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MAJOR ARTICLE

Early Detection of Severe Acute Respiratory Syndrome Coronavirus 2 Antibodies as a Serologic Marker of Infection in Patients With Coronavirus Disease 2019

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Background. Thousands of medical staff have been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with hundreds of deaths reported. Such loss could be prevented if there were a serologic assay for SARS-CoV-2–specific antibodies for serological surveillance of its infection at the early stage of disease.

Methods. Using Chinese hamster ovarian (CHO) cell–expressed full-length SARS-CoV-2 S1 protein as capturing antigen, a coronavirus disease 2019 (COVID-19)/SARS-CoV-2 S1 serology enzyme-linked immunosorbent assay (ELISA) kit was developed and validated with negative samples collected prior to the outbreak or during the outbreak and positive samples from patients confirmed with COVID-19.

Results. The specificity of the ELISA kit was 97.5%, as examined against total 412 normal human samples. The sensitivity was 97.1% by testing against 69 samples from hospitalized and/or recovered COVID-19 patients. The overall accuracy rate reached 97.3%. The assay was able to detect SARS-CoV-2 antibody on day 1 after the onset of COVID-19 disease. The average antibody levels increased during hospitalization and 14 days after discharge. SARS-CoV-2 antibodies were detected in 28 of 276 asymptomatic medical staff and 1 of 5 nucleic acid test–negative "close contacts" of COVID-19 patients.

Conclusions. With the assays developed here, we can screen medical staff, incoming patients, passengers, and people who are in close contact with the confirmed patients to identify the "innocent viral spreaders," protect the medical staff, and stop further spread of the virus.

Keywords. SARS-CoV-2; COVID-19; serological assay for SARS-CoV-2 antibodies.

As of 20 April 2020, there were 2 319 066 confirmed cases of coronavirus disease 2019 (COVID-19) with 157 970 deaths worldwide [1]. Infections among healthcare providers were even more alarming, with 4826 Italian doctors and nurses reported to be infected over a short period due to the lack of appropriate medical protection gear and quick screening of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections [2, 3]. Making the issue even worse, the virus can be widely transmitted by asymptomatic viral carriers to people in close contact [4], with some patients reportedly becoming sick once again after their initial recovery and yielding a positive nucleic acid test (NAT) [5]. There is an urgent need to develop rapid, fast, and simple screening tools to find "moving viral carriers" and quarantine them.

Several serological kits for measuring SARS-CoV-2 immunoglobulin M (IgM) and immunoglobulin G (IgG) have been approved by the Chinese National Medical Products Administration with the restriction that they may only be used as companion tests for NAT, not for general screening of SARS-CoV-2 infection due to a lack of required specificity and sensitivity. The cause may be the poor quality of the detecting antigens used. Three different types of antigens have been reported to be used: (1) the recombinant N protein from SARS-CoV-2, which is highly conserved among all 7 human coronaviruses and led to poor specificity in tests of the general population; (2) Chinese hamster ovarian (CHO)–expressed S1 protein from SARS-CoV, which has very different antigenicity

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from its counterpart in SARS-CoV-2; or (3) the receptor binding domain of SARS-CoV-2 S1, which is about 200 amino acids long with only 1 glycosylation, compared to the full-length S1, which has 7 glycosylation sites. These latter 2 can result in poor sensitivities. Misdiagnosis of nonlethal human coronaviruses (eg, OC43, NL63, 229E, and HKU1) as SARS-CoV-2 could send thousands of people to already overloaded hospitals and increase the risk of real infection by SARS-CoV-2 during the unnecessary hospital visit. On the other hand, missed detections of SARS-CoV-2 infections can deny patients the opportunity to receive early preventive care before the disease progresses into acute respiratory distress syndrome, which has a mortality rate of > 60%. Therefore, it is extremely important to develop serological tests using the right detecting antigen: fully glycosylated, full-length SARS-CoV-2 S1 recombinant protein(s).

The full-length SARS-CoV-2 S1 protein has previously been difficult to express at a commercially viable level, but using our patented technology, we have improved the expression level by close to 1000-fold (~80 mg/L) using either CHO or 293F mammalian cells. Using the CHO-expressed SARS-CoV-2 S1-6×. His protein as the detecting antigen, we have developed a very sensitive and highly specific serological assay for screening the healthcare staff at the hospitals to reduce the in-hospital infections, as well as checking the incoming visitors from the epidemic areas, the returning work forces, and the general population for SARS-CoV-2 viral infection.

