Simultaneous detection of antibody responses to multiple SARS-CoV-2 antigens by a Western blot serological assay

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Abstract

The nucleic acid test is still the standard assessment for the diagnosis of coronavirus disease 2019 (COVID-19), which is caused by human infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In addition to supporting the confirmation of disease cases, serological assays are used for the analysis of antibody status and epidemiological surveys. In this study, a single Western blot strip (WBS) coated with multiple *Escherichia coli* (*E. coli*)-expressed SARS-CoV-2 antigens was developed for comprehensive studies of antibody profiles in COVID-19 patient sera. The levels of specific antibodies directed to SARS-CoV-2 spike (S), S2, and nucleocapsid (N) proteins were gradually increased with the same tendency as the disease progressed after hospitalization. The signal readouts of S, S2, and N revealed by the multi-antigen-coated WBS (mWBS)-based serological assay (mWBS assay) also demonstrated a positive correlation with the SARS-CoV-2 neutralizing potency of the sera measured by the plaque reduction neutralization test (PRNT) assays. Surprisingly, the detection signals against the unstructured receptor-binding domain (RBD) purified from *E. coli* inclusion bodies were not observed, although the COVID-19 patient sera exhibited strong neutralizing potency in the PRNT assays, suggesting that the RBD-specific antibodies in patient sera mostly recognize the conformational epitopes. Furthermore, the mWBS assay identified a unique and major antigenic epitope at the residues 1148, 1149, 1152, 1155, and 1156 located within the 1127–1167 fragment of the S2 subunit, which was specifically recognized by the COVID-19 patient serum. The mWBS assay can be finished within 14–16 min by using the automatic platform of Western blotting by thin-film direct coating with suction (TDCS WB). Collectively, the mWBS assay can be applied for the analysis of antibody responses, prediction of the protective antibody status, and identification of the specific epitope.

Key points

- A Western blot strip (WBS) coated with multiple SARS-CoV-2 antigens was developed for the serological assay.
- The multi-antigen-coated WBS (mWBS) can be utilized for the simultaneous detection of antibody responses to multiple SARS-CoV-2 antigens.
- The mWBS-based serological assay (mWBS assay) identified a unique epitope recognized by the COVID-19 patient serum.

Keywords COVID-19 · SARS-CoV-2 · Serological assay · Western blot strip · Antibody profile

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its emerging variants of concern have spread worldwide, resulting in the exponential growth of the global pandemic of coronavirus disease 2019 (COVID-19). The diagnosis of SARS-CoV-2 infection is largely relied on nucleic acid tests, rapid antigen tests, and serological assays (Ravi et al. 2020; Roberts et al. 2021; Singh et al. 2021; Yuce et al. 2021). Currently, the real-time reverse transcription polymerase chain reaction (RT-qPCR) is still the standard assessment for the confirmation of SARS-CoV-2 infection; however, it usually takes 2–4 h to accomplish a testing circle (Asif et al. 2021; Teymouri et al. 2021). During the disease progression of COVID-19 in patients, serological
assays have the best overall characteristics to detect and monitor specific antibodies in different stages and provide useful information and an indication of the severity of the disease (Post et al. 2020). Serological assays are of critical importance to define previous exposure to SARS-CoV-2 and the prevalence of COVID-19 in populations, evaluate the susceptibility or resistance to subsequent reinfection, identify highly reactive human donors for convalescent plasma therapy, and investigate the correlation of protection obtained through natural infection or vaccination (Tantuoyir and Rezaei 2021). In addition, serological assays provide the testing algorithms to indicate the presence of protective antibodies, which cannot be accessed by nucleic acid tests (Castillo-Olivares et al. 2021; GeurtsvanKessel et al. 2020; Liu et al. 2021; Patil et al. 2021; Tang et al. 2020).

Enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), rapid diagnostic test (RDT), and neutralization assay are the major types of serological assays (Espejo et al. 2020). ELISA typically uses a surface coated with specific viral antigen(s) to bind to or capture the corresponding antibodies, which are subsequently detected by a secondary antibody conjugated with an enzyme, which can digest a substrate to generate color or fluorescent signals quantitated by a spectrophotometer. ELISA assays can be finished in 2–5 h and deployed in different formats such as direct, indirect, competitive, and sandwich assays. CLIA basically adopts a similar concept as ELISA but uses chemical probes for the generation of light emission through a chemical reaction that greatly increases the sensitivity and lowers the detection limit. It usually takes 1–2 h to obtain CLIA results. ELISA and CLIA are both high-throughput laboratory-based immunoassays. RDT is mainly based on lateral flow immunoassay (LFIA) technology. Typically, RDT can be administered as a point-of-care (POC) test or self-test by using a strip to detect the presence of patient antibodies against specific viral antigen(s). RDT is simple to use and can obtain the test result within 10–30 min. Therefore, RDT has the potential to be applied in large-scale serological surveys without requiring a special demand for laboratory devices. Neutralization assay is a cell-based assay, which uses a pseudotyped or authentic virus to determine if protective antibodies can prevent viral infection in vitro and must be performed in laboratories with designated biosafety certificates. The neutralizing assay takes 3–5 days and is therefore not a common approach for large-scale serological surveys.

The SARS-CoV-2 genome encodes four major structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Naqvi et al. 2020). The S protein, which is composed of an N-terminal domain (NTD), a receptor-binding domain (RBD), and C-terminal S2 subunits, plays a critical role in host cell binding, fusion, and entry (Huang et al. 2020). It has been demonstrated that blocking of S protein binding to angiotensin-converting enzyme 2 (ACE2) receptor by the convalescent sera or the neutralizing antibodies derived from the recovered COVID-19 patients can inhibit SARS-CoV-2 infection (Cao et al. 2020; Chen et al. 2020; Hansen et al. 2020; Ju et al. 2020; Liu et al. 2020; Wan et al. 2020; Wang et al. 2020; Wu et al. 2020; Zost et al. 2020). Both of the M and the E proteins play important roles in virus assembly (Satarker and Namboothiri 2020). The primary function of the N protein is packing of the viral RNA genome into a helical nucleocapsid structure during viral replication (Bai et al. 2021). Furthermore, the high immunogenicity and abundant expression of N proteins during SARS-CoV-2 infection result in the induction of large amounts of specific antibodies (Dai et al. 2020). Consequently, S and N proteins are the major diagnostic antigens for serological assays (Li and Li 2021; Marien et al. 2021).

As reported previously, ELISA, CLIA, and RDT have been designed for COVID-19 serological assays (Espejo et al. 2020), but the Western blot-based method was rarely applied. In addition, the immunoassays for analyzing specific antibodies in the COVID-19 patient sera against various regions of S protein and the different functional domains of the S2 subunit remain scarce. In this study, we aimed to develop a serological assay which was performed on a Western blot strip (WBS) coated with multiple SARS-CoV-2 antigens, including S protein, NTD, RBD, S2, and N protein, for parallel characterization of the antibody profiles against these specific antigens. The serological assay using the multi-antigen-coated WBS (hereinafter referred to as mWBS) can obtain the results in 2–3 h. The mWBS-based serological assay (mWBS assay) was also used to screen the sera which were sequentially collected from the COVID-19 patients since the day they were admitted to the hospital for up to 31 days. The test results of mWBS assays can provide useful information on humoral immune responses to SARS-CoV-2 antigens in different stages of disease progression and are suitable for the implementation of epidemiological surveys of past infection and vaccination. We also tried to establish a diagnostic algorithm by comparing the antibody profiles of the S protein- and N protein-based mWBS assays with the results of neutralization assays for evaluation of the presence of protective antibodies in COVID-19 patient sera. In addition, the mWBS assay is applicable for epitope mapping experiments.

Materials and methods

Plasmid construction

The cDNA encoding for the full-length S protein (GenBank: BCN86353.1; residues 16–1208) with R682A, R683A,
and R685A substitutions, NTD (residues 16–310 of S protein), RBD (residues 331–520 of S protein, or S2 (residues 686–1206 of S protein) was amplified by PCR using a mammalian codon-optimized S gene sequence (GenScript, Cat. No. MC_0101081) as the template for subcloning into the pET30a vector (Novagen, Merck Group) with a C-terminal hexa-histidine tag (His-tag). The cDNA encoding for N protein (Sino Biological, Cat. No. VG40588-CH) was subcloned into the pET30a vector with a C-terminal His-tag. The cDNA encoding for two tandem repeats of the ZZ domain of Staphylococcus aureus protein A (designated as Z4 in the study) was synthesized (GenScript) and subcloned into the pET28a vector (Novagen, Merck Group) with a C-terminal His-tag. The gene sequence encoding for the S(732–894), S(816–855), S(909–1041), S(1042–1167), S(1168–1208), S(1042–1126), and S(1127–1167) was respectively subcloned into the pET30a vector (Novagen, Merck Group) with a C-terminal His-tag and an additional N-terminal superfolder GFP (sfGFP) fusion tag (Pedelacq et al. 2006). The plasmid construction of the sfGFP-CD proteins with a series of individual alanine substitutions at the residues 1144–1157 was described in the previous study (Li et al. 2022).

