 gene), E2 (377–434 aa, S2 gene), E3 (647–671 aa, S3 gene), and E4 (951–971 aa, S4 gene), and two T-cell epitopes (Table 1). We screened recombinant fusion proteins and compared their predicted properties, such as antigenicity, hydrophilicity, and surface accessibility. The amino acid sequences of the selected B cell and T cell epitopes were 94.83% to 100% identical to those in the NCBI database. Sequence assembly involved arranging the four fragments in tandem in the following order: T–E1–E2–E3–E4–T. A model of the three-dimensional structure of this protein was made using SWISS-MODEL (PyMOL). The optimized gene was cloned into pET-28α.

Identification of the recombinant plasmid

The constructed recombinant plasmid was subjected to double digestion with *Bam*HI and *Xho*I, which led to the generation of two bright bands of approximately 5000 bp and 600 bp (Fig. 1). These sizes were consistent with those of the plasmid (5334 bp) and target band (639 bp), respectively.
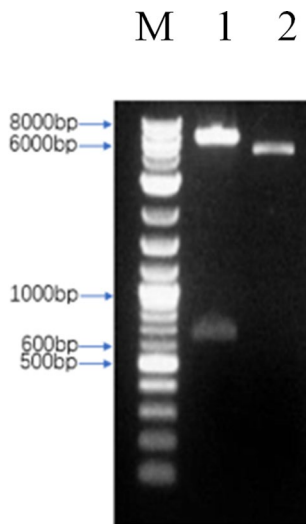
Fig. 1 Agarose gel electrophoresis of the recombinant plasmid pET28α-S1. Lane M, DNA molecular weight marker; lane 1, *Bam*HI and *Xho*I double-digested product; lane 2, uncut pET28α-S1 plasmid DNA

Expression and identification of the recombinant protein

### SDS-PAGE, Western blotting, and purification of rSP

In positive colonies, protein expression was induced using 1 mM IPTG, followed by incubation at 37°C for 8 h. The samples were then subjected to SDS-PAGE. We verified that pET28α-S1 was mainly expressed in an insoluble form in inclusion bodies and was present in large quantities. The size of rSP was approximately 25 kDa (Fig. 2).

Liquid chromatography – mass spectrometry

Using mass spectrometry analysis, the pertinent peptide was identified to be the purified target protein. The peptides identified were NDVQQRPLLK, YLGTLPPSVKEIAISK, and SLYVIYEEGDNIVGGGGGASILPSHNSK (Fig. 3).

