Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19)

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Summary:

The time kinetics of humoral responses against the novel coronavirus (SARS-CoV-2) are characterized in COVID-19 patients by nucleocapsid-based ELISA. The antibody testing can aid to the diagnosis of COVID-19 when combined with qPCR, including in subclinical cases.

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Abstract:

Background Emergence of coronavirus disease 2019 (COVID-19) is a major healthcare threat. Current method of detection involves qPCR-based technique, which identifies the viral nucleic acids when present in sufficient quantity. False negative results can be achieved and failure to quarantine the infected patient would be a major setback in containing the viral transmission. We here aim to describe the time kinetics of various antibodies produced against the 2019 novel coronavirus (SARS-CoV-2) and evaluate the potential of antibody testing to diagnose COVID-19.

Methods The host humoral response against SARS-CoV-2 including IgA, IgM and IgG response were examined by using an ELISA based assay on the recombinant viral nucleocapsid protein. Total 208 plasma samples were collected from 82 confirmed and 58 probable cases (qPCR negative but had typical manifestation). The diagnostic value of IgM was evaluated in this cohort.

Results The median duration of IgM and IgA antibody detection were 5 days (IQR 3-6), while IgG was detected on 14 days (IQR 10-18) after symptom onset, with a positive rate of 85.4%, 92.7% and 77.9% respectively. In confirmed and probable cases, the positive rates of IgM antibodies were 75.6% and 93.1%, respectively. The detection efficiency by IgM ELISA

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is higher than that of qPCR method after 5.5 days of symptom onset. The positive detection rate is significantly increased (98.6%) when combined IgM ELISA assay with PCR for each patient compare with a single qPCR test (51.9%).

Conclusions Humoral response to SARS-CoV-2 can aid to the diagnosis of COVID-19, including subclinical cases.

Key words: novel coronavirus; COVID-19; antibody; ELISA; diagnosis

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Introduction

Novel coronavirus (SARS-CoV-2) causing coronavirus disease in 2019 (COVID-19) emerged as a major pandemic[1, 2]. With more than 110 thousand confirmed cases and over 4000 deaths as of March 11, 2020, this pandemic surpassed the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) of 2003[3].

Coronaviruses are frequent causes of respiratory infections where six major species are known to cause human infections besides the SARS-CoV-2. These species include highly pathogenic SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), along with less virulent species that include NL63, 229E, OC43 and HKU1[4]. The epidemiology, etiology and clinical characteristics of COVID-19 have recently been described in detail[5-11]. The current diagnostic of COVID-19 includes detection of virus by genomic techniques using either PCR-based method or deep sequencing[7, 12, 13]. However, these detection methods heavily rely on the presence of viral genome in sufficient amounts at the site of sample collection that can be amplified. Missing the time-window of viral replication can provide false negative results. Similarly, an incorrect sample collection can limit the usefulness of qPCR-based assay. A false negative diagnosis can have grave consequences, specially at this stage of the pandemic by allowing infected patients to spread the infection and hampering the efforts to contain the spread of virus[14]. In such conditions,

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additional screening methods that can detect the presence of infection despite lower viral titers can be highly beneficial to ensure timely diagnosis of all infected patients. Detecting the production of antibodies, especially IgM, which are produced rapidly after the infection can be such a tool that can be combined with PCR to enhance detection sensitivity and accuracy.

However, currently, the extent and the time kinetics of humoral response against SARS-CoV-2 are not known. In this study, we demonstrate the time kinetics of antibody response to SARS-CoV-2 in infected patients. We further demonstrate that combining the antibody testing with qPCR can significantly improve the diagnosis of COVID-19.

Materials and Methods

Expression and purification of SARS-CoV-2 nucleocapsid proteins

The full-length nucleocapsid (N) genes of SARS-CoV-2 was amplified from a bronchoalveolar lavage fluid (BAL) specimen of a patient infected with SARS-CoV-2 and cloned into prokaryotic expression vector pET30a (+) (Novagen, San Diego, CA, USA). The resultant plasmids were transformed into *E. coli* BL21 (DE3) to express the recombinant N proteins (rNPs) according to the manufacturer's protocol. The 6×Histidine-tagged proteins were then purified by using HiTrap SP FF and HisTrap HP columns (GE Healthcare, Waukesha, WI, USA) to >90% purity. The identity of the purified protein was confirmed by Western blot analysis using an anti-6×Histidine monoclonal antibody (Sigma, St. Louis, MO, USA). The rNPs of human CoV-229E, -NL63, -OC43, -HKU1, SARS-CoV, and MERS-CoV were expressed and purified as described previously[15].