MATERIALS AND METHODS

Regents and Supplies

High-binding 96-well enzyme-linked immunosorbent assay (ELISA) plates were purchased from Corning. Goat antihuman IgG (H+L) peroxidase conjugate was sourced from Jackson Immunoresearch. Monkey anti-SARS-CoV-2 S1 polyclonal antibody and mouse anti-6×His monoclonal antibody (mAb) 6E2 were produced by AbMax, China. HEK 293F and CHO cells were provided by ZhenGe Biotech., Shanghai, China.

Protein Expression and Purification

The full-length SARS-CoV-2 S1 gene (GenBank accession number: QIC53204.1) was synthesized by Genewiz, China, and inserted into mammalian cell expression vector with 6×His tag. CHO and 293F cells were transfected with purified plasmid DNA by lipofection using a liposome transfection kit from Invitrogen. The transfected mammalian cells were grown at 37°C and 5% carbon dioxide for a few days prior to harvesting, and culture supernatant was collected by centrifugation at 4000 rpm for 10 minutes. The recombinant S1-6×His protein was purified by immobilized metal affinity chromatography. Protein concentration was determined by optical density (OD) absorbance at 280 nm. The purity of the S1 protein was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) Coomassie brilliant blue staining.

Serum Samples for Assay

Strong negative and negative human plasma samples were collected prior to or during the COVID-19 outbreak, respectively. Positive plasma samples were obtained from hospitalized and/or recovered COVID-19 patients. Informed consent was obtained from all of the human subjects who participated in the study and the protocols were approved by the institutional ethical committee. The serum samples were inactivated at 56°C for 30 minutes and stored at −20°C until use.

SARS-CoV-2 Virus Serology ELISA Kit

In brief, recombinant SARS-CoV-2 S1-6×His was diluted in phosphate-buffered saline (PBS; 10 mM, pH 7.4) and 100 µL of the solution was added to each well of 96-well ELISA plates and incubated overnight at 2°C–8°C. The wells were emptied and washed twice with PBS and unsaturated sites were blocked with 3% bovine serum albumin (BSA) in PBS by incubating for 1 hour at room temperature. Coated plates were air-dried and sealed in plastic bags and stored at 2°C–8°C until use.

Monkey anti–SARS-CoV-2 S1 antisera or human plasma samples were first diluted in negative human sera (pooled from 8 normal human sera). For ELISA, each serum sample was tested in duplicate. Prior to test, human samples or the standards were diluted 1:20 in sample dilution buffer. Then, 100 µL of appropriately diluted sample was added to each well of the S1-6×His-coated plates and incubated for 0.5 hour at 37°C with constant shaking. The wells were emptied and washed twice with washing buffer, and 100 µL of appropriately diluted horseradish peroxidase–conjugated goat antihuman IgG (H+L) was added to each well and incubated for another 15 minutes at 37°C with constant shaking. The wells were emptied and washed 5 times before addition of TMB substrate solutions. The chromogenic development was stopped using 0.1 M sulfuric acid after 5–10 minutes of incubation in the dark. OD was measured at 450 nm wavelength in a microplate spectrophotometer (Thermo Scientific, Multiskan MK3).

For data analysis, calculate the mean value (AVG1) of negative control, and times the lot-specific converting factor as the negative cutoff point (N-Cut); calculate the mean value (AVG2) of positive control, use AVG2 as the positive cutoff point (P-Cut). If the absorbance value of the sample is greater than or equal to the positive cutoff point (P-Cut), the result of the sample is positive, indicating that the sample has detected antibodies that recognize the SARS-CoV-2; if the absorbance value of the sample is less than the negative cutoff point (N-Cut), the result of the sample is negative, it means that no antibody that recognizes the SARS-CoV-2 is detected in the sample; if the absorbance value of the sample is less than the positive critical point value (P-Cut) and greater than or equal to the negative critical point value (N-Cut), the result of the

sample falls into a gray area and needs further experimental confirmation.

