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COVID-19 diagnosis and study of serum SARS-CoV-2 specific IgA, IgM and IgG by a quantitative and sensitive immunoassay

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Highlights

- Developed a highly quantitative and sensitive serologic immunoassay for SARS-CoV-2-specific IgA, IgM and IgG in COVID-19 patients.
- Showed the inclusion of IgA to the conventional IgM + IgG in a serological test improves the performance.
- Revealed the kinetics of three antibody isotypes in COVID-19 patients.
- Observed that serum IgA level positively correlated with COVID-19 disease severity.

Abstract

Background

The current pandemic of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused a great loss in lives and economy. Detecting viral RNAs on nasopharyngeal and throat swabs is the standard approach for SARS-CoV-2 diagnosis with variable success. Currently, there are only a few studies describing the serological diagnostic methods that involve the detection of SARS-CoV-2-specific IgM and IgG. Here, we aimed to develop a more quantitative and sensitive serological test for COVID-19 diagnosis, monitoring and clinical investigation, based on the detection of antigen-specific IgA as well as IgM and IgG in blood in response to SARS-CoV-2 infection.

Methods

In this investigation, we report the development of a set of validated diagnostic kits for detecting serum IgA, IgM, and IgG specific to SARS-CoV-2 nucleocapsid protein (NP) and receptor-binding domain (RBD) of the spike protein by chemi-luminescence immuno-analysis. The kits were tested with a cohort of 216 sera from 87 laboratory-confirmed COVID-19 patients, and 483 sera from SARS-CoV-2 negative or healthy individuals as negative controls. A standard receiver operating characteristic (ROC) analysis was conducted to evaluate the diagnostic accuracy. Using the kits, serum levels of IgA, IgM, and IgG were analyzed, in response to SARS-CoV-2 infection and COVID-19 pathogenesis.

Findings

The diagnostic kits based on the RBD antigen outperformed those based on the NP. RBD-specific IgA, IgM, and IgG detection kits showed sensitivities of 98.6%, 96.8%, and 96.8%, and specificities of 98.1%, 92.3%, and 99.8%, respectively. In addition, using purified RBD-specific immunoglobulins from a serum pool of COVID-19 patients as standards, the serum concentrations of RBD-specific IgA, IgM, and IgG proteins were determined. The concentrations varied widely among different patients. Median concentration of IgA and IgM reached peaks at 16-20 days after illness onset at 8.84 μg/mL and 7.25 μg/mL, respectively, while median concentration of IgG peaked during 21-25 days after illness onset at 16.47 μg/mL. Furthermore, the serum IgA level positively correlates with COVID-19 severity.

Interpretation

Our immunoassay of measuring SARS-CoV-2 specific antibodies IgA, IgM, and IgG in serum provides a better serological testing with improved sensitivity and specificity. Data of IgA, IgM, and IgG responses in blood of COVID-19 patients may provide novel insight for the monitoring and treatments of COVID-19. The kits are also suitable for epidemiological studies and vaccine validations.

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Research in context

Evidence before this study

On April 13th, 2020, we searched PubMed and preprint depositories with the key words COVID-19, SARS-CoV-2, antibody, IgM, IgG, or IgA. Ten studies described serum antibody responses in COVID-19 patients; most of them only measured IgM and IgG. Only three of them tested IgA levels. Relationship of serum IgA and disease severity has not been reported. *The Lancet* published an article on April 4th 2020, calling for "developing antibody tests for SARS-CoV-2".

Added value of this study

We present serum profiles of IgA, IgM and IgG responses in a cohort of 87 COVID-19 patients. We found the RBD of the SARA-CoV-2 spike protein to be a better viral antigen than the nucleocapsid protein for diagnostic kits. IgA detection provides additional values for diagnosing and monitoring COVID-19. The combination of IgA/IgG or IgA/IgM/IgG provides improved diagnostic reliability as compared to conventional IgM/IgG combinations. In addition, we observed that IgA levels in serum correlate positively with COVID-19 severity.

Implications of all the available evidence

Highly sensitive and quantitative immunoassays to measure serum antibodies will improve clinical diagnosis and epidemiology study of COVID-19. It is of great interest that the serum IgA levels positively correlate with illness severity. Further studies on the role of IgA in disease progress are warranted.

Introduction

At the end of 2019, a novel coronavirus (2019-nCoV or SARS-CoV-2) emerged in Wuhan, Hubei Province in China, causing a new type of coronavirus disease now named as COVID-19.[1] The virus spread globally and became a public health emergency and pandemic declared by the World Health Organization.[2] The quickly determined genetic sequence and virologic studies indicate that it is an enveloped RNA virus belonging to the corona virus superfamily.[3] Among the seven coronaviruses known to cause human diseases, the severe acute respiratory syndrome (SARS) virus broke out in 2003[4] and Middle East Respiratory Syndrome (MERS) virus broke out in 2012[5]. COVID-19 which is pathologically related to but different from SARS is expected to cause great impact on human society since World War II.[6] Reliable and effective diagnostics of SARS-CoV-2 and treatment of COVID-19 are urgently needed.

Detections of SARS-CoV-2 viral RNA by methods such as RT-qPCR supplemented by CT imaging are the primary methods for clinical diagnosis of COVID-19.[7, 8] However, this method has inherent limitations. The difficulty to obtain high-quality and consistent throat swab samples, as well as the low viral load at the late stage of infection, are limiting factors in clinical practice. Both challenges result in

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a sensitivity below 70%.[9-13] Therefore, there is an urgent need for more reliable and rapid diagnostic methods to screen SARS-CoV-2 infected people including those who do not have overt symptoms. A serological test of virus-induced antibody production has unique advantages in clinical diagnostics, especially for identifying people who acquired immunity against pathogens without noticeable symptoms.[14] When the virus invades host, the body produces large amounts of immunoglobulin (Ig) by the immune system and released into blood, among them, IgG, IgM, and IgA isotypes.[15] It has been widely believed that IgM is the first antibody to be transiently synthesized in response to the virus invasion.[15] IgG is a major class of immunoglobulins found in the blood, comprising 75% of total serum immunoglobulins and has long-term immunity and immunological memory.[15, 16] Therefore, a combination of IgM and IgG has been used in various serological tests for detecting infection of SARS-CoV-2 as previously used for SARS and other coronaviruses.[10, 11, 14, 17-21] In contrast, IgA, which is mainly produced in mucosal tissues to hinder virus invasion and replication but also detected in blood (~15% of total immunoglobulins in blood),[22] has not been widely used in serological tests for detecting coronavirus infection. IgA's production kinetics and roles in anti-viral immunity of IgA are even less known. Currently, only a few published studies reported diagnosis of COVID-19 by using ELISA or "flow immunoassay" for detection of serum IgM and IgG with limited accuracy,[10, 11, 17-20] although SARS-CoV-2 specific IgA in serum was also detected in recent papers or a preprint. [12, 23, 24] The kinetics of antibody responses in COVID-19 remains undefined, specifically for IgA production.

In this study, we designed and evaluated a set of sensitive and quantitative kits to measure serum IgA, IgM, and IgG for detection of SARS-CoV-2 infection. Comprehensive data of RBD specific IgA, IgG, and IgM antibody levels in 216 serum samples of 87 COVID-19 patients and 483 negative controls are presented.

Methods

Patients and clinical samples

This study was reviewed and approved by the Medical Ethical Committee of the First Affiliated Hospital of USTC (approval number: 2020-XG(H)-014) and the First Affiliated Hospital of Anhui Medical University (approval number: Quick-PJ 2020-04-16). Patient information is listed in **supplementary table 1**.