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Patients and plasma specimens

In this study, a total of 208 blood sample were collected from two cohorts. In the first cohort, we recruited a total of 101 inpatients from Wuhan hospitals during the early phase of the pandemic in January 2020. Paired throat swab and blood samples were taken from each patient. Among them, 43 (20 severe and 23 mild to moderate) were confirmed viral positive [referred as "confirmed cases" (CCs)] by deep sequencing or a qPCR assay with a detection limit of 1 copy/μL as previously reported[1,2]; while 58 (5 severe and 53 mild to moderate) suspected to be infected with SARS-CoV-2 based on clinical manifestation, chest radiography imaging and epidemiology but no virus was detected by deep sequencing or a qPCR assay [referred as "probable cases" (PCs)]. A total of 69 blood sample were taken from the 43 CCs (two serial samples from 26 patients with a 4-day interval and one sample from remaining17 patients); while 100 plasma samples were collected from 58 PCs (two blood samples from 42 PCs and single blood samples from 16 PCs). The second cohort included a total of 39 hospitalized CCs recruited from Beijing hospitals (8 severe and 31 mild to moderate cases) which provided one blood sample from each patient. All the blood samples were taken between 1-39 days of the disease onset.

In addition, a family cluster including 6 individuals of three generations were enrolled to validate our detection method.

Another 135 plasma samples collected in 2018 from adult patients with acute lower respiratory tract infections (ALRTIs) and 150 plasma samples obtained from healthy adults in 2018-2019 for regular health check-ups in Wuhan city were used as controls. The plasma positive for human CoV-229E, -NL63, -OC43, -HKU1, and SARS-CoV were obtained as previously reported[15].

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Western blot analysis

Purified rNPs of human CoV-229E, -NL63, -OC43, -HKU1, and SARS-CoV were separated by 12% SDS-PAGE gels and transferred to a nitrocellulose membrane (Pall, Port Washington, NY, USA). Human plasma positive for these CoVs were applied to detect cross reactivity between these N proteins and antibodies. Goat anti-human IRDye Fluor 800-labeled IgG secondary antibody was used at a dilution of 10,000 (Li-Cor, Lincoln, NE, USA). The membranes were scanned by using Odyssey Infrared Imaging System (Li-Cor).

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Enzyme-linked immunosorbent assay (ELISA)

We developed an indirect ELISA protocol for detecting IgM, IgA, and IgG antibodies against SARS-CoV-2 using purified rNPs as coating antigens. The ELISA protocol was developed as previously reported[16]. The concentration of the coated rNPs and plasma dilutions for this ELISA assay were optimized using chessboard titration tests. To determine the cut-off values for the ELISAs, we determined the mean values and standard deviation (S.D.) of healthy individual plasma. The optimal coating concentration of antigen and optimal plasma dilutions were 0.1μg/mL and 1:200, respectively. The cut-off values were determined by calculating the mean absorbance at 450 nm (A450) of the negative sera plus three folds of the SD values which were 0.13, 0.1 and 0.30 for IgM, IgA, and IgG, respectively.

Phylogenetic analysis

The viral nucleocapsid gene sequences were aligned using the ClustalW program using MEGA software (version 7.0.14). Phylogenetic trees were constructed by means of neighbor-joining methods with the use of MEGA software. The viral full genome sequences were deposited in GISAISD (no. EPI_ISL_402123) and the Genome Warehouse in National Genomics Data Center, Beijing Institute of Genomics (BIG), Chinese Academy of Sciences (accessible at https://bigd.big.ac.cn/gwh).

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Ethics Approval

This study was approved by the Ethical Review Board of Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College. Written informed consent was waived for in the light of this emerging infectious disease of high clinical relevance. For healthy volunteers and patient suffering from common respiratory infections, the written informed consent was obtained from each person before enrollment.

Statistical analysis

Plasma antibody titers were expressed as geometric mean titers (GMTs), and compared using the Student's t-test by using SPSS software program version 19.0. We used Nonlinear Regression (Third order polynomial) model to fit the data of antibody positive rate. $P<0.05$ was considered to be statistically significant.

Results

Cross-reactivity between SARS-CoV-2 and other human coronaviruses

To evaluate the potential cross-reactivities of N proteins between SARS-CoV-2 and other human CoVs, we examined the reactivities of the SARS-CoV-2 rNP and human plasma with positive antibodies against NL63, 229E, OC43, HKU1, and SARS-CoV, respectively, by using Western blot and ELISA assays.

Western blot analysis showed that there were no cross-reactivity of SARS-CoV-2 rNP with human plasma positive for IgG antibodies against NL63, 229E, OC43, and HKU1. However, a strong cross-reactivity was observed between SARS-CoV positive human plasma and SARS-CoV-2 rNP, and specific bands of 45 kD in size were observed **(Figure 1A**). The

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cross-reactivities were also confirmed by using ELISA assays where SARS-CoV positive human plasma presented high A450 with SARS-CoV-2 rNP **(Figure 1B)**. These results indicate cross reactivity of antibodies to N proteins between the SARS-CoV and SARS-CoV-2. Pair-wise analysis showed the SARS-CoV-2 nucleocapsid gene had 46.1%, 27.6%, 26.5%, 20.0% and 19.1%, amino acid (aa) sequence homology with MERS-CoV, HKU1, OC43, NL63, and 229E, respectively. Interestingly, 90.5% aa sequence homology was observed with SARS-CoV, which may explain the cross-reactivity between the two viruses (**Figure 2**).