RESULTS

Production of Recombinant SARS-CoV-2 S1-6×His Proteins

The spike protein S1 plays a key role in virus binding and entering host cells via human ACE2. It has 685 amino acids with 7 potential glycosylation sites, and its heavy glycosylation makes its antigenicity very distinguishable from its close family members severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS); indeed, no significant cross-reactivity was observed with existing neutralizing mAbs to SARS or MERS. The DNA sequence corresponding to the full-length SARS-CoV-2 S1 protein was chemically synthesized and inserted into 1 mammalian cell expression vector with 6×His tag to produce recombinant SARS-CoV-2 S1-6×His protein (Figure 1A).

The recombinant S1-6×His protein was purified from the culture supernatants using Ni column. As shown in Figure 1B, a defused band was observed around 120 kD in elution (lanes 5 and 6), which is much larger than the expected size of S1-6×His, suggesting that heavy glycosylation took place.

Multiple batches of expressions and purifications of recombinant S1-6×His proteins using 2 mammalian cell systems have been carried out. The expression levels were examined by ELISA using mouse anti 6×His tag mAb 6E2. Using our patented technology, the transient expression levels of S1-6×His in either CHO cells or 293F cells reached 36.3–71.7 mg/L (Supplementary Table 1). Stable cell lines were established recently.

To verify the true identity, the purified recombinant S1-6×His protein was coated on to 96-well plate and examined with the SARS-CoV-2–positive plasma samples collected from recovered COVID-19 patients. As shown in Supplementary Figure 1, all of the positive plasma samples reacted strongly with the purified recombinant SARS-CoV-2 S1 protein, indicating not only the right sequence but also the correct conformation.

The sera collected from 2 SARS-CoV-2 S1 protein–immunized monkeys were mixed and used to spike the human normal sera for preparation of the positive controls.

Development of Serological Assays for SARS-CoV-2 Antibodies

To determine the coating concentration of S1-6×His protein for capturing anti–SARS-CoV-2 antibodies in testing samples, each well of a 96-well enzyme immunoassay plate was coated with 100 µL of S1-6×His protein at 8 different concentrations (0.1, 0.2, 0.4, 0.5, 0.8, 1.0, 1.2, and 1.5 µg/mL) in 10 mM PBS (pH 7.4) at 2°C–8°C overnight.

The solid phase-bound S1-6×His protein was probed using appropriately diluted mouse anti–S1-6×His mAb 6E2 (0, 2, 5, or 20 µg/mL). As shown in Supplementary Table 2, at all 8 different coating concentrations of S1-6×His, the OD values showed dose dependency, while the maximal OD values increased with increasing coating concentrations of S1-6×His protein. Since the background OD values did not change significantly, 1.5 µg/mL