Confirmed COVID-19 cases and clinical classifications were defined according to the New Coronavirus Pneumonia Prevention and Control Program (7th edition) published by the National Health Commission of China. This study enrolls a total of 87 cases of confirmed COVID-19 patients, who were admitted to the First Affiliated Hospital of USTC Hospital or the First Affiliated Hospital of Anhui Medical University between Jan 26 and Mar 5, 2020. Their blood samples were collected during routine clinical testing. All enrolled cases were confirmed to be infected with SARS-CoV-2 by use of a standard RT-qPCR assay on throat swab samples from the respiratory tract. For all of the enrolled patients, the date of illness onset, clinical classifications of severity, RNA testing results during the hospitalization period, and the personal demographic information, were obtained from the clinical records.

Among them, five patients were admitted to the ICU, one was died of cerebral hemorrhage after stroke. Twenty-two patients had severe COVID-19, and all of whom required oxygen supplementation. Fifty-six patients had moderate and nine patients had mild COVID-19. The median age of patients was 48 years (range 21–91), and the average age of patients was 47.4 years. Thirty seven (42 5%) patients had

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underline illnesses; the most common one was hypertension in eighteen patients (20.7%). A total 216 serum samples were taken from the 87 COVID-19 patients.

Negative controls and potentially interfering non-COVID-19 patient serum samples were collected in order to evaluate the reliability of the kits. This cohort contains 330 sera from obviously healthy people, fifteen sera from once suspected cases (RT-qPCR negative but had typical manifestation of pneumonia) and 138 sera from other patients with different underlying diseases. All sera were stored at -20 ℃.

Molecular cloning, protein expression and purification

The viral nucleocapsid protein (NP) was expressed and purified from *E. coli*. Briefly, special treatment during the addition of high salt in lysis buffer and a hydrophobic interaction column was used to completely remove non-specific nucleic acid contamination. Our final protein was homogeneous and free of nucleic acid contamination as revealed by gel filtration and UV-Vis spectrum.

To make recombinant SARS-CoV-2 RBD in mammalian cells, an IFNA1 signal peptide DNA sequence, and DNA sequences encoding receptor binding region of spike protein and a human IgG1 Fc were fused together and cloned into pTT5 vector. The constructed expression vector was used to transiently transfect human HEK293F cells by polyethylenimine. After three days of expression, fusion protein was purified from cell supernatant using a Protein A column.

Diagnostic kit preparation and testing

Briefly, the purified NP or RBD viral antigens were coated to magnetic particles to catch SARS-CoV-2 specific IgA, IgM, and IgG in patient sera. Then a second antibody that recognizes IgA, IgM, or IgG is conjugated with acridinium (which can react with substrates to generate a strong chemiluminescence) was added for detection of IgA, IgM, and IgG, respectively. The detected chemiluminescent signal over background signal was calculated as relative light units (RLU). Serum samples were collected by centrifugation of whole blood in test tubes at room temperature for 15 min. Prior to testing, a denaturant solution was added to each serum to a final concentration of 1% TNBP, 1% Triton X-100. After adequate mixing by inverting, the samples were incubated at 30 ℃ for 4 hours to completely denature any potential viruses. Such solvent/detergent (1% TNBP + 1% Triton X-100) treatment is recommended by WHO guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products (*https://*www.who.int/bloodproducts/*publications/WHO_TRS_924_A4.pdf*) [25]. Virus deactivated serum samples were then diluted 40 times with dilution buffer and subjected to testing at room temperature. Then RLU was measured using a fully automatic chemical luminescent immunoanalyzer, Kaeser 1000 (Kangrun Biotech, Guangzhou, China).

SARS-CoV-2 RBD-specific IgA, IgM, and IgG antibodies were purified from a serum pool of recovering patients (a manuscript in preparation) to be used as standards. The concentrations were determined using Bradford method (using bovine serum albumin protein as a standard). These antibodies were used to make a standard curve for each antibody detection kits to quantify the absolute antibody amounts in serum.

Statistical analysis

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Based on the clinical RT-qPCR diagnosis results of SARS-CoV-2 infection, receiver operating characteristic (ROC) analysis was conducted using MedCalc software to determine the optimal cut-off value (criterion) and evaluate the diagnostic value of NP- or RBD-specific IgA, IgM, and IgG kits. The specificity and sensitivity of the antibody kits were calculated according to the following formulas:

Specificity (%) = 100 x [True negative / (True Negative + False Positive)];

Sensitivity (%) = 100 x [True Positive / (True Positive + False Negative)];

Overall agreement (%) = (True negative + True Positive) / Total tests.

In order to analyze the correlation of serum antibody levels and age with disease severity, we first used the Kruskal Wallis test[26] to test if there is any significant difference of IgA among the three groups (Mild, Moderate, Severe). Then Dunn's test[27] was used to perform a pair-wise test between each group, and Benjamini-Hochberg procedure[28] was used to adjust p-values. All the above analyses use R software version 3.6.1[29]. A p value less than 0.05 was judged statistically significant.

Results

Highly purified SARS-CoV-2 NP and RBD proteins (supplementary figure 1) were employed to develop a series of serological test kits, to detect the presence of NP- and RBD-specific IgA, IgM, and IgG, respectively (hereinafter referred to as "NP kit" and "RBD kit"). A cohort of 216 sera from 87 SARS-CoV-2 infected patients was tested with both NP and RBD kits, together with 20 sera from 20 non-SARS-CoV-2 infected patients as negative controls initially. The NP kits for IgA, IgM, and IgG showed diagnostic sensitivities of 89.8%, 78.2%, and 95.8%, and specificities of 85.0%, 95.0%, and 100% respectively (supplementary figure 2A-C). However, the RBD kits for detecting IgA, IgM, and IgG showed higher diagnostic sensitivities of 97.2%, 93.1%, and 96.8%, and specificities of 100%, 90.0%, and 100%, respectively (supplementary figure 2D-F). We conclude that the RBD based kits provide a better diagnostic accuracy than those based on NP, and thereafter used RBD kits in further studies.

To further evaluate the diagnostic accuracy of the RBD-based antibody detection kits, 330 sera from healthy people, 138 interfering sera from non-SARS-CoV-2 infected patients with different underlying diseases, and 15 sera from once suspected cases (RT-qPCR negative but had typical pneumonia symptoms) were included as negative controls. All samples were measured using RBD-based IgA, IgM, and IgG kit, respectively. The testing results were shown in figure 1A-C. With few exceptions in patients with other diseases (see supplementary table 2 for details), our detection of antibodies binding to SARS-CoV-2 RBD viral antigen is highly specific as well as sensitive. Overall, RBD-based IgA, IgM, and IgG kits show sensitivities of 98.6%, 96.8%, and 96.8%, and specificities of 98.1%, 92.3%, and 99.8%, respectively (figure 1D-F). The sensitivities, specificities and overall agreements of the RBD based IgA, IgM, or IgG kit, and their combinations are also summarized in table 1. When combining the IgA and IgG kits, the sensitivity, specificity and overall agreement elevate to 99.1%, 100%, and 99.7%, respectively. This is much better than when IgM and IgG were combined. When IgA, IgM, or IgG individual kit was used, we observed a total of 9 (0.61% to 6.67%), 37 (5.54% to 40.0%), and 1 (0 to 0.73%) false positive cases in the three types of “negative controls”, respectively, as shown in supplementary table 2. IgA is second after IgG in yielding few false positive, but much better than IgM. Few false-positive IgA results were mainly found in non-COVID-19 patients who had pneumonia or other underlying diseases. Very few cases of RBD IgA and IgG positive results in 330 healthy individuals

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and 153 non-COVID-19 patients also indicate that our RBD-based detection kits did not cross-interact with antibodies raised against other human coronaviruses presenting in ~15% of common cold cases and also causing pneumonia. Taken together, our detection systems are highly specific to SARS-CoV-2 RBD.