Establishment of indirect ELISA for detecting CCoV antibodies

Using a matrix titration test, the optimal concentration of the antigen rSP was found to be 2 μg/mL, and the optimal serum dilution was 1:200 (Table 2); 5% non-fat milk was the best blocking buffer, with the best incubation temperature being 37°C, with an incubation time of 60 min. Furthermore, ELISA test results were optimal when the secondary antibody was diluted to 1:10,000, the color development time was 10 min, and the incubation temperature was 37°C.
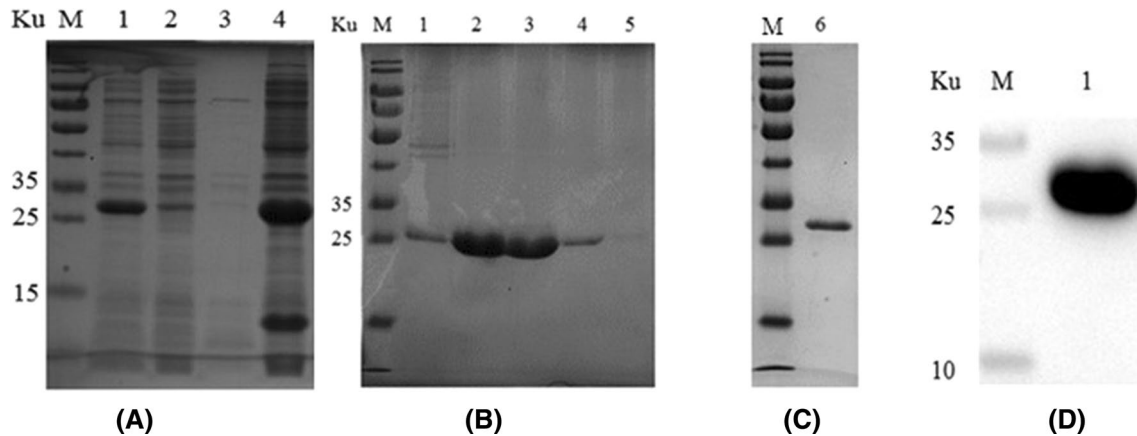
Fig. 2 Protein expression, purification, and identification. (A) Induction of expression of the recombinant protein. M, protein molecular weight marker; lane 1, protein expression induced by 1 mM IPTG; lane 2, protein expression without IPTG induction; lane 3, expression of the target protein in the supernatant; lane 4, expression of the target protein in inclusion bodies. (B) Analysis of the purified protein using affinity chromatography. M, protein molecular weight marker; lane 1, impurities in the nickel affinity column eluted by binding buffer; lanes 2-5, target protein after purification by nickel affinity chromatography. (C) The protein after renaturation. M, protein molecular weight marker; lane 6, purified rSP. (D) Identification of the recombinant protein via Western blotting. M, protein Molecular Weight Marker. Lane 1, target protein identified by Western blotting
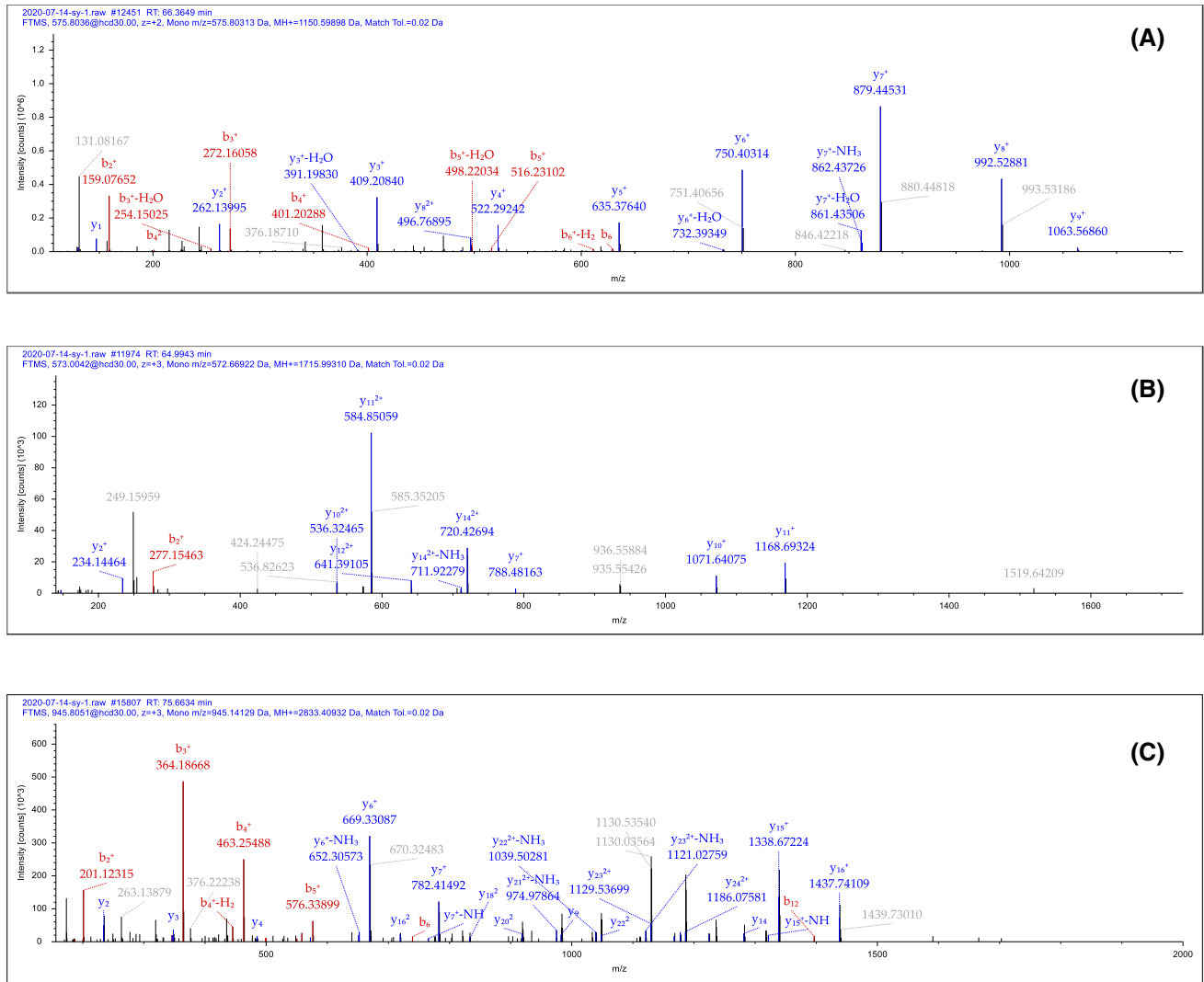
Fig. 3 Mass spectrometry results. A. Results pertaining to the peptide NDVQQRPLLK. B. YLGTLPPSVKEIAISK. C. SLYVIYEEGDNIVGGG GGASILPSHNSK