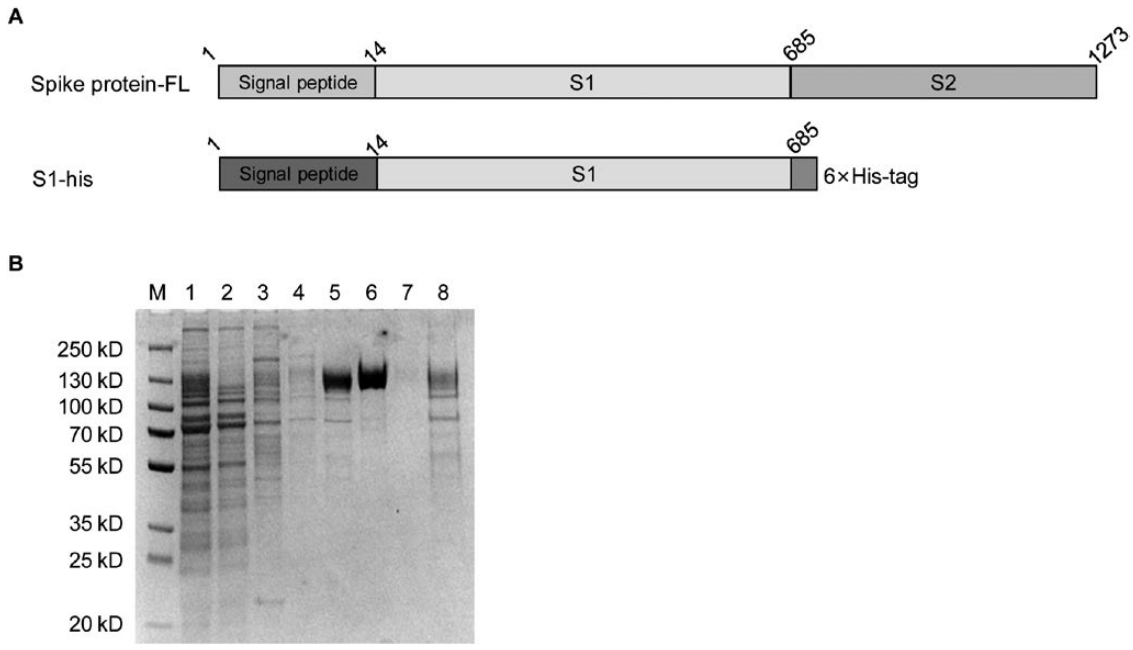

Figure 1. Construction and expression of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S1 protein. *A*, Domain structures of SARS-CoV-2 spike proteins, including full-length (FL) spike S1 protein with 6×His. *B*, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of S1-6×His expression and purification. Lane M, MW markers; lane 1, culture supernatant; lane 2, flow-through; lane 3, first wash with buffer 1; lane 4, second wash with buffer 2; lanes 5–8, 3 different fractions eluted with buffers containing 50 mM MES, 250 mM imidazole, and 150 mM NaCl pH 7.4. Abbreviations: MW, molecular weight; MES, 2-(N-morpholino) ethanesulfonic acid; NaCl, sodium chloride.

of S1-6×His protein was considered optimal for coating of the ELISA plate for kit manufacture to ensure the highest sensitivity.

A set of 8 strong negative plasma samples were tested at 5 different dilutions to experimentally determine the assay's optimal dilution. As shown in Supplementary Table 2, if the dilution is not high enough, such as 1:5 or 1:10, the background is too high. At 1:20 or higher dilutions, the background was acceptable. Based on suggestions from the clinicians, 1:20 dilution is more practical.

To balance between preservation of the detection of low affinity SARS-CoV-2 antibodies and reduction of background, we have tried different washing buffers and sample dilution buffers. Addition of detergent will certainly reduce the nonspecific binding, but too much of it will also remove some of the blocking of the plate and give more opportunity for nonspecific binding. As shown in Supplementary Figure 2, with detergent Tween-20 in the washing buffers, the background was significantly reduced.

Human plasma or serum contains an extremely high level of antibodies that will nonspecifically bind to the wells and could increase the background significantly. In this study, both 3% BSA-PBS and 20% calf serum (CS)–PBS were used as the sample dilution buffers. As shown in Supplementary Figure 3*A*, 20% CS-PBS as the sample dilution buffer significantly reduced the background. At the same time, 20% CS-PBS also produced better signal-to-noise ratio (S/N) for detection of anti–SARS-CoV-2 S1 antibodies in the sera of S1-Fc–immunized monkeys (Supplementary Figure 3*B*). Although taping the plates every 10 minutes produced similar results as constant shaking, it is still highly recommended to use a temperature-controlled microplate shaker.

Based on the above data, the best manufacturing and key testing parameters for the SARS-CoV-2 serological ELISA kit were selected as (1) 1.5 μg/mL SARS-CoV-2 S1-6×His for plate coating; (2) 1:20 dilution of human sera with 20% CS-PBS as sample and enzyme diluent; (3) PBS-T (0.1% Tween 20 in PBS) as the washing buffer; and (4) incubation with constant rotation using a temperature-controlled microplate shaker.

Several batches of the SARS-CoV-2 serology ELISA kits were manufactured at 3 different locations, and were tested using positive monkey sera at different dilutions in human sera for assessing the reproducibility of manufacturing and assay precisions.

Summarized in Supplementary Table 4, all 3 batches' intra–coefficient of variations (CVs) were in the range of 4.32%–12.05%, meeting the acceptable criterion. The intra-assay imprecision of samples (CV) was around 10.38%.

Specificity of the Serological ELISA Assay

The assay specificity of the kit was demonstrated by testing 412 human samples including 257 samples collected prior to (strong negatives) and 155 samples collected during (negatives) the outbreak of COVID-19.