We attempted to analyze the kinetics of all the three isotypes of antibodies when multiple serum samples were collected from individual patients. Data from nine patients were showed in supplementary figure 3. To better understand the trends of antibody levels detected in all the 87 COVID-19 patients (some of them contributed multiple samples), data of 216 sera samples were divided into 6 groups according to the time windows of collection after illness onset. As shown in table 2, at 4-10 days after symptom onset, the RBD IgA kit showed the highest positive diagnostic rate as 88 2% (15/17), which is 76 4% (13/17) and 64 7% (11/17) for IgM and IgG kit, respectively. The 2 sera diagnosed as negative at the 4-10 days group by the IgA kit were collected at the 4th day after illness onset, which could be too soon for detecting viral-specific antibodies of any types. In the group of 11-41 days after symptom onset, both IgA and IgG kit showed the same positive diagnostic rate as 99 5% (198/199). In contrast, IgM kit somehow showed a relatively lower positive diagnostic rate as 98 5% (196/199). These results suggest that including IgA in a test kit would provide better diagnostic outcome. We also plotted the quantitative data of all the three antibody levels as a function of the time windows when sera were collected after illness onset (figure 2A).

Because the detection sensitivity would vary among IgA, IgM, and IgG due to different secondary antibodies used, we used highly purified RBD-specific IgA, IgM, and IgG proteins from pooled sera of COVID-19 patients as standards (standard curves were shown in supplementary figure 4). In this way, we can convert RLU measured for clinical samples into absolute antibody concentrations (amounts per mL). To simplify a plot from large numbers of samples, we only plotted median and interquartile range values of antibody concentrations as a function of time windows. As shown in figure 2B, the median concentration of IgA reached the highest (8 8 μg/mL) during 16 to 20 days after illness onset, and then began to decline but remained at about 3 6 μg/mL until 41 days. The median concentration of IgG was the lowest in early stages but raised at 15 days post illness onset. IgG concentration reached peak during 21-25 days after illness onset as 16 5 μg/mL, and stayed at a relatively high concentration (11 4 μg/mL) until 41 days, suggesting that IgG is more suitable for later stage of COVID-19 diagnosis. Although IgM reached its peak at early stages, its detecting sensitivity is lower than that of IgA and IgG. Our data suggest that IgM has the lowest diagnostic power among the three types of antibodies for diagnosing SARS-CoV-2. Adding IgA into a diagnostic kit that contains IgG and IgM improves the serologic testing power at both early and late stages.

To explore if a simple laboratory test such as measuring antibody levels in serum could serve as a quantifiable indicator for COVID-19 severity, we divided the 87 patients into three severity groups based on established clinical classifications. Consistent with previous studies [30], we found that disease severity is correlated positively with age in our cohort (supplementary figure 5). Patients with severe symptoms were significantly older (median age of 62 5) than those patients with moderate (median age of 46) and mild symptoms (median age of 30). Remarkably, we found that IgA concentrations in severe cases were significantly higher than mild or moderate cases (figure 3A). IgG levels in moderate and severe COVID-19 patients were also higher than mild cases ($p < 0\ 0001$) (figure 3C). The observation that serum IgG levels were higher in severe and moderate than mild COVID-19 patients have been previously reported

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[10, 21]. We also provided here a novel observation that serum IgA levels correlate with COVID-19 severity (figure 3A), how the levels and roles of different types of antibodies as related to COVID-19 severity remain to be determined.

Discussion

Compared to sampling of nasopharyngeal or throat swabs, blood extraction is more convenient and reliable. Furthermore, serum antibody test is more convenient, fast and accurate, and with other advantages over the detection of viral RNA.[9, 17] We report here an improved serological kit that can sensitively and quantitatively detect serum levels of IgA as well as IgM and IgG. Together with recent reports by others[9-12, 17-20, 23, 24], the serological data that we obtained from 216 serum samples of 87 COVID-19 patients and 483 negative controls provide valuable information for all of us to use in the coming months, for diagnostics, treatment, epidemiological studies and vaccine validations of COVID-19.

RBD-based serologic kits is better than NP-based kits for detecting IgA as well as IgM and IgG

The nucleocapsid protein (NP) is the most abundant protein in coronaviruses, which was reported to be highly immunogenic and often used as a diagnostic marker for coronaviruses such as SARS-CoV[31]. The RBD of the spike protein on viral surface is the ligand binding to the major host receptor ACE2; therefore RBD could be a main target for neutralization antibodies.[32, 33] In this study, we explored the possibility of using either NP or RBD as an immobilized antigen in for developing a clinical COVID-19 diagnostic kit. Our data (supplementary figure 2) showed RBD-based diagnostic kits were better performed than that of NP in detecting all the three types of antibodies. A few previous studies reported that RBD-based IgM and IgG detection is better than NP once a comparison was made[19, 23], and the measurement is agreeable with the titers measured by virus neutralization assays[23, 24]. We provided here the evidence that RBD as an immobilized antigen is also better than NP in detection serum IgA from COVID-19 patients. The exact mechanisms of difference between the use of two types of viral antigens remain to be resolved. It could be that the NP as a highly basic protein interacts with acidic residues in complementarity determining region in antibodies is less specific. It could also be due to the fact that the NP antigen is expressed in bacteria as most investigators do, and the RBD protein we used is expressed in a human cell line enabling critical glycosylation and high-affinity binding to antibodies raised in COVID-19 patients. Nonetheless, we showed that our serological kits based on SARS-CoV-2 spike protein RBD as an immobilized antigen provide a high sensitivity and specificity for detecting IgA, IgM, and IgG in a quantitative manner.

Our serological kits have overall good performance

Our kits have much higher accuracy than RT-qPCR (sensitivity less than 70%) for detecting viral RNA[9-13], and published immune-assays such as " flow immunoassay" and ELISA in earlier studies[10-12, 17-21, 23, 24]. When we combined RBD-specific IgA and IgG kits together, the sensitivity, specificity and overall agreement elevate to 99·1%, 100%, and 99·7%, respectively (table 1). In addition, this RBD-based detection kit may also help to screen and detect neutralization antibodies targeting SARS-CoV-2 RBD, because this peptide domain is exposed on viral surface and functions as a ligand binding to the host cell surface receptor ACE2.

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Detection of three isotypes of SARS-CoV-2 induced antibodies

Although one serum collected at the 4th day after illness onset was diagnosed as positive by our IgM kit (not IgA or IgG kits in this study), the IgM kit overall showed a lower diagnostic specificity of 92.3% compared to that of IgG and IgA (figure 1). IgM is known to have relatively lower affinity toward antigens compared with that of IgG or IgA. In addition, IgM often causes false positive signals as we also observed (supplemental table 2), due to its pentameric structure[34]. To the contrary, IgA or IgG antibody does not have this problem. Our RBD-specific IgG kit showed high specificity of 99.8% (figure 1) but relatively low sensitivity of 96.8%. This is expected, because that most (6/7) false negative cases were samples collected at 4-10 days after illness onset when IgG production is likely very low.

Our RBD-based IgA kit showed high sensitivity and specificity of 98.6% and 98.1%, except two sera collected at the 4th day after illness onset. All other sera (2 at the 6th day, 3 at the 7th day, 1 at the 8th day, 6 at the 9th day, and 3 at 10th day after illness onset) were diagnosed as positive. Based on our results, IgA and IgM are produced nearly simultaneously in early stage of infection, while IgA test has less false positives. As a result, IgA should be included in a serological test, which may provide higher diagnostic accuracy for COVID-19. Therefore, we highly recommend the use of RBD-specific IgA/IgG or IgA/IgM/IgG combinational serological test supplementing nucleic acid detection to provide a more accurate diagnosis of COVID-19.