**Protein expression and purification**

The prokaryotic expression vector pET30a or pET28a was transformed into E. coli BL21(DE3) competent cells and then cultured in Luria–Bertani medium with kanamycin (50 μg/mL) at 37°C on an orbital shaker. Expression of the recombinant protein was induced at an A600 of 0.6 by adding isopropyl-1-thio-beta-D-galactopyranoside to a final concentration of 1 mM for 4 h. Cells were collected by centrifugation at 6000 g for 10 min. The cell pellet was washed three times with PBS and then homogenized by a cell disruptor (Constant Systems Limited, UK). The His-tagged S, NTD, RBD, or S2 was expressed in the inclusion body, which was further dissolved with 8 M urea in phosphate-buffered saline (PBS) and then purified using HisTrap FF column (GE Healthcare) pre-equilibrated with a binding buffer (20 mM sodium phosphate, 10 mM imidazole, 0.5 M NaCl, pH 7.4). Bound proteins were eluted with a 20–500 mM gradient of imidazole in a buffer containing 20 mM sodium phosphate (pH 7.4) and 0.5 M NaCl. All of the purified proteins were then exchanged buffer with PBS by using the PD-10 desalting column (GE Healthcare). Protein purity was examined by SDS-PAGE, and protein concentration was determined by the Bradford dye-binding method (Bradford 1976).

**Antibodies**

The animal experiments were approved by the Institutional Animal Care and Use Committee of National Taiwan University (approval number: NTU-109-EL-00051) and implemented in accordance with animal care and ethics guidelines. The NTD-specific monoclonal antibody (mAb) NTD-3F, the RBD-specific mAb 3D10, and the S2-specific mAb S2-8A were generated through the conventional hybridoma technology as described previously (Chiang et al. 2021; Li et al. 2021) by immunization of the BALB/c mice with the recombinant NTD, RBD, and S2 proteins expressed in the Exp293 cells. The anti-N protein polyclonal antibody (pAb) was generated by immunization of the BALB/c mice with the recombinant N protein (25 µg) four times at a two-week interval. The antisera of the anti-N protein pAb were collected and filtered through a 0.22 µm membrane disc. The non-specific human IgG (hIgG) was purchased from Merck (Cat. No. I4506).

**Human sera**

Serum samples were collected from hospitalized patients diagnosed with COVID-19 by RT-qPCR in the National Taiwan University Hospital. Written informed consent was obtained from all individual patients in the study, which was approved by the Institutional Review Board of National Taiwan University Hospital. Every patient was subjected to three times of serum collections at a one or two-week interval. The collected sera were heated at 56°C for 30 min and then stored at 4°C.

**The mWBS assay**

The mWBS is composed of a PVDF membrane (5 cm×0.4 cm) attached to the surface of a supporting plastic backboard (6 cm×0.4 cm). The recombinant S (0.16 µg), NTD (0.16 µg), RBD (0.36 µg), S2 (0.16 µg), N (0.24 µg), and Z4 (0.08 µg) were coated as separate lines on the mWBS. The mWBS was blocked with blocking buffer (5% non-fat milk in phosphate buffer saline with 0.05% Tween-20 (PBST)) for 15 min and then incubated with the serum sample (1:1000 in blocking buffer) at room temperature for
1 h. The mWBS was subsequently washed three times for 10 min each with PBST and then incubated with the HRP-conjugated goat anti-human IgG secondary antibody (Bethyl Laboratories, CAT # A80-119) for 30 min. The mWBS was then washed three times for 10 min each with PBST. The chemiluminescent substrate (VisGlow, Visual Protein Biotechnology, Taipei, Taiwan) was used to detect the signal, which was then visualized by the UVP BioSpectrum Imaging System (Upland, CA, USA). The signal density of S, S2, N, and Z4 revealed on the mWBS was measured by ImageJ. Since IgG molecules in the patient’s serum can bind to Z4 to obtain the detection signal, the value of Z4 was considered as the internal control. To process the signal density as a relative scale, the values of S, S2, and N on each mWBS were normalized against the value of Z4, which was designated as 100%.