Characteristic of plasma antibodies in patients infected with SARS-CoV-2

Firstly, we examined the specificity of the ELISA by using 135 plasma samples from ALRTIs patients and 150 healthy individuals whose samples were collected in 2018-2019 in Wuhan city. No anti-SARS-CoV-2 IgM, IgA, and IgG antibodies were detected in these samples (data not shown). The antibody levels were then evaluated in the plasma samples of confirmed and probable cases.

The production of IgM, IgA and IgG antibodies against SARS-CoV-2 were positive as early as day 1 after the symptom onset (**Figure 3A**). Of the 208 samples we tested, 41 were collected between 1–7days, 84 samples between 8 –14 days, and 83 samples were collected > 14 days post symptom onset (PSO). The total positive numbers of IgM and IgA were 188 (90.4 %) and 194 (93.3 %) of 208 plasma samples, respectively. As IgM is a marker for acute infection, we specially examined the positivity of anti-SARS-CoV-2 IgM in the 41 plasma samples which were collected within seven days PSO, which may represent acute phase samples. Of these samples, anti-SARS-CoV-2 IgM was detected in 35 samples (85.4%), 27 of which were detected at plasma dilution of 1:400 and 8 samples were detected at 1:200 dilution. IgA antibodies were detected in 38 samples of these early samples (92.7%), with antibody dilution of 1:400. Based on these acute phase samples, the IgM and IgA antibodies

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were both detectable at day 5 (median, IQR 3-6), respectively. The anti-SARS-CoV-2 IgG antibodies were positive in 162 (77.9 %） plasma samples. The median time of the IgG appeared at day 14 (IQR 10-18) PSO (Figure 2A). The detection time of IgM, IgA, and IgG against SARS-CoV-2 ranged from day 1 to 39 PSO in our study (Data not shown).

The end-point titration for antibodies against SARS-CoV-2 rNP were determined by calculating the A450 of 2-fold serial dilution of each plasma sample for IgM, IgA, and IgG (**Figure 3B**). Compared to the titers at days 0–7 (GMT 400), the IgM antibodies levels increased between days 8–14 (GMT 535.8, p=0.000, student's *t* test); but did not increase further between days 15–21 (GMT 536.31, p=0.992) or after day 21 (GMT 565.69, p=0.719). IgA antibody levels increased from days 0–7 (GMT 400) to days 8–14 (GMT 597.24, p=0.000), however, there was no increase after this time point (GMT at day 15-21=723.28, p=0.156 or GMT=831.41, p=0.538 at day > 21). The IgG antibodies were detected on days 0–7 (GMT 490.45), and it increased on days 8–14 (GMT 1325.6, p=0.000), and continued to rise until days 15-21 (2690.87, p=0.000), and plateaued by day 21 (GMT 2974.83, p=0.72).

IgM ELISA assay used for early diagnosis of patients infected with SARS-CoV-2

The IgM-positive rate to SARS-CoV-2 was 75.6% (62/82) in CCs, and 93.1% (54/58) in PCs (**Figure 4A**). Further, we generated curve fits of the PCR-positive rate, IgG and IgM ELISA positive rates on different days after symptom onset (**Figure 4B**). The detections between days 26-39 PSO are not shown due to the limited number of patients on each day. Based on the fitted curve, the PCR-positive rate was >90% on 1-3 days PSO, then declined to less than 80% (95% CI, 57.1%-95.7%) at day six and less than 50% (95% CI, 23.7%-59.5%) after 14 days PSO. Overall, the detection rate by qPCR was higher than the IgM ELISA assay before 5.5 days PSO, while the detection efficiency by IgM ELISA is higher than that of PCR method after 5.5 days of symptom onset. Overall, the positive detection rate is only 51.9% in

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a single PCR test, but significantly increased (98.6%) when applied IgM ELISA assay to PCR-negative patients (**Figure 4C**). To further evaluate the performance of anti-SARS-CoV-2 IgM in COVID-19 diagnosis, we analyzed the data from the 26 CCs who had two serial plasma samples together with paired throat swabs within a four-day interval. Among them, seven patients showed PCR negative when testing the first swab, but six patients showed IgM positive. All the seven patients showed PCR positive for the second swab. These results suggest that IgM ELISA assay can increase the positive detection rate when combined with the PCR method and can be used for the early diagnosis of COVID-19 infections.