Kinetics of antibody production during COVID-19

We also provide a data set of absolute antibody levels and their production kinetics for all three isotypes of antibodies in serum. Our results revealed that both IgM and IgA have early responses (peaked around 20th day after illness onset), while IgG showed up later (peaked around 25th day after the onset). Rapid increase of the three isotypes of serum RBD-specific antibodies started at about 10 days after illness onset (supplementary figure 4A-C), which is consistent with other reports describing the trends of IgM and IgG levels in serum[14-19]. The early appearance of IgA in COVID-19 patients' sera is probably due to the initial infection of this virus at the respiratory system, which is rich of mucosal immune cells. Due to the low basal level of IgA in serum, it makes SARS-CoV-2 specific IgA detection highly sensitive at early stage of infection. When we analyzed IgA, IgM, or IgG concentrations in the patients' serum with different COVID-19 severity, we observed that disease severity is positively correlated with IgA antibody concentrations (figure 3A). The underlying mechanisms of this novel observation need to be further investigated in the future.

Clinical implications of high-levels of virus-induced IgA in COVID-19

We observed the presence of high-level of RBD-specific IgA in COVID-19 patients' sera. It is widely believed that mucosal plasma cells are a major production source of IgA, which is rapidly transported across adjacent epithelial barriers into external secretions. In normal situations, very little IgA enters the blood[35]. During infection, high-level of pathogen specific IgA has been reported in mucosal for EV-71[2], influenza[36], and SARS[37], suggesting the importance of IgA in immune responses to viral infection. It has also been reported that IgA is present in serum of COVID-19 patients, although a small number of patients was involved and study on IgA detection was so far limited[12, 23, 24].

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IgA is traditionally recognized to play an anti-inflammatory role and prevent tissue damage at mucosal sites. However, recent reports also demonstrated that serum IgA is involved in the formation of immune complexes to amplify inflammatory responses.[38] Serum IgA induced proinflammatory cytokine production by macrophages, monocytes and Kupffer cells in non-mucosal tissues including liver, skin and peripheral blood.[39] In this study, we observed that IgA was present in COVID-19 patients' serum, and its levels positively correlated with COVID-19 severity (figure 3A). In our cohort, we also observed that IgG levels were associated with worse clinical outcomes (figure 3C), as previously described[10, 21]. The latter phenomena has been suggestive of possible antibody-dependent enhancement (ADE) of infection. The immunopathological effects of ADE have been observed in various viral infections, characterized as antibody- mediated enhancement of viral entry and induction of a severe inflammatory response.[40] It is unclear currently if IgA as well as IgG contributed directly (e.g. via ADE) or indirectly (e.g. leading to a pathogenic inflammatory storm[41]) to the worse clinical outcome in severe COVID-19 patients. If a high-level of IgA indeed contributes to aggravations in COVID-19 severe patients, blocking of IgA-Fc alpha Receptor I (FcαRI, CD89, an IgA receptor) interaction could mitigate ADE or inflammatory storms, thus providing a novel treatment strategy.

While the exact origin of serum IgA after SARS-CoV-2 infection remains to be determined, we suspect that lung and gut are probable places of producing large-quantity of IgA by abundant mucosal immune cells at these sites. Interestingly, several reports are available on the detection of viral RNA in stool or anal swabs[42, 43]. In fact, abdomen abnormality/diarrhea is often complained from COVID-19 patients.[43] These observations suggest that gut may be an important place for anti-viral response to coronaviruses, and large amounts of secretory IgA could be detected in these mucosal tissues in addition to that in blood.

Weakness of this study

The current study at the present form has several limitations. We used 216 serum samples from 87 confirmed COVID-19 patients in this study, and serum samples were not available every day for each patient. The earliest collected serum is at the 4th day after self-reported illness onset, and the last one was collected at the 41th day after illness onset. There are only 17 cases of serum samples collected within the first 10 days after illness onset; consequently the accuracy of early diagnosis requires further verification using larger and controlled samples. Similarly, there were only 23 cases of serum samples taken after 30 days post illness onset, hampering an analysis of long-term antibody levels in recovered patients. Most patients enrolled in this study were with clinically moderate symptoms (56/87, 64.4%). There were 17 severe and five critical cases, respectively, accounting for 19.5% and 5.75% respectively. There were also few cases of COVID-19 patients whose symptoms remained mild and serum samples were collected during hospitalization. Therefore, this study of the correlation between antibody levels and disease severity needs further verification.

In summary, this study reports a novel sensitive and quantitative serological testing kit of detecting IgA as well as IgM and IgG, for the diagnostics of COVID-19. Due to its high specificity and sensitivity, this kit could sensitively and quantitatively measure levels of IgA in blood and other tissues. The serological study also provides valuable information for monitoring and understanding of COVID-19.

Acknowledgements

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Conflict of interest

Dehua Jiang and Weihuang He are employees of Kangrun Biotech LTD (Guangzhou, China). Tengchuan Jin, Huan Ma, Weihong Zeng in USTC and Dehua Jiang have applied a joining patent related to the antibody detecting kits. Other authors declare that they have no conflicts of interest.

Author Contributions

Tengchuan Jin, Yajuan Li and Xiaoling Ma provide funding, designed the study, participated in data analysis, and wrote the manuscript. Huan Ma, Weihong Zeng and Hongliang He designed the study, performed the majority of experiments, analyzed the data and drafted the manuscript. Other authors participated in the experiments and/or writing of the manuscript.

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Tables

Table 1. Comparisons of sensitivity, specificity and overall agreements of RBD-based IgA, IgM, and IgG detection kits and their combinations for diagnosing SARS-CoV-2.

Antibody type	Sensitivity		Specificity		Overall agreement	
	%	n/total	%	n/total	%	n/total
IgA	98.6	213/216	98.1	474/483	98.3	687/699
IgM	96.8	209/216	92.3	446/483	93.7	655/699
IgG	96.8	209/216	99.8	482/483	98.9	691/699
IgA and IgM	95.8	207/216	90.7	438/483	92.3	645/699
IgA and IgG	96.3	208/216	97.9	473/483	97.4	681/699
IgM and IgG	94.9	205/216	92.1	445/483	93.0	650/699
IgA and IgM and IgG	94.4	204/216	90.5	437/483	91.7	641/699
IgA or IgM	99.5	215/216	99.8	482/483	99.7	697/699
IgA or IgG	99.1	214/216	100	483/483	99.7	697/699
IgM or IgG	98.6	213/216	100	483/483	99.6	696/699
IgA or IgM or IgG	99.5	215/216	100	483/483	99.9	698/699

Table 2. Sensitivity of RBD-based IgA, IgM, and IgG detection kits in serum samples obtained at different periods after illness onset.