Plaque reduction neutralization test (PRNT)

All experiments related to the authentic SARS-CoV-2 virus were done in the biosafety level 3 (BSL-3) laboratory. COVID-19 patient sera with different dilution ratios were incubated with 150 plaque formation unit (PFU) of SARS-CoV-2 strain hCoV-19/Taiwan/NTU13/2020 (GISAID ID: EPI_ISL_422415) in the presence of DMEM containing with 8 µg/mL TPCK-trypsin for 1 h at 37°C. Serum-virus mixtures were subsequently added to Vero E6 cell monolayers in the 24-well plates. After 1 h, cells were washed with PBS and overlaid with 2% (w/v) methylcellulose in DMEM supplemented with 2% FBS and then incubated at 37°C in the 5% CO₂ incubator. Five to six days later, cells were fixed with 10% formaldehyde in PBS and stained with 0.5% crystal violet to reveal the plaques. The concentration of the serum to reduce the number of plaques in the PBS-virus control experiment by 50% was denoted as the PRNT50.

Western blotting by thin-film direct coating with suction (TDCS WB)

The TDCS WB was conducted as described previously (Liu et al. 2016; Yen et al. 2014) with slight modifications. The mWBS (5 cm × 0.4 cm) was placed on the stage of the TDCS machine. The serum sample was fed into the slot die coater by using a pipette. The coater was mounted on the vertical axis of a translational stage to control the coating gap between the coater and the membrane by using a linear encoder. The stage running speed was set as 50 mm/s. The mWBS was coated with 20 µL of the diluted serum sample (1:100 in PBST) and then incubated at room temperature for 5–6 min. The PBST washing step was carried out within 90 s. The HRP-conjugated goat anti-human IgG secondary antibody (diluted 1:200 in PBST) was coated by using the same experimental procedures of the serum sample. The final PBST washing step was performed, and the chemiluminescent substrate was used to detect the signal, which was then visualized within 1 min by the UVP BioSpectrum Imaging System as described previously. The total operation duration of TDCS WB for the mWBS assay is about 14–16 min.

Results

The characteristics of the SARS-CoV-2 mWBS assay

As mentioned previously, the S and N proteins of SARS-CoV-2 have been utilized for various ELISA-based serological assays. In the study, multiple recombinant S, NTD, RBD, S2, and N proteins were prepared (Fig. 1a) and coated onto a PVDF membrane with a specially designed arrangement (Fig. 1b) to obtain the antibody profiles in sera of COVID-19 patients. In addition, two tandem repeats of protein A ZZ domain (Z4 protein) were utilized as the experimental control (Fig. 1b) since it can capture the human IgGs in the serum sample to develop a detection signal and determine whether the serological assay was valid or not. The mWBS was subjected to WB analysis with the positive control antibodies such as the anti-His tag mAb, anti-NTD mAb (NTD-3F), anti-RBD mAb (3D10), anti-S2 mAb (S2-8A), and anti-N protein pAb. The results showed that the anti-His tag mAb can recognize all the His-tagged antigens coated on the mWBS. NTD-3F, 3D10, and S2-8A can recognize S protein and their corresponding antigen fragments, the NTD, RBD, and S2, respectively (Fig. 1c). The anti-N protein pAb can only recognize N protein without exhibiting any cross-reaction with other S protein-related antigens (Fig. 1c). Certain unknown antibodies derived from mice receiving N protein immunization can bind to Z4 and yielded some detection signals (Fig. 1c). Notably, Z4 can be applied as the experimental control to capture the non-specific human IgG and then be developed as a single band on the mWBS (Fig. 1c).