Diagnosis of a family clustered cases using IgM ELISA

To prospectively evaluate the validity of IgM ELISA in the diagnosis of COVID-19, we used the IgM ELISA to diagnose a family cluster involved two patients and four close contacts (**Table 1**). The grandparents returned to Beijing from Wuhan on January 22, 2020. The grandmother started having symptoms including fever (38.0℃), dry cough, and chest computed tomography (CT) showed bilateral patchy shadow on February 2nd. The grandfather suffered stuffy and runny nose on February 3rd. The other four members in the family had no significant clinical symptoms. PCR assay and chest CT of their close contacts, including their daughter, son-in-law, grandson, and granddaughter were performed on February 5. The grandmother was found to be positive on viral RNA detection by qPCR. Grandfather and the daughter only presented abnormal findings on chest CT but were negative for viral RNA tested by PCR. The grandson presented with PCR positivity with a normal chest CT. The son-in-law and granddaughter were PCR negative and had normal lungs by CT imaging. The grandfather, daughter, son-in-low, and granddaughter were also negative for SARS-CoV-2 after the repeat PCR detection on the next day. However, the IgM antibodies were detected in grandmother, grandfather, daughter, grandson, and

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granddaughter. These findings indicate the significant and improved efficacy of COVID-19 detection when IgM measurements are used to assess subclinical subjects.

Discussion

COVID-19 emerged as a major healthcare challenge globally[13, 14, 16, 17]. A key aspect of limiting this virus spread is to ensure early and accurate diagnosis of the viral infection and appropriate quarantine for those infected. The current method of diagnosis by qPCR or deep sequencing-based technologies rely on the presence of replicating virus in sufficient amount to ensure sufficient quantitates of virus is collected[7]. This method often fails to detect the viral infection if collection procedure is not optimal, or if the patient has low viral load due to early stage of the disease or suppressed by host immunity, or if the samples were obtained at a late stage in the course of infection.

In this study, we first demonstrated that current SARS-CoV-2 positive plasma did not show any cross reactivity with other coronaviruses with the exception of SARS-CoV. It is highly unlikely that these patients were pre-infected with SARS-CoV during the last epidemic in 2002, as we measured IgM reactivity, which is unlikely to last that long. Secondly, the number of infections with SARS-CoV were limited to 8,096 worldwide[18], which only represent a tiny fraction of the Chinese population. However, given the sequence homology between these two viruses (>90%), the cross reactivity is not surprising.

We propose to conduct antibody testing when qPCR test is negative despite other indications of COVID-19 including symptoms and epidemiology. Our data show that the supplementary IgM test can provide a better sensitivity than qPCR-based method alone. This is especially important at this stage of the pandemic, where proper diagnosis is essential to limit the viral spread. Although, a more sensitive qPCR may improve detection efficacy, but the efficacy may still be limited due to various biological reasons as discussed above.

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However, higher sensitive qPCR assays can even further improve the detection method when combined with IgM ELISA assays.

We used primarily a cross-sectional sample of specimens to determine the kinetics of SARS-CoV-2 antibodies. This is one of the limitations of our study because each patient has different kinetics for the development of antibodies. The median time of appearance of antibodies is therefore affected by factors such as when the specimen was collected and when the symptom onset took place in each individual patient. Further studies are warranted that use longitudinal sample collection in an unbiased manner to test the kinetics of the antibody response.

It is worth noting that approximately 22.0% (18/82) of the patients who were confirmed to be positive by qPCR were found to be negative by the IgM antibody tests. Among the 18 patients, 13 cases were enrolled within less than 7 days after symptoms onset which is probably when the antibodies were not produced in sufficient amounts. Two patients were severe cases and their samples were collected at 19 and 22 days after symptoms onset respectively, suggesting a possible failure of these patients to generate the antibody response, which may have contributed to the disease severity. The remaining three patients were sampled between days 9-17 after symptoms onset. These data suggest that the time course as well as host factors can contribute toward the antibody response to the virus.

To confirm the applicability of our method, we prospectively tested a cluster of family which had two members that were confirmed to be infected by SARS-CoV-2, while the remaining members tested negative using PCR. In contrast, the antibody testing confirmed the viral infections in the two patients and the three of the four close contacts, which have been clinically corroborated in three of the family members. Moreover, after a variable period of time, one expects the PCR result to become negative as people stop shedding. Presumably,

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IgG antibodies will remain positive. This would permit the use of serological tools to better understand the overall rate of infection in the community - including the rate of asymptomatic infections. This could potentially enable us to identify healthcare workers who have been infected in the past and might be less prone to becoming infected on the job. Our data also show its ability to detect subclinical infections effectively, a much-needed tool to stop the spread of infection, which is currently spreading with an alarming rate in the Europe and the USA. In addition, the higher speed of antibody testing can be useful and applied at remote areas where qPCR assays cannot be performed.