Days after illness onset	Positive serum samples diagnosed by RBD-based kits					
	IgA		IgM		IgG	
	%	n	%	n	%	n
4-10	88.24	15/17	76.47	13/17	64.71	11/17
11-15	100	30/30	100	30/30	96.67	29/30
16-20	100	55/55	100	55/55	100	55/55
21-25	98.21	55/56	100	56/56	100	56/56
26-30	100	35/35	100	35/35	100	35/35
31-41	100	23/23	86.96	20/23	100	23/23

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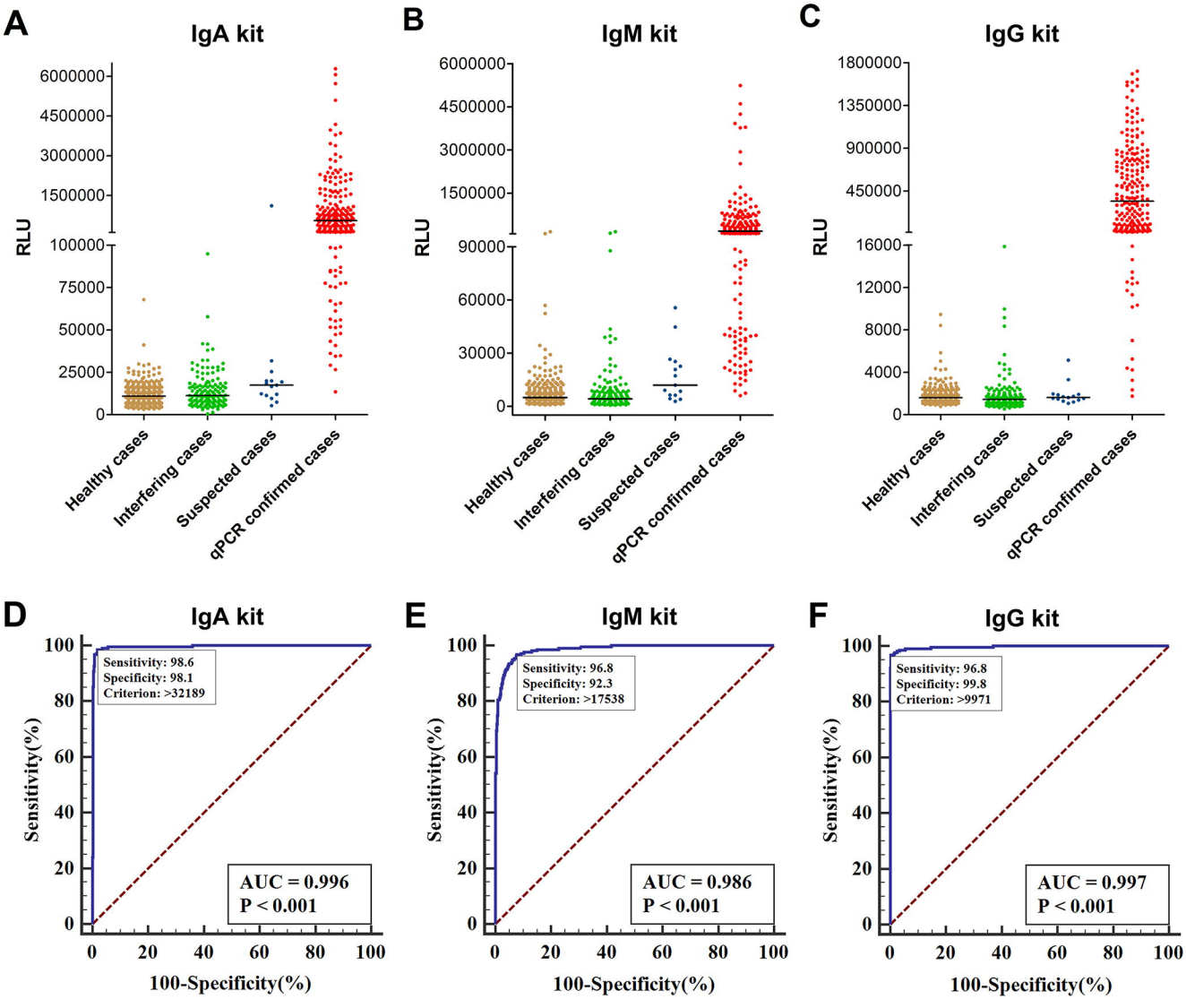
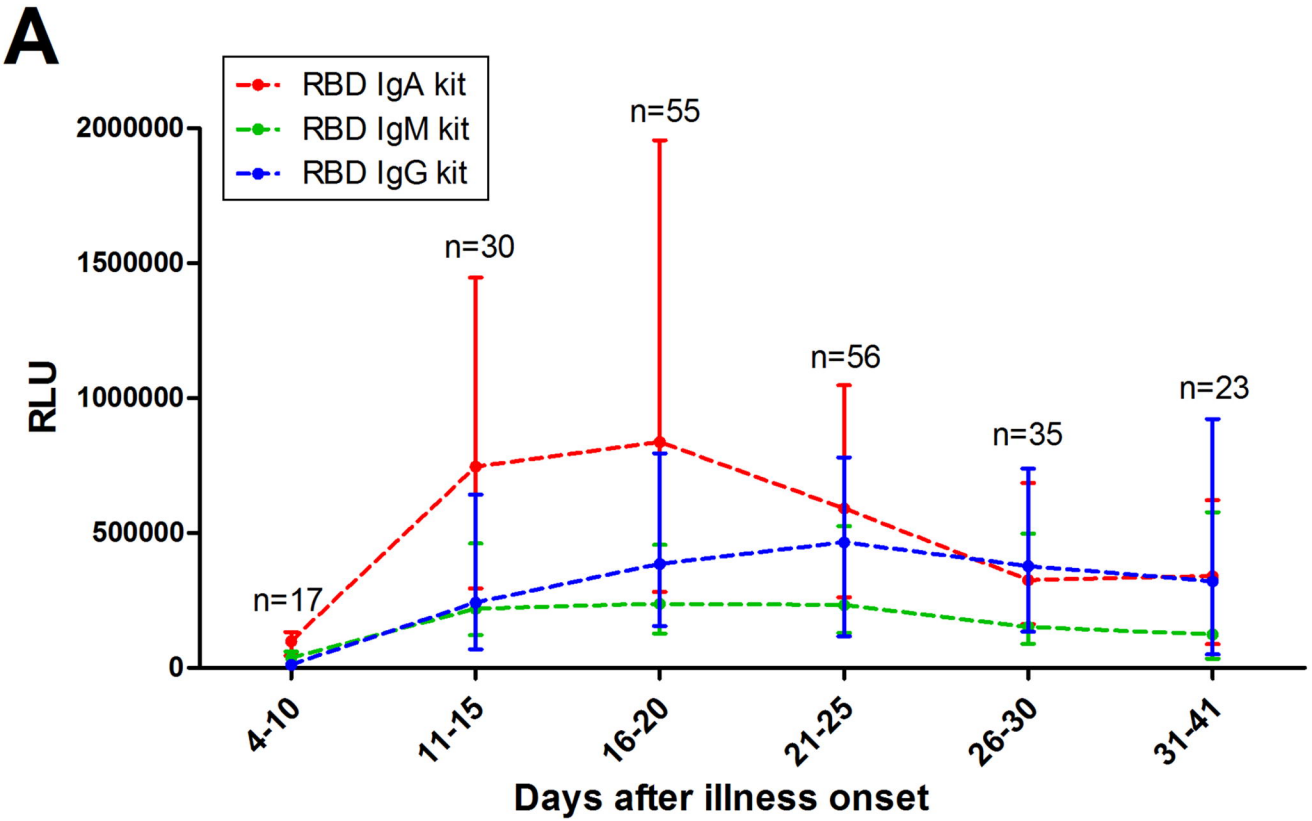
Figure legends