Determination of the cutoff value

The average OD_{450} values of negative and positive sera were 0.2117 and 1.6611; the average S/P of negative serum samples ($\bar{x}$) was 0.1315, and the standard deviation was 0.0360. The cutoff value was $0.1315 \pm 3 \times 0.0360 = 0.2395$ and $0.1315 \pm 2 \times 0.0360 = 0.2035$, respectively. When the S/P value was ≥ 0.2395, the sample was considered positive, and when the S/P value was ≤ 0.2035, the sample was considered negative. If the S/P value was between these two values, the sample was considered negative. If the result was ambiguous, the test was repeated, and if the S/P value was still ≤ 0.2035, the sample was regarded as negative.
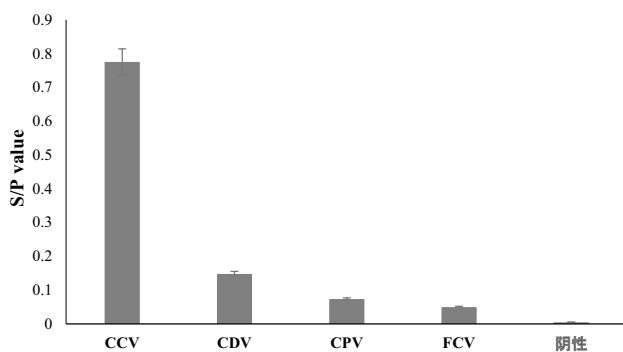
Specificity of the rSP ELISA

We used established methods to test CCoV, CDV, canine adenovirus, CPV, and FCV-positive sera, and data were analyzed statistically. The S/P values of CDV-, CPV-, and FCV-positive sera were all lower than the critical value; no cross-reactivity was observed with S1 antigen, and the specificity was good (Fig. 4).

Sensitivity of the rSP ELISA

Sensitivity analysis indicated that the titer of the standard CCoV-positive serum was 1:800, indicating that the method had good sensitivity (Fig. 5).

Table 2 Optimization of serum dilution and antigen concentration (??OD$_{450}$ values??)