Conclusions: We demonstrated the time kinetics of humoral response during COVID-19 and provided evidence that this can aid to the diagnosis of COVID-19, including in subclinical cases.

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Author contributors: JWW, LHW, LS and LLR conceived and designed experiments. LG, CW, YX, YYW, LLH, SYD, JCZ and CW performed the experiments. SYY, MX, FY, QJ and LHW contributed clinical samples and clinical data collection. LLZ, LLR, DC, CDC and LG analyzed the data. LG, LLR, DC, LLZ, LS, CDC and JWW wrote the manuscript. All authors reviewed the manuscript.

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Conflict of Interest Disclosures: All authors declare no competing interests.

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Table 1. Diagnosis of a family clustered cases using IgM ELISA

Relationship	Age (year)	PCR	IgM titers	IgG titers	Computer tomography	Clinical diagnosis
Grandma	61	+	400	-	+	Case
Grandpa	64	-	800	3200	+	Case
Daughter	37	-	400	-	+	Close contact
son-in-law	40	-	-	-	-	Close contact
Grandson	6	+	200	-	-	Close contact
Granddaughter	2	-	400	-	-	Close contact

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Figure legends

Figure 1. Cross-reactivity between human plasma against NL63, 229E, OC43, and HKU1 with N protein of SARS-CoV-2. (A) Western blot analysis to determine the reactivity of human plasma containing antibodies against NL63, 229E, OC43, and HKU1. Plasma were diluted at 1:400 and incubated with the N protein of NL63, 229E, OC43 HKU1 and and SARS-CoV which was loaded at 250 ng/well. The N protein of SARS-CoV-2 was also loaded in each gel to determine the cross reactivity (B) ELISA assay showing reactivity of human plasma against NL63, 229E, OC43, HKU1, and SARS-CoV. Plasma samples were diluted at 1:400 to test the cross reactivity against N protein of SARS-CoV-2. The coating amount of N proteins was 10 ng/well. The absorbance (OD) values at 450 nm are shown on the y axis.

Figure 2. Characteristics of SARS-CoV-2 nucleocapsid gene. (A). Phylogenic analysis of viral nucleocapsid gene. The SARS-CoV-2 is labeled red in the phylogenetic trees. Other human coronaviruses from public database are listed as outgroup. Evolutionary distances were calculated with the Neighbor-joining method. (B) Amino acid sequence comparison of the nucleocapsid gene of SARS-CoV-2, IPBCAMS-WH-01/2019 (EPI_ISL_402123) compared with SARS-CoV (NC004718).

Figure 3. Characteristic of plasma antibodies in patients infected with SARS-CoV-2. (A) Time of appearance of IgM, IgA, and IgG antibodies to SARS-CoV-2, determined by ELISA of plasma samples obtained from inpatients with SARS-CoV-2 infection. As we only want to show the early time points, the plasma used for detection of IgM and IgA antibodies were

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selected from patients that had fever or respiratory infectious symptoms within 7 days. The plasma used for detection of IgG antibodies were selected from all the patients whose IgG antibodies were positive. (B) Levels of IgM, IgA, and IgG antibodies against SARS-CoV-2 in plasma samples after symptom onset. Antibody titers were expressed as geometric mean titers (GMTs).

Figure 4. IgM ELISA assay to diagnose patients infected with COVID-19. (A) IgM antibody detection using ELISA assay in patients that were either PCR positive (82 cases) or that were tested negative for virus by qPCR (58 cases). Red is IgM positive while blue is IgM negative. (B) Fitted curve of the positive detection by PCR, IgM or IgG ELISA on different days after symptom onset. The fitted cure are created by Fit Spline program of Graphpad software. The 95% confidence interval are shown for each curve. The dots in the upper panel represent positive rates of PCR, IgM or IgG ELISA at each time point. The lower table show the number of samples tested positive at each time point. The first intersection of real-time PCR method and IgM ELISA assay was found to be 5.5 days. (C) The positive detection rate of cases using PCR method vs PCR plus IgM ELISA assay.