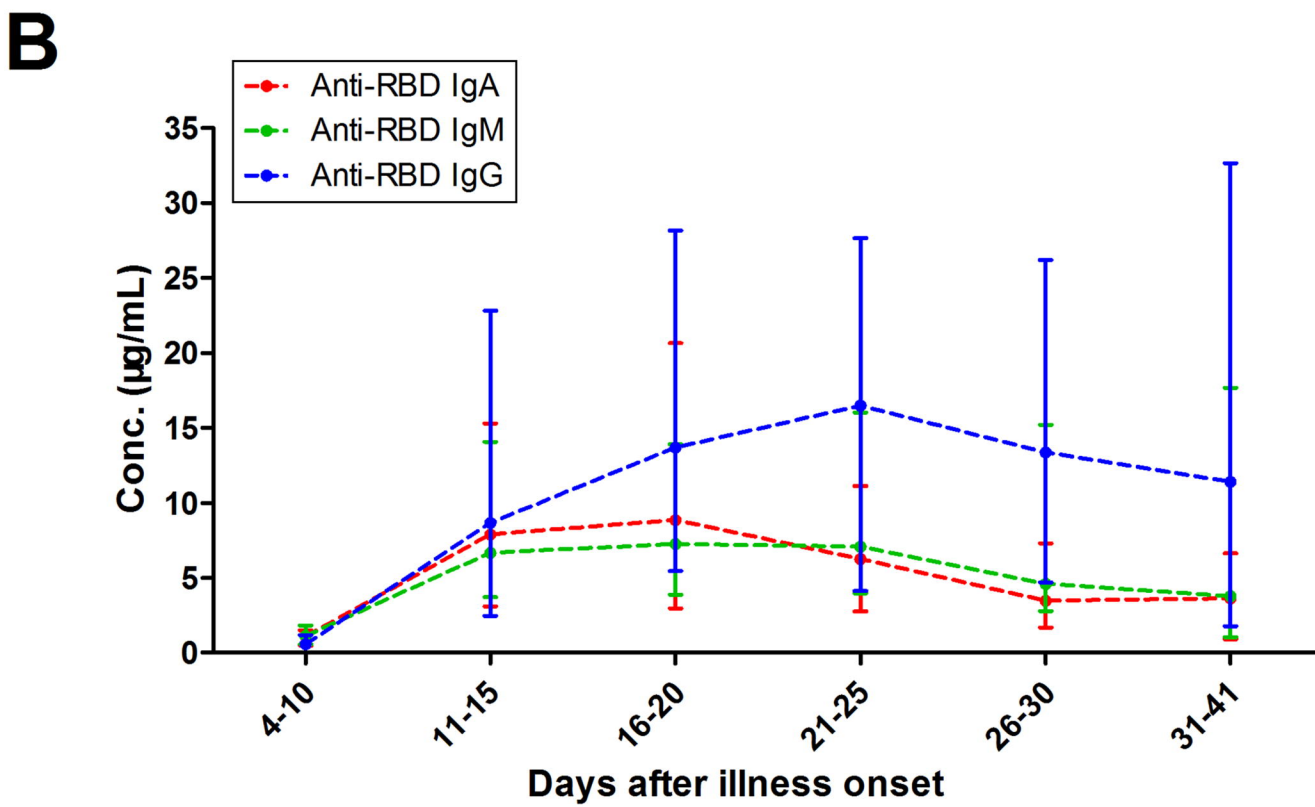
Figure 1. Detection results and analyses of RBD-specific IgA, IgM, and IgG kits. Testing results of RBD-specific IgA (A), IgM (B), and IgG (C) kits using 330 sera from healthy people, 138 interfering sera from other patients with different diseases, 15 sera of once-suspected pneumonia patients that were tested negative for SRAS-CoV-2, and 216 sera of 87 qPCR-confirmed COVID-19 patients. RLU: relative light units. Black bar indicates median values. D-F: The receiver operating characteristic (ROC) curve analysis for SARS-CoV-2 diagnosis by RBD-specific IgA, IgM or IgG kit (D, E and F, respectively) using 483 sera of SARS-CoV-2 negative individuals and 216 sera of SARS-CoV-2 infected patients. AUC, area under the curve of ROC.

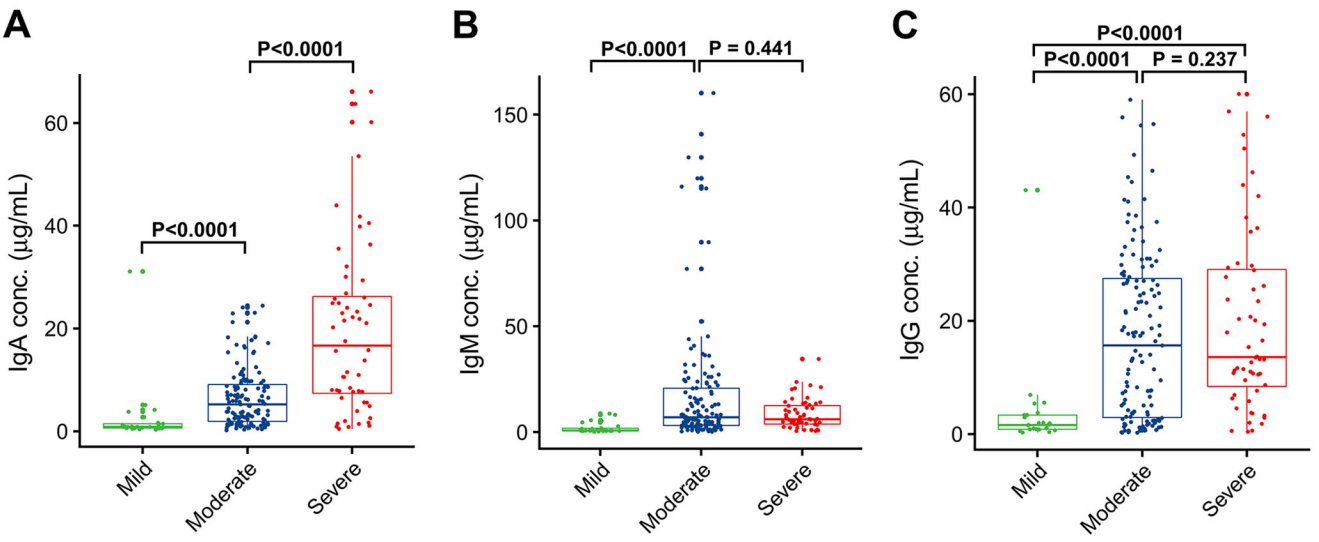
Figure 2. The kinetics of anti-RBD IgA, IgM, and IgG levels in sera of COVID-19 patients at different time windows. The median values of RLU (A) or calculated antibody mass concentrations (B) were plotted for each isotypes of three antibodies, IgA (red), IgM (green), and IgG (blue). Bars indicate interquartile ranges.

Figure 3. Serum antibody levels in three distinct severity groups of COVID-19 patients; mild: 25 sera from 9 patients; moderate: 135 sera from 56 patients; and severe: 56 sera from 22 patients (see methods for clinical classifications and supplemental Figure 5 for age distributions). Antibody levels in serum samples were collected from confirmed patients at 4 - 41 days post illness onset and presented as scatter plots. For IgA (A), levels in mild, moderate and severe patients were sequentially increased (p values indicated). Results for IgM are shown in B. For IgG (C), levels in moderate and severe patients were significantly higher than mild patients.


A
IgA kit
RLU
6000000
4500000
3000000
1500000
100000
75000
50000
25000
0
Healthy cases
Interfering cases
Suspected cases
qPCR confirmed cases
B
IgM kit
RLU
6000000
4500000
3000000
1500000
90000
60000
30000
0
Healthy cases
Interfering cases
Suspected cases
qPCR confirmed cases
C
IgG kit
RLU
1800000
1350000
900000
450000
16000
12000
8000
4000
0
Healthy cases
Interfering cases
Suspected cases
qPCR confirmed cases
D
IgA kit
Sensitivity: 98.6
Specificity: 98.1
Criterion: >32189
AUC = 0.996
P < 0.001
Sensitivity(%)
100-Specificity(%)
E
IgM kit
Sensitivity: 96.8
Specificity: 92.3
Criterion: >17538
AUC = 0.986
P < 0.001
Sensitivity(%)
100-Specificity(%)
F
IgG kit
Sensitivity: 96.8
Specificity: 99.8
Criterion: >9971
AUC = 0.997
P < 0.001
Sensitivity(%)
100-Specificity(%)


A
RBD IgA kit
RBD IgM kit
RBD IgG kit
RLU
2000000
1500000
1000000
500000
0
n=17
n=30
n=55
n=56
n=35
n=23
4-10
11-15
16-20
21-25
26-30
31-41
Days after illness onset


B
Anti-RBD IgA
Anti-RBD IgM
Anti-RBD IgG
Conc. (μg/mL)
35
30
25
20
15
10
5
0
4-10
11-15
16-20
21-25
26-30
31-41
Days after illness onset


A
P<0.0001
P<0.0001
IgA conc. (μg/mL)
0
20
40
60
Mild
Moderate
Severe
B
P<0.0001
P = 0.441
IgM conc. (μg/mL)
0
50
100
150
Mild
Moderate
Severe
C
P<0.0001
P<0.0001
P = 0.237
IgG conc. (μg/mL)
0
20
40
60
Mild
Moderate
Severe

Supplementary table 1. Information of the 87 RT-qPCR confirmed COVID-19 patients.