Performance of the SARS-CoV-2 mWBS assay

The sera collected from five RT-qPCR-confirmed COVID-19 patients were used to determine the performance of the mWBS. For each patient, three serum samples were collected at different time points after being diagnosed with COVID-19. The day being admitted to the hospital was denoted as day 1. The data showed that the antibodies against S, S2, and N in the sera collected on day 1 can be detected by the mWBS assay even though the signal density remained weak at this time point (Fig. 2a). Seven days after hospitalization, the signal densities were all largely increased and can be clearly observed on the mWBS (Fig. 2a). Moreover, the levels of the antibodies against S, S2, and N in the sera
collected from patients 3, 4, and 5 were still in the plateau period after four weeks (Fig. 2a). The antibodies against NTD can be detected during 15–31 days in all of the serum samples (Fig. 2a). Surprisingly, the detection signals for antibodies against RBD were very weak and could only be detected in some serum samples such as patient 1 on day

Fig. 1 Design of the mWBS for COVID-19 serological assays. a The purified His-tagged S, NTD, RBD, S2, N, and Z4 were analyzed by SDS-PAGE and coomassie staining. The numbers labeled on the left of the small ticks are the molecular weight standards in the kDa unit. b The mWBS was composed of a PVDF membrane (5 cm×0.4 cm) attached to the surface of a supporting plastic backboard. The purified His-tagged S, NTD, RBD, S2, N, and Z4 were coated on the indicated bold lines as illustrated in the schematic diagram. c Several antibodies were utilized to reveal the target antigens on the mWBS. Anti-His, anti-His tag mAb. NTD-3F, NTD-specific mAb. 3D10, RBD-specific mAb. S2-8A, S2-specific mAb. Anti-N, anti-N protein pAb. hIgG, non-specific human IgG.

Fig. 2 Characterization of the antibody profiles of COVID-19 patient sera by mWBS assays. a The COVID-19 patient sera were collected sequentially on the indicated days after hospitalization. The serum samples and the control non-specific human IgG (hIgG) were diluted (1:1000) with blocking buffer and then incubated individually with a single mWBS at room temperature for 1 h. After washing three times with PBST, the HRP-conjugated goat anti-human IgG secondary antibody was utilized to develop the signal which was further detected by an imaging system. b The signal density of S, S2, N, and Z4 detected by the mWBS assay shown above was measured by ImageJ. To process the signal density as a relative scale, the values for S, S2, and N were normalized against the values of Z4. The 100% was marked with a red dotted line for easy reading. The day(s) after hospitalization for collecting the serum was plotted against the relative signal values derived from the corresponding mWBS assays.
21, patient 2 on day 8, patient 3 on day 15, patient 4 on days 17 and 31 days, and patient 5 on day 15 (Fig. 2a). In order to compare the antibody levels against S, S2, and N derived from different serological assays in a statistical way, the detection signals were measured by ImageJ software and normalized with the values of the Z4 bands on each mWBS. The measurement results were shown in Fig. 2b. As expected, the antibodies against S, S2, and N in patients 1–5 were all induced with the same tendency to reach the highest levels within 15–31 days after hospitalization (Fig. 2b).

We also found that more and more S-, S2-, and N-specific antibodies were detected as the disease progressed (Fig. 2b). Since these patients were all diagnosed with COVID-19 by RT-qPCR, the present SARS-CoV-2 mWBS can also be applied for diagnosis of COVID-19 and screening of the antibody status during the period of disease progression.

Analysis of the SARS-CoV-2 neutralizing potency of the COVID-19 patient sera by PRNT

To determine the SARS-CoV-2 neutralizing potency of the COVID-19 patient sera mentioned above, the sera collected from patients 1–5 were serially diluted with PBS and then subjected to PRNT assays. The serially diluted sera of patients 1, 2, 3, and 5 on the day being admitted to the hospital (day 1) did not exhibit enough neutralizing potency against SARS-CoV-2 (Fig. 3a–c,e, gray dashed lines). The sera of patient 1 or patient 2 collected on day 7 or day 8 exhibited stronger neutralizing potency that the serum dilution ratio for reaching PRNT50 was $10^{-2}$–$10^{-3}$ (Fig. 3a,b, black lines). After day 15, the neutralizing potency for all of the sera derived from patients 1–5 was greatly increased and exhibited the PRNT50 with the dilution ratios of $10^{-3}$–$10^{-4}$ (Fig. 3a–e).

Notably, we found that the tendency of the conversion of the serum neutralizing potency was highly correlated to the results observed in the mWBS assay (Fig. 2). Collectively, if the relative signal density of S protein over Z4 measured by the mWBS assay was more than 100% (Fig. 2b, P1: day 21; P3: day 15; P4: days 17 and 31; P5: day 28, blue bars), the PRNT50 of the patient serum can reach $10^{-3}$–$10^{-4}$ (Fig. 3a,c–e). If the relative signal density of S protein over Z4 was 75–100%, the PRNT50 of the patient serum was very close to $10^{-3}$. In general, a higher relative signal density of S protein over Z4 was detected by the mWBS assay, and more SARS-CoV-2 neutralizing potency was observed in the PRNT assay. Interestingly, the relative signal density of N protein over Z4 also has a similar tendency, but the threshold was slightly higher than that of S protein, although the anti-N protein antibodies may not play the major roles in neutralizing SARS-CoV-2. Therefore, the mWBS assay can be applied in the COVID-19 serological assay to reveal the antibody responses and also provide a useful indication for the prediction of the SARS-CoV-2 neutralizing potency.