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Figure 1

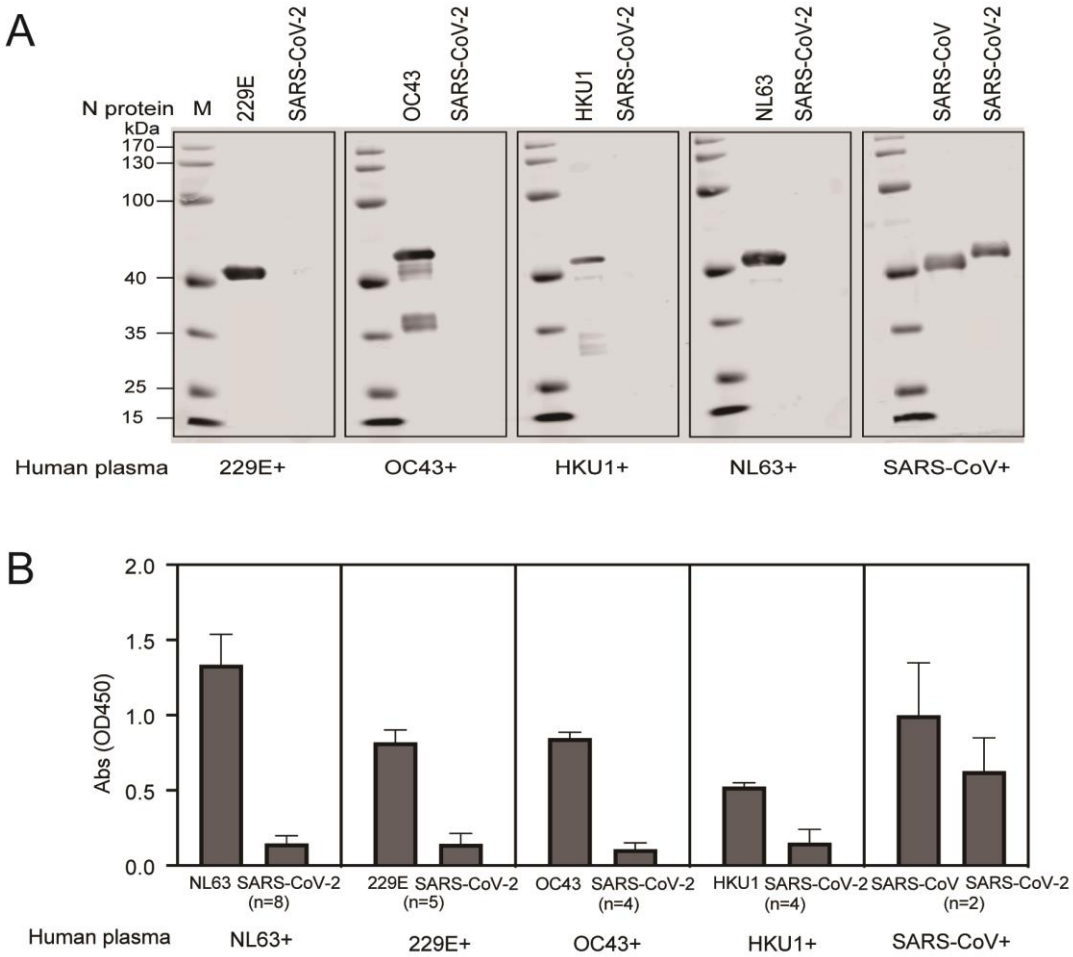

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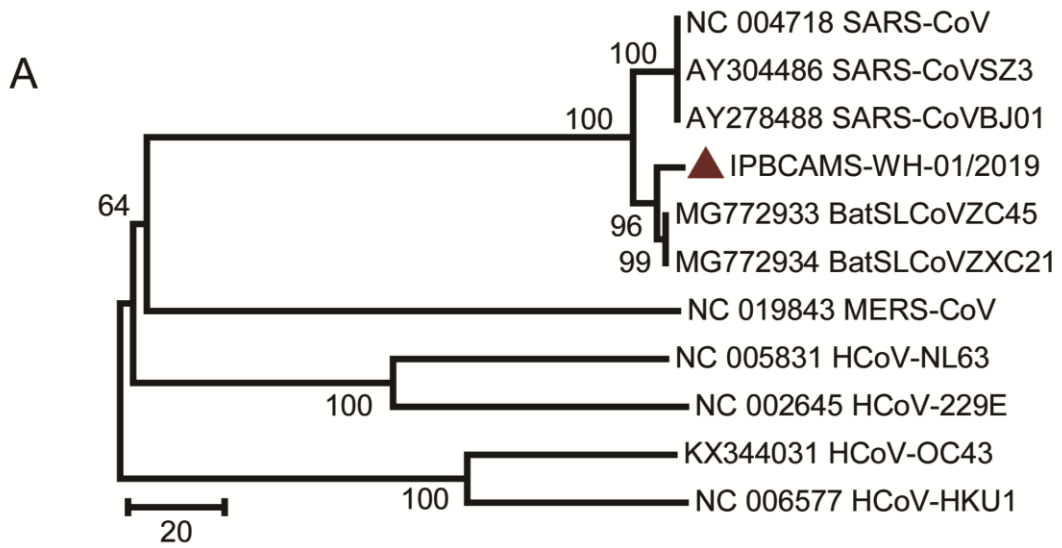
Figure 2



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IPBCAMS-WH-01 MSDNGPQ-NQ RNAPRITFGG PSDSTGSNQN GERSGARSKQ RRPQGLPNNT ASWFTALTQH  59
SARS-CoV      .......S.. .S........ .T...DN... .G.N...P.. .......... ..........  60