Patients No.	Disease severity	Underlying disease
1	High severe	1, 2, 3
2	High severe	4, 5, 6
3	High severe	7, 4, 8, 9
4	High severe	10, 11
5	High severe	5, 12
6	Severe	4
7	Severe	none
8	Severe	none
9	Severe	4
10	Severe	none
11	Severe	none
12	Severe	4, 13
13	Severe	1
14	Severe	none
15	Severe	none
16	Severe	4, 7, 10, 14, 15
17	Severe	4
18	Severe	4
19	Severe	4, 10, 16
20	Severe	none
21	Severe	none
22	Severe	17
23	Moderate	none
24	Moderate	none
25	Moderate	none
26	Moderate	none
27	Moderate	18
28	Moderate	none
29	Moderate	19, 5
30	Moderate	none
31	Moderate	20, 21, 22
32	Moderate	4 , 10, 19
33	Moderate	none
34	Moderate	none
35	Moderate	19
36	Moderate	4
37	Moderate	10, 1, 23
38	Moderate	none
39	Moderate	none
40	Moderate	none
41	Moderate	19, 20

42	Moderate	4
43	Moderate	24
44	Moderate	25
45	Moderate	none
46	Moderate	none
47	Moderate	3, 4, 10, 26
48	Moderate	none
49	Moderate	none
50	Moderate	none
51	Moderate	27
52	Moderate	4, 28
53	Moderate	none
54	Moderate	none
55	Moderate	4, 10, 29
56	Moderate	none
57	Moderate	none
58	Moderate	4,30
59	Moderate	none
60	Moderate	none
61	Moderate	none
62	Moderate	none
63	Moderate	none
64	Moderate	31, 32
65	Moderate	10
66	Moderate	none
67	Moderate	none
68	Moderate	33
69	Moderate	none
70	Moderate	1
71	Moderate	none
72	Moderate	none
73	Moderate	none
74	Moderate	1
75	Moderate	none
76	Moderate	none
77	Moderate	none
78	Moderate	none
79	Mild	none
80	Mild	none
81	Mild	none
82	Mild	none
83	Mild	4,34
84	Mild	4
85	Mild	none

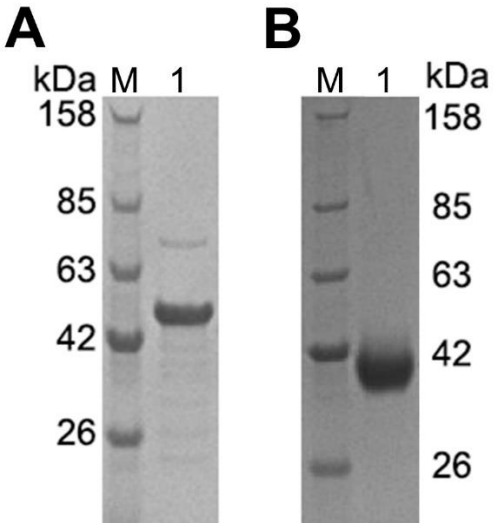
86	Mild	none
87	Mild	none

Underlying illness: 1. Hepatitis B; 2. Postoperative hepatocellular carcinoma; 3. Respiratory failure; 4. Hypertension; 5. Cerebral infarction; 6. Upper gastrointestinal bleeding; 7. Coronary heart disease; 8. Cholecystectomy; 9. Benign prostatic hyperplasia; 10. Diabetes; 11. 1. Hepatitis B; 2. Postoperative hepatocellular carcinoma; 3. Respiratory failure; 4. Hypertension; 5. Cerebral infarction; 6. Upper gastrointestinal bleeding; 7. Coronary heart disease; 8. Cholecystectomy; 9. Benign prostatic hyperplasia; 10. Diabetes; 11. Rheumatoid Arthritis; 12. Hernia surgery; 13. Lower extremity arterial occlusion; 14. Heart valve disease; 15. Atrial fibrillation; 16. Expansion; 17. Car accident caused multiple soft tissue, intracranial hemorrhage, and improved after conservative; 18. Chronic gastritis; 19. Hyperlipidemia 20. Leukopenia; 21. Anemia; 22. Sinus tachycardia; 23.Fatty Liver; 24. Proteinuria; 25. Hyperthyroidism; 26. Hypoproteinemia; 27. Uremia; 28. Left nephrectomy; 29. Breast cancer; 30. Parkinson; 31. Frozen shoulder; 32. Rheumatism; 33. Nasal drip syndrome; 34. Hyperuricemia.

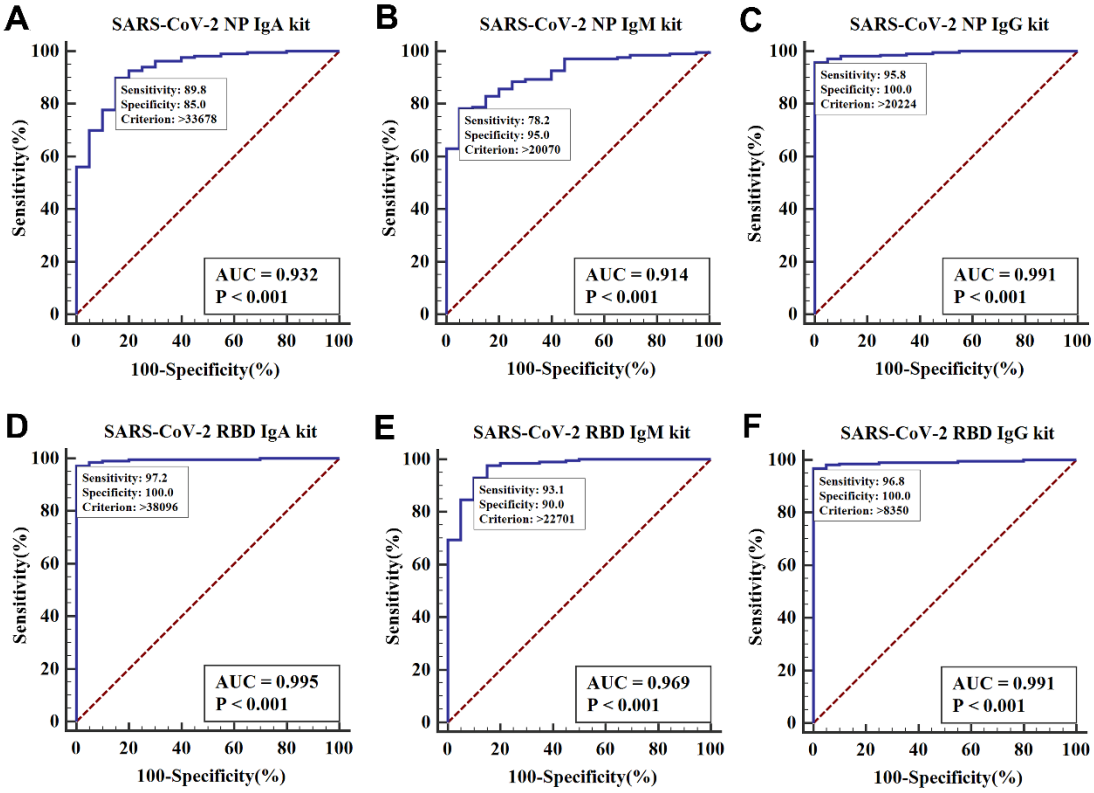
Supplementary table 2. False positive cases diagnosed by the current RBD-based kits.