Analysis of the S2-specific antibody profile by the mWBS assay

It has been demonstrated that the mWBS assay can be applied for the analysis of the specific antibodies against different functional domains of S protein (Fig. 2a). It is also observed that the signal density of S2 detected by the mWBS assay was nearly as strong as that of S or N (Fig. 2a), indicating that there is a large population of S2-specific antibodies in the COVID-19 patient serum. In order to investigate the specific antibodies against different subdomains
of S2, several recombinant S2 fragments, such as Core-N: S(732–894), FP: S(816–855), Core-C: S(895–1041), HR1: S(909–988), CD: S(1042–1167), and HR2: S(1168–1208) (Fig. 4a) were expressed as the sfGFP-fusion proteins with a C-terminal His-tag for preparing the target antigens. The His-tagged S2 fragments were coated on the WBS and then probed with the anti-His tag mAb or the COVID-19 patient sera. The S2 fragments and Z4 were detected by the anti-His tag mAb, and only Z4 was detected by the non-specific human IgG control (Fig. 4b). HR1 and HR2 were not detected by the sera of patients 1–5 (Fig. 4b), indicating that the immunogenicity of HR1 and HR2 might be very low for provoking an immune response through natural infection of SARS-CoV-2 in COVID-19 patients. In addition, Core-N and Core-C can be detected by the sera of patient 4 (days 17 and 31) and patient 5 (days 15 and 28) (Fig. 4b). FP, which is located within Core-N, was clearly detected by the sera of patient 4 (days 17 and 31), but it was not detected by the sera of patient 5 although Core-N was clearly detected by the same mWBS assay. This result suggested that the content of the FP-specific antibodies may not adhere strictly to the content of the Core-N-specific antibodies in the COVID-19 patient sera. Surprisingly, the signal density of CD was markedly detected by the sera of patient 1 (days 7 and 21), patient 3 (days 15 and 30), patient 4 (days 17 and 31), and patient 5 (days 15 and 28) and was highly co-related to the signal density of S2 (Fig. 4b). To further determine the key region in the CD region which was detected by the COVID-19 patient serum, the CD fragment was divided into two parts of S(1042–1126) and S(1127–1167) and iteratively subjected to the mWBS assay with the serum of patient 3 collected on day 15. The result showed that the COVID-19 patient serum (P3: day 15) can only detect S(1127–1167) without exhibiting any cross-reactivity to S(1042–1126), suggesting that S(1127–1167) was the major antigenic epitope for induction of the CD-specific antibodies (Fig. 4c).

**Application of the mWBS assay in the epitope mapping experiment**

In the previous study (Li et al. 2022), we have found that the S2-specific mAbs S2-4D, S2-5D, S2-8D, and S2-4A, which recognized the key residues of E1144, F1148, L1152, and F1156 within the epitope peptide S(1144–1156) and exhibited strong SARS-CoV-2 neutralizing activities. Here, we showed that the COVID-19 patient serum (P3: day 15) also exhibited cross-reactivity to S(1127–1156) and S(1144–1157) were utilized to determine the key residues recognized by the patient 3 (day 15) serum sample. The results of the mWBS assay showed that the signal density for detecting the series of the sfGFP-CD mutant proteins by using the anti-His antibody was very similar (Fig. 5a) and the negative control serum can only bind to Z4 (Fig. 5b). However, the signal density for detecting the sfGFP-CD proteins with the F1148A, K1149A, L1152A, Y1155A, or F1156A mutation by using the patient 3 (day 15) serum sample was largely decreased (Fig. 5c), indicating that these five residues are the critical antigenic determinants recognized by the majority of antibodies in the patient serum. Without doing the SDS-PAGE and the electric transfer of the target antigens to the

![Fig. 4](image-url)