Table 1. Specificity of the Assay Against Strong Negative Samples

Sources	SARS-CoV-2 Ab Negative	SARS-CoV-2 Ab Positive	Subtotal	Specificity
Rabies vaccinated	47	1	48	97.9%
Commercial laboratory 1	20	0	20	100.0%
Commercial laboratory 2	119	5	124	96.0%
Hospital 3	43	2	45	95.6%
Clinical laboratory	20	0	20	100.0%
Total	249	8	257	96.9%

Samples were collected prior to the outbreak of coronavirus disease 2019 from different origins.
Abbreviations: Ab, antibody; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

As shown in Table 1, for the strong negatives, obtained from different sources including 48 samples from rabies-vaccinated patients, 144 samples purchased from commercial sources, and 65 from hospital and clinical laboratory, showed very similar specificities between 95.6% and 100%. In the group of commercial laboratory 2, they were sera collected from 50 black white individuals, 30 white individuals, 24 Asian females, and 20 Asian males; no significant difference in background was observed between different races or sexes. The specificity for strong negative was determined at 96.9% (249/257).

For the negatives, group 1 was collected from Beijing, and groups 2–4 were collected from Zhejiang province, both areas with confirmed COVID-19 cases. In the initial test, 2 of the 15 samples from Beijing's group were tested SARS-CoV-2 S1 antibody positive (Table 2). We performed the antigen competition assay using the recombinant S1-6×His proteins at very high molar ratio, and found that the signals could not be blocked, suggesting those 2 were false negatives. No positive was detected in the other 3 groups. The specificity was 98.7%. Combine the data from the strong negative samples, the overall specificity of the ELISA kit was 97.5% (402/412).

Table 2. Specificity of the Assay Against Negative Samples

Sources	SARS-CoV-2 Ab Negative	SARS-CoV-2 Ab Positive	Subtotal	Specificity
Group 1	13	2	15	86.7%
Group 2	9	0	9	100.0%
Group 3	123	0	123	100.0%
Group 4	8	0	8	100.0%
Total	153	2	155	98.7%

Samples were collected during the outbreak of coronavirus disease 2019 from different cities in China.
Abbreviations: Ab, antibody; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