Antigen coating concentration	Serum dilution			
	1:50	1:100	1:200	1:400
4 µg/ml	2.9352	2.6511	2.0264	1.7232
	0.4661	0.4494	0.2534	0.1720
P/N	6.2974	5.8991	7.9968	10.0186
2 µg/ml	2.1513	1.9798	1.5192	1.3733
	0.2784	0.2601	0.1507	0.1357
P/N	7.7274	7.6116	**10.0809**	10.1201
1 µg/ml	1.8111	1.5715	1.0424	0.7762
	0.2074	0.1903	0.1380	0.1229
P/N	8.7324	8.2580	7.5536	6.3157
0.5 µg/ml	1.5134	1.1750	0.8290	0.6313
	0.2005	0.1617	0.1281	0.1227
P/N	7.548	7.2665	6.4715	5.1450
0.25 µg/ml	1.4028	1.2084	0.7576	0.3267
	0.1900	0.1554	0.1280	0.1227
P/N	7.3831	7.7760	5.9187	2.6626

Fig. 4 Specificity analysis of rSP ELISA
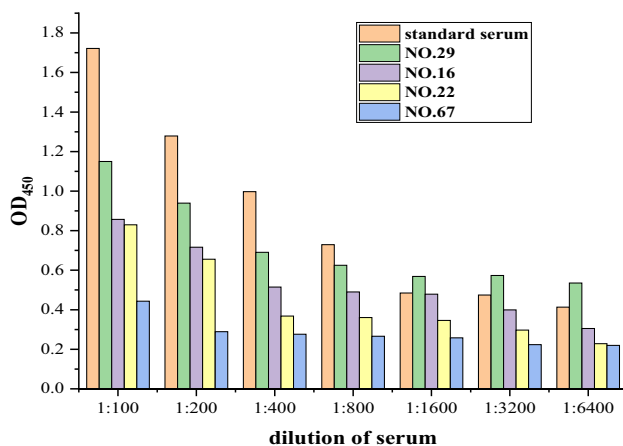
Fig. 5 Sensitivity analysis of rSP ELISA

Table 3 Test results using clinical samples

Method	Positive	Negative	Total	Positive rate
Indirect ELISA test	53	11	64	82.81%
Immunochromatographic test	54	10	64	84.38%
Total	107	21	128	98.44%

Comparison of rSP ELISA with clinical serum samples

For the 64 clinical serum samples obtained from Beijing, Hebei, and Shandong regions, the positive rate was 84.37%. When our indirect rSP ELISA method was used, the positive rate was 82.81%. The total coincidence rate was 98.44% (Table 3).

Discussion

ELISA is a convenient technique for evaluating the response to vaccination, particularly in the case of viruses such as CCoV that have various serotypes, because this type of assay can identify different antibodies via coated antigens. Usually, the coating antigen is either an inactivated virus or a recombinant protein. In recent years, multiple antigens or synthetic peptides have been used for ELISA, and such methods have shown improved sensitivity and specificity. In this study, one of the reasons for using CCoV SP in the form of a multiepitope antigen was to assess whether it can serve as an alternative to full-length SP. SP is encoded by ORF2, has multiple glycosylation sites, exists in the form of a trimer, is located in the outermost layer of the coronavirus particle, and is the main protein of the viral envelope [24, 25]. Because SP is very large, it is difficult to express full-length SP. Using bioinformatics software, we screened all of the dominant epitopes, using epitopes originally described in other studies as references [26–29]. In this manner, we synthesized the entire gene encoding the epitope, thereby overcoming the issues associated with the high molecular weight of SP and its potential weak reactogenicity. Different serotypes and genotypes of CCoV several epitopes in common, as well as some unique epitopes, and these are of great significance for cross-immunization.

The selected epitope was constructed using pET28α, and competent *E. coli* BL21 (DE3) cells were used for expression. At 37°C, expression was observed in the form of inclusion bodies. We attempted to lower the culture temperature to 20°C to obtain soluble protein; however, soluble protein was not obtained, although we found that the expression levels at the two temperatures were similar. Western blotting revealed that rSP showed good antigenicity.

In conclusion, we have successfully established an ELISA-based method using multiepitope rSP instead of full-length SP as the coating antigen. This method showed good sensitivity and specificity for detection of antibodies against CCoV, and it can thus serve as a new, efficient diagnostic assay. We believe that our technique can be used in further studies for developing genetic recombinant vaccines and therapeutic monoclonal antibodies.

Declarations

Conflict of interest None.

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