**Fig. 4** Analysis of the antibody profiles of COVID-19 patients against the subdomains of the S2 subunit by mWBS assays. a The schematic diagram of the subdomains of the S2 subunit, S(686–1208). FP: fusion peptide, S(816–855). HR1: heptad repeat 1, S(909–988). CD: connection domain, S(1042–1167). HR2: heptad repeat 2, S(1168–1208). b The S2 subdomains and Z4 were coated on a WBS and then detected by the anti-His tag mAb, the non-specific human IgG (hIgG), or COVID-19 patient serum (P1–P5), as described previously. c CD, S(1042–1126), S(1127–1167), and Z4 were coated on a WBS and then detected by the anti-His tag mAb, control human IgG, or the serum of P3 collected on day 15 after hospitalization (P3(15)).
PVDF membrane, the mWBS assay provides a reliable way to perform the epitope mapping experiment and a simple way to demonstrate the results.

The conventional WB usually takes 2–3 h to obtain the analysis result. In order to shorten the operation duration of the mWBS assay, Western blotting by thin-film direct coating with suction (TDCS WB) (Liu et al. 2016; Yen et al. 2014) was applied in the epitope mapping experiment. The mWBS was mounted on the platform of a TDCS WB instrument and subjected to the antibody probing and the PBST washing step under the highly repeatable process control. The results of the mWBS assay conducted by TDCS WB exhibited the same results as that of the conventional WB (Fig. 5d). Notably, it only took 14–16 min to finish the mWBS assay.

Discussion

Both nucleic acid tests and serological assays are commonly used for the screening and diagnosis of infectious diseases. Serological assays can also be conducted by asymptomatic individuals for which RT-PCR was either not performed or for which nasopharyngeal or throat swab result was negative at the time of examination. More importantly, serological assays are of advantage for epidemiological studies and investigations of the immune response to vaccination. It has been suggested that accurate SARS-CoV-2 seroprevalence surveys require robust multi-antigen assays to increase specificity and improve overall performance (Ayoub et al. 2020; den Hartog et al. 2020; Fotis et al. 2021). Furthermore, because the testing readouts were derived from the simultaneous detection of antibody responses against SARS-CoV-2 N, S1, and RBD antigens, the serological assay can increase the specificity of diagnosis for borderline cases and provide a more comprehensive picture for a low seroprevalence epidemiological study (Marien et al. 2021; Ni et al. 2020; Qu et al. 2020). Based on the same concept, here, we developed the mWBS assay using the SARS-CoV-2 S, S1, RBD, S2, and N proteins as the antigens, which can be respectively detected by the anti-His tag mAb, anti-NTD mAb (NTD-3F), anti-RBD mAb (3D10), anti-S2 mAb (S2-8A), and anti-N protein pAb (Fig. 1c). Our data further demonstrated that the specific antibodies in COVID-19 patient sera against S and N proteins were both gradually induced as the disease progressed after hospitalization. The detection signals against S2 subunits were also clearly found in the mWBS assays. To our surprise, the detection signals against RBD were not revealed by the mWBS assays (Fig. 2) even though the serum samples exhibited strong SARS-CoV-2 neutralizing potency in the PRNT assays (Fig. 3). It has been known that the SARS-CoV-2 neutralizing potency of the serum samples was largely derived from the anti-RBD antibodies (Castillo-Olivares et al. 2021; GeurtsvanKessel et al. 2020). Therefore, our data suggested that the recombinant RBD proteins, which were expressed in E. coli as inclusion bodies and purified by using the buffer containing with 8 M urea, were not suitable for serological assays. On the other hand, these findings suggested that the RBD-specific antibodies in COVID-19 patient sera mostly recognize the conformational epitopes (Ladner et al. 2021;
Li et al. 2020) which shall be maintained in the correctly folded structure. Similar results were also observed in the mWBS assays using the E. coli-expressed NTD as the antigen (Fig. 2a), implying that the NTD-specific antibodies in COVID-19 patient sera may not be abundantly induced after SARS-CoV-2 infection or they also need to bind to the natively folded NTD. In contrast, the mWBS assays using the E. coli-expressed S and S2 antigens can still obtain very strong signals. After performing a second round of mWBS assay using the S2 truncated fragments as antigens, the results showed that the detection signal against the CD domain, S(1042–1167), was clearly observed using the serum sample of patient 3 (Fig. 4b). The third round of mWBS assay further found that the majority of anti-S2 antibodies in the serum sample of patient 3 specifically bind to a unique epitope located in the region of S(1127–1167) (Fig. 4c). These data showed that the mWBS assay using multiple SARS-CoV-2 antigens and their truncated fragments can be applied for investigation of the binding epitope recognized by a specific group of antibodies in the COVID-19 patient serum. To our knowledge, this is the first study to use multiple S2 truncated fragments for COVID-19 serological assays (Guo et al. 2022; Han et al. 2021; Ilkhani et al. 2021). More importantly, our data showed that the COVID-19 patient serum (P3: day 15) seemed to have a unique antibody profile against the S(1127–1167) fragment. The detailed epitope mapping experiments performed by the additional mWBS assay further demonstrated that the antibodies in the patient 3 (day 15) serum sample have the major binding epitopes at the residues F1148, K1149, L1152, Y1155, and F1156, which are very similar with several S2-specific SARS-CoV-2 neutralizing antibodies (Li et al. 2022; Pinto et al. 2021; Sauer et al. 2021; Zhou et al. 2022). Notably, patient 3 (day 15) serum sample also performed very strong SARS-CoV-2 neutralizing activity (Fig. 3c).

