A Peptide-Based Magnetic Chemiluminescence Enzyme Immunoassay for Serological Diagnosis of Coronavirus Disease 2019

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Abstract

The coronavirus disease 2019 (COVID-19), once an unknown acute respiratory disease, is caused by a novel coronavirus (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]). Given the substantial increase in daily confirmed global cases, on January 30, 2020, the World Health Organization (WHO) officially declared the COVID-19 outbreak a public health emergency of international concern. Furthermore, on March 12, 2020, the WHO declared the global coronavirus crisis a pandemic. As of April 21, 2020, a total of 2,395,822 confirmed cases have been reported in 200 countries, territories, and areas around the world.

Conclusions. Therefore, combining our immunoassay with real-time RT-PCR might enhance the diagnostic accuracy of COVID-19.

Keywords. chemiluminescence immunoassay; COVID-19; SARS-CoV-2; serological test.
Such an immunoassay can obtain a diagnosis within a relatively short time and provides the ability to probe an active immune response to the virus.

Similar to SARS-CoV and Middle East respiratory syndrome (MERS) CoV, SARS-CoV-2 are enveloped, positive-sense, single-stranded RNA viruses [1, 2]. Genomic analysis of SARS-CoV-2 revealed 4 major structural proteins, including spike (S) protein, nucleocapsid (N) protein, envelope (E) protein, and membrane (M) protein, as well as several accessory open reading frame (ORF) proteins [1, 2]. In this study, we developed a magnetic chemiluminescence enzyme immunoassay (MCLIA), which showed high specificity and sensitivity in detecting serum immunoglobulin (Ig)G and IgM against SARS-CoV-2.

METHODS

Human Sera
A total of 276 sera samples were collected from 276 patients from 3 designated hospitals: Chongqing Three Gorges Central Hospital, Yongchuan Hospital Affiliated to the Chongqing Medical University (CQMU), and the Public Health Center, in Chongqing, China. Through real-time RT-PCR, these patients were confirmed to be infected with SARS-CoV-2 by the detection of viral RNA. Among these sera samples, 168 patients displayed pyrexia symptoms. The time points of sampling ranged from day 2 to day 27 from the onset of the fever. In addition, 99 patients reported recent exposure to persons with a confirmed infection. The 200 normal human sera were collected from healthy people more than 1 year before the SARS-CoV-2 outbreak. In total, 167 sera from patients with an infection from other pathogens were collected from the Second Hospital Affiliated to CQMU and the Children’s Hospital Affiliated to CQMU. The pathogens identified were as follows: influenza A virus (25), respiratory syncytial virus (7), parainfluenza virus (8), influenza B virus (5), adenovirus (6), Klebsiella pneumonia (8), Streptococcus pneumonia (3), Mycoplasma (5), Acinetobacter baumannii (10), Candida albicans (2), Staphylococcus aureus (3), Mycobacterium tuberculosis (4), hepatitis B virus (33), hepatitis C virus (22), syphilis (23), and Saccharomyces (3).

All sera samples were inactivated at 56°C for 30 minutes. This study was approved by the Ethics Commission of Chongqing Medical University (CQMU-2020-01). Written informed consent was waived by the Ethics Commission of the designated hospital for emerging infectious diseases.

A confirmed COVID-19 case was defined as a positive infection by real-time RT-PCR assay