IPBCAMS-WH-01 GKEDLKFPRG QGVPINTNSS PDDQIGYYRR ATRRIRGGDG KMKDLSPRWY FYYLGTGPEA 119
SARS-CoV      ...E.R.... .........G .......... ....V..... ...E...... ..........  120

IPBCAMS-WH-01 GLPYGANKDG IIWVATEGAL NTPKDHIGTR NPANNAAIVL QLPQGTTLPK GFYAEGSRGG 179
SARS-CoV      S.......E. .V........ .......... ..N....T.. .......... ..........  180

IPBCAMS-WH-01 SQASSRSSSR SRNSSRNSTP GSSRGTSPAR MAGNGGDAAL ALLLIDRLNQ LESKMSGKGQ 239
SARS-CoV      .......... ..GN...... .....N.... ..SG..ET.. .......... ....V.....  240

IPBCAMS-WH-01 QQQGQTVTKK SAAEASKKPR QKRTATKAYN VTQAFGRRGP EQTQGNFGDQ ELIRQGTDYK 299
SARS-CoV      .......... .......... .......Q.. .......... .......... D.........  300

IPBCAMS-WH-01 HWPQIAQFAP SASAFFGMSR IGMEVTPSGT WLTYTGAIKL DDKDPNFKDQ VILINKHIDA 359
SARS-CoV      .......... .......... .......... ....H..... .....Q...N ..........  360

IPBCAMS-WH-01 YKTFPPTEPK KDKKKKADET QALPQRQKKQ QTVTLLPAAD LDDFSKQLQQ SMS--SADST 417
SARS-CoV      .......... ......T..A .P........ P......... M....R...N ...GA.....  420

IPBCAMS-WH-01 QA* 420
SARS-CoV      ..* 423
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Figure 3