Recent studies have shown that the seroconversion rates reach 100% in both hospitalized COVID-19 patients and self-quarantine patients with mild disease symptoms after 10–14 days, and the antibody levels of the patient sera were correlated with clinical severity (Iyer et al. 2020; Long et al. 2020; Okba et al. 2020; Patil et al. 2021; Xu et al. 2021). By using the mWBS assays to detect the specific antibodies in COVID-19 patient sera, we also found the same tendency that the antibody levels were very low in the first few days after being admitted to the hospital and then largely increased as the disease progressed (Rezaei et al. 2021). Eight to fifteen days after hospitalization, the antibody levels may reach plateau periods (Fig. 2a). Notably, the detection signal in the mWBS assays can be clearly observed on day 1 for patients diagnosed with COVID-19 by RT-qPCR (Fig. 2a), indicating that the mWBS assays can also support the diagnosis of COVID-19.

The excellent correlation between the ELISA titers against the spike protein and the SARS-CoV-2 neutralization potency was reported previously (Amanat et al. 2020; Okba et al. 2020). In the study, we also tried to find out the possible correlation between the signal readouts of the mWBS assay and the PRNT assay. We found that if the relative signal density of S protein over Z4 detected by the mWBS assay was more than 100% (Fig. 2b), the PRNT50 of the patient serum can reach a dilution ratio of 10^3–10^4. In addition, if the relative signal density of S protein over Z4 detected by the mWBS assay was between 75 and 100%, the PRNT50 of the patient serum can reach a dilution ratio of 10^3 (Fig. 3). These findings demonstrated that the readouts of the mWBS assay can be converted to the potential neutralizing potency with a positive correlation as they have been shown in the ELISA-based assays (Iyer et al. 2020; Long et al. 2020; Patil et al. 2021; Xu et al. 2021). Notably, the testing duration for finishing the mWBS assay is only 1–2 h, unlike performing the standard neutralization assay for several days in a BSL-3 laboratory.

Although the mWBS assay provides a simple method to support the diagnosis of COVID-19, screen the potential neutralizing antibody, and identify the specific antibody biomarker in the patient sera, its operating turnaround time needs to be further improved to fit the demand of large-scale and high-throughput epidemiological surveys. Our group has previously invented an easy and fast Western blotting method named TDCS WB (Liu et al. 2016; Yen et al. 2014), which can completely finish a high-quality WB within 15 min. Here, we also demonstrated that the mWBS assay was finished within 14–16 min by using the TDCS WB platform (Fig. 5d). It is worth mentioning that the operation duration of the mWBS assay in combination with TDCS WB is as short as that of LFIA, which usually takes 15 min to read out the test result. Thus, the power of the mWBS-TDCS WB technology can be applied for performing automatic high-throughput serological assays, epitope mapping experiments, diagnostic analysis during the period of disease progression, and large-scale epidemiological surveys. The volume of the diluted serum sample (1:100 in PBST) for finishing the mWBS-TDCS WB only needs 20 μL, so this newly developed technology can be integrated into the general serological examination and the specific detection of infectious diseases, including COVID-19, as demonstrated in the study.

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Data availability All data associated with this study are included in the paper.

Declarations

Ethics approval The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (grant numbers NTU-109-EL-00051) and implemented in accordance with the paper.

Conflict of interest The authors declare no competing interests.

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