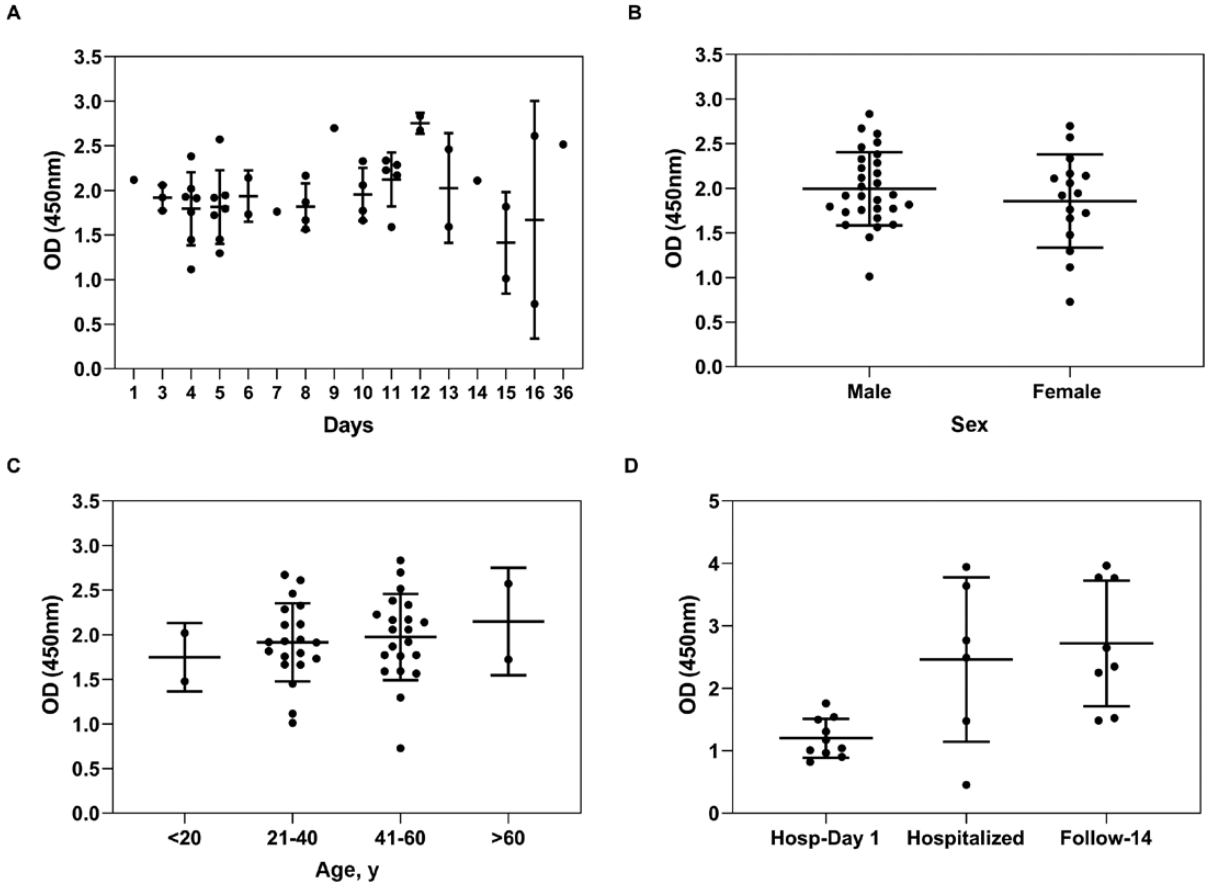

Figure 2. Sensitivity of the serological assay. Group 1 patient samples were sorted by days after onset of disease (*A*), by sex (*B*), and by age (*C*). *D*, Group 2 samples were sorted by day of admission (Hosp-Day 1), during the hospitalization (Hospitalized), and day 14 after release from the hospital (Follow-14). Abbreviation: OD, optical density.

Sensitivity of the Serological ELISA Assay

In collaboration with the Chinese Center for Disease Control and Prevention (CDC), the ELISA kits were sent to several hospitals including 2 in Beijing and 1 in Wuhan to examine its sensitivity against the real clinic samples. Some of the data were presented in Figure 2 and Table 3. One study group encompassed 45 clinic samples from COVID-19–confirmed patients at different clinical stages. As shown in Figure 2A, of the 45 samples, 44 tested positive for SARS-CoV-2 antibodies with a sensitivity of 97.8%. There were 21 samples collected within 1 week (1 on day 1; 3 on day 3; 7 each on days 4 and 5; 2 on day 6; and 1 on day 7) of onset of COVID-19; all of them tested positive for SARS-CoV-2 antibodies. So far, no significant difference in antibody levels were observed between different sexes or ages (Figure 2B and 2C).

In another study group, shown in Figure 2D, 23 of 24 clinic samples tested positive for SARS-CoV-2 antibodies. We sorted the samples by collecting times: (1) 1 day after hospitalization; (2) any time during the hospitalization; and (3) follow-up on day 14 after release from the hospital. Clearly, the patients just arriving at the hospital had the lowest levels of SARS-CoV-2 antibodies. The antibody levels increased during treatment and after the patients were released from hospital.

As summarized in Table 3, the overall sensitivity of the serological assay for SARS-CoV-2 total antibodies was 97.1%. More works will be carried out to examine the levels of IgG and IgM of those positive samples, respectively, by simply changing the goat-antihuman IgG (H+L) secondary antibodies, which will detect both IgG and IgM, to human IgG Fc and IgM μ chain–specific secondary antibodies.

Using the assay as screening tool for an epidemiologic study, as shown in Figure 3 and Table 4, SARS-CoV-2

Table 3. Sensitivity of the Assay Against Positive Samples

Sources	SARS-CoV-2 Ab Negative	SARS-CoV-2 Ab Positive	Subtotal	Specificity
Hospital 1	1	44	45	97.8%
Hospital 2	1	23	24	95.8%
Total	2	67	69	97.1%

Positive samples were collected from coronavirus disease 2019–infected or recovered patients. Two different hospitals completed the test independently.
Abbreviations: Ab, antibody; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