Kit type	False-positive cases					
	Healthy cases		Interfering cases		Once suspected cases	
	%	n	%	n	%	n
IgA kit	0.61	2/330	4.35	6/138	6.67	1/15
IgM kit	5.45	18/330	9.42	13/138	40.0	6/15
IgG kit	0	0/330	0.73	1/138	0	0/15

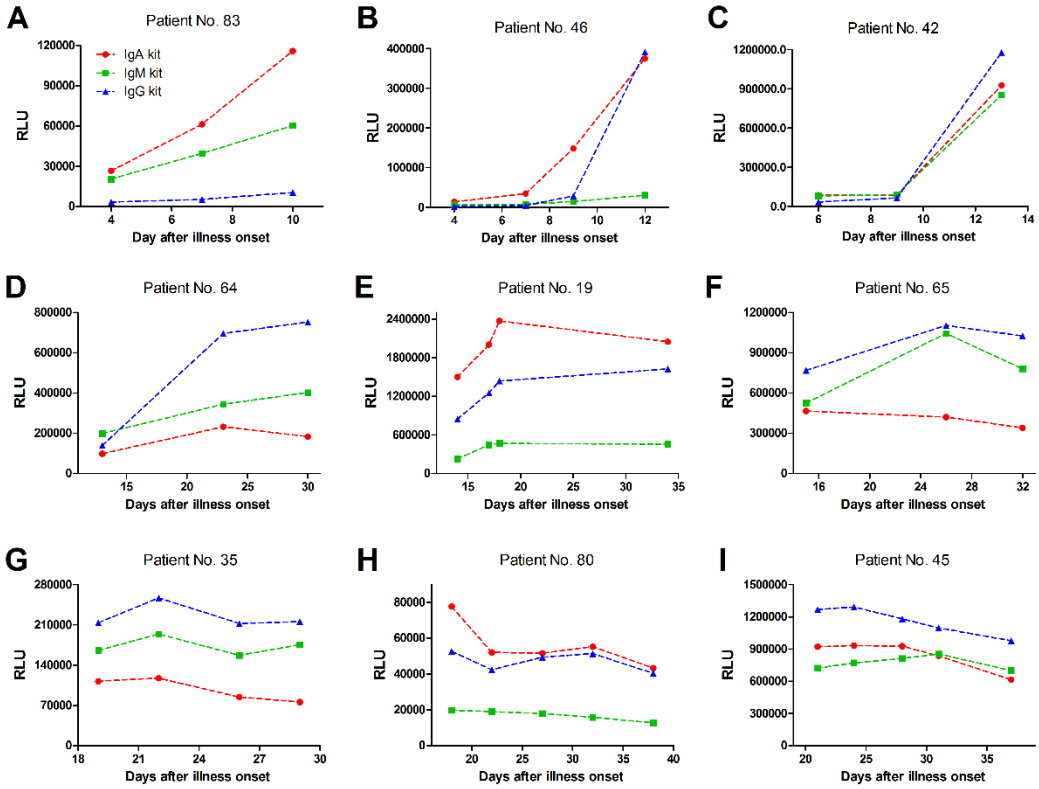
Most of false-positive outcomes in the IgA and IgG tests are from patients who were tested negative for SARS-CoV-2 viral RNA and had other underlying diseases. For example, the four of six “false-positive” samples by the RBD IgA test included two patients who had kidney diseases and high-level self-antibodies, one had liver cirrhosis, and another had breast cancer.



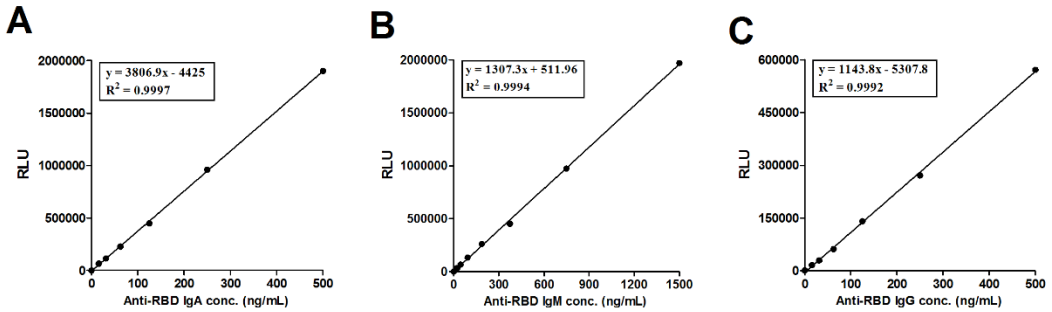
Supplementary figure 1. Purification of recombinant SARS-CoV-2 nucleocapsid protein (NP, A) and receptor-binding domain (RBD, B) of the spike protein. After SDS-PAGE separation based on molecular weight (kDa) together with protein size markers (M), the proteins were stained with Coomassie Blue. A is staining of purified NP (predicted molecular mass of 49.5 kDa). B is staining of purified RBD (predicted molecular mass of 30.3 kDa).



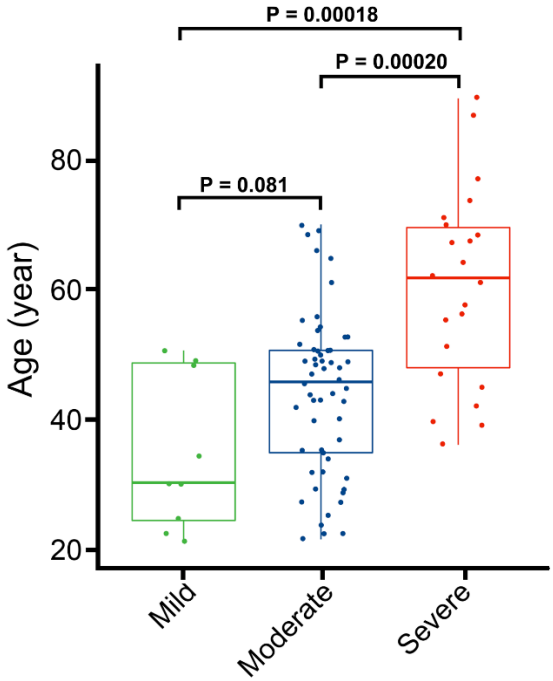
Supplementary figure 2. The receiver operating characteristic (ROC) curve analysis for SARS-CoV-2 diagnosis by NP-specific IgA, IgM, and IgG kit (A, B, and C, respectively), and RBD-specific IgA, IgM, and IgG kit (D, E, and F), respectively. Twenty sera of 20 SARS-CoV-2 negative patients and 216 sera of SARS-CoV-2 infected patients were used. AUC, area under the curve of ROC.



Supplementary figure 3. Representative testing results of sera collected at consecutive time points after illness onset by RBD-based kits (IgA, red; IgM green; and IgG, blue). A-I were testing results of sera from nine individual COVID-19 patients.



Supplementary figure 4. Standard curves for antibody concentration vs. RLU. Standard curves were constructed based on immunoassay of 7-8 concentrations of anti-RBD IgA (A) or IgM (B) or IgG (C) antibodies purified from a serum pool of SARS-CoV-2 infected patients.



Supplementary figure 5. Correlation between patient age and disease severity. Mild: 9; moderate: 56, and severe: 22 patients.