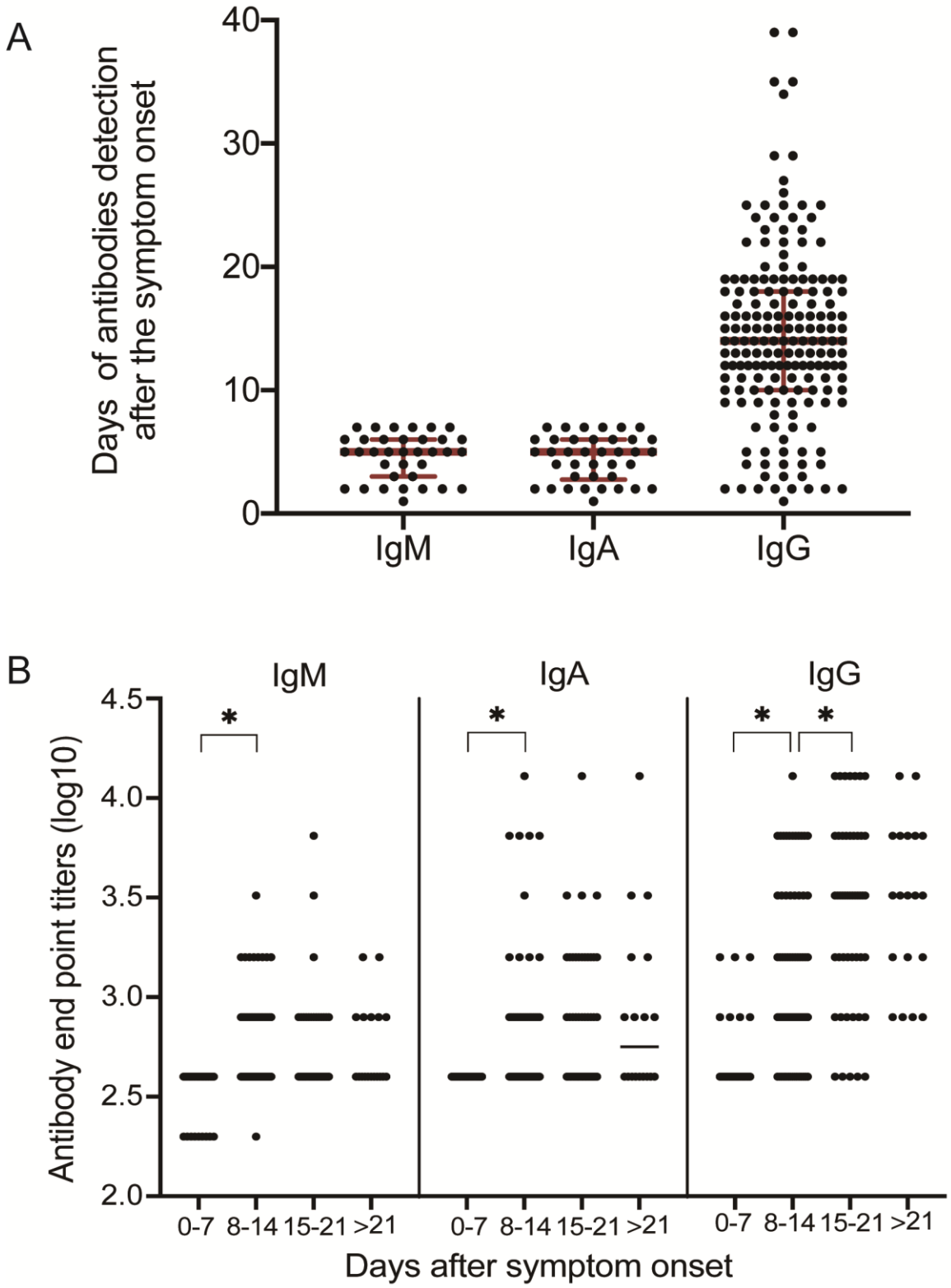

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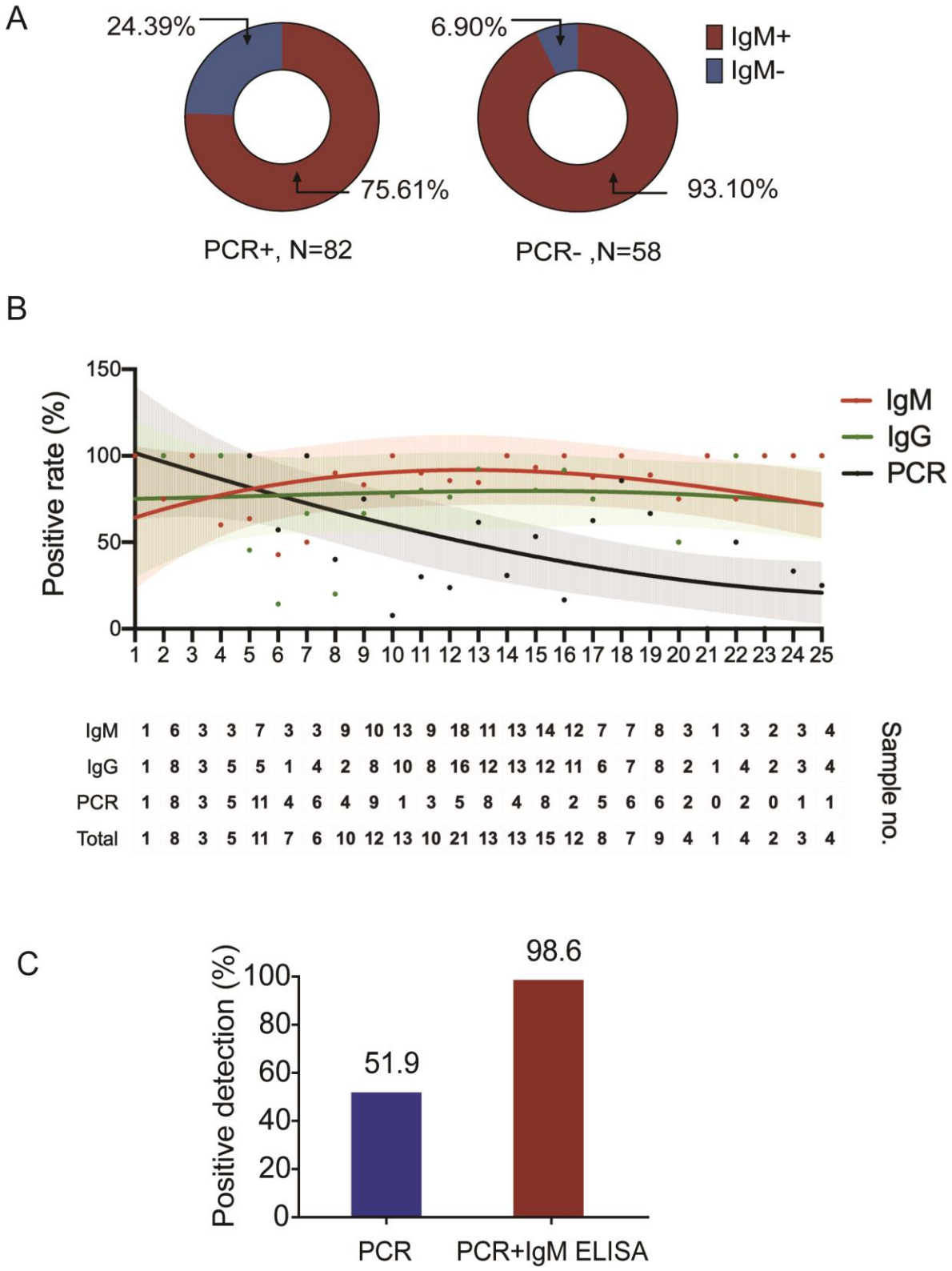
Figure 4



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