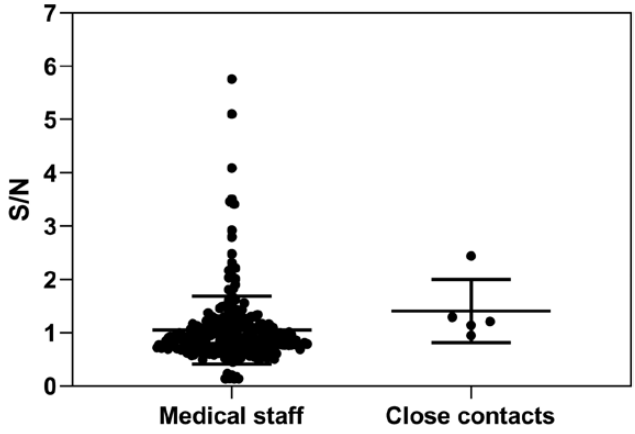

Figure 3. Screening of asymptomatic medical staff and close contacts. Samples were collected from asymptomatic medical staff or close contacts of patients with confirmed coronavirus disease 2019. Signal-to-noise ratio (S/N) was used for analysis. If the sample's S/N was < 1.5, it was considered negative for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibody (Ab); if S/N was 1.5–2, it was considered weak positive for SARS-CoV-2 Ab; if S/N was > 2, it was strong positive for SARS-CoV-2 Ab.

antibodies were detected in 10.1% (28/276) of the asymptomatic medical staff working at a hospital in Wuhan, China. In another case, 5 persons were in close contact with confirmed COVID-19 patients and they had been quarantined for 14 days, showed no sign of sickness, tested negative twice by NAT, and were released. One of them tested positive for SARS-CoV-2 antibodies by this serological ELISA kit.

This ELISA assay may offer a tool for the Chinese CDC to search the clues for those recently surfaced COVID-19 cases who had no clear connection with any confirmed COVID-19 patients.

DISCUSSION

The S1 protein binds to ACE2 protein on the surface of the human cells and plays a critical role in virus infection. Our data showed that the S1 protein of SARS-CoV-2 virus is heavily glycosylated, evidenced by the purified S1-His protein, which had an apparent molecular weight of 120 kD on SDS-PAGE gel, while its calculated molecular weight should be just around 70 kD. Glycosylation not only helps the protein fold correctly, but also contributes greatly to protein's affinity to its receptor. For example, the binding affinity of IgG1 to FcγRs on effector cell surfaces is highly dependent on the N-linked glycan at asparagine 297 (N297) in its CH2 domain [6, 7], with a loss of binding to the FcγRs observed in N297A point mutants [8, 9]. Even the nature of the carbohydrate attached to N297 modulates the affinity of the FcγR interaction as well [10, 11].

In this study, full-length SARS-CoV-2 S1 proteins were expressed using both human 293F cells and CHO cells to ensure the recombinant proteins have the correct glycosylation profiles to resemble the native conformation on the surface of virus. Using our patented technology, we have successfully increased the expression levels of the full-length recombinant SARS-CoV-2 S1 proteins up to 70 mg/L. Using the CHO cell–expressed full-length SARS-CoV-2 S1-His protein as the capturing antigen, we have been able to develop a COVID-19 serological ELISA kit with high specificity (97.5%) and great sensitivity (97.1%). With an accuracy of 97.3%, the assay we developed here will be well suited for screening of healthcare staff to reduce in-hospital transmission of SARS-CoV-2 virus.

Table 4. Screening of Asymptomatic Medical Staff and Close Contacts

Signal-to-Noise Ratio	Medical Staff	Close Contacts
< 1.5	248	4
1.5–2	10	0
> 2	18	1
Total	276	5

Samples were collected from asymptomatic medical staff or close contacts of patients with confirmed coronavirus disease 2019. If the sample's signal-to-noise ratio (S/N) was < 1.5, it was considered negative for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies; if S/N was 1.5–2, it was considered weak positive for SARS-CoV-2 antibodies; if S/N was > 2, it was considered strong positive for SARS-CoV-2 antibodies.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. R. Z. and L. S. report a patent pending for expression of SARS-CoV-2 S1 proteins using mammalian cells and their applications, unrelated to the submitted work. L. S. reports a patent pending for a method to express viral glycoproteins using mammalian cells, unrelated to the submitted work. All other authors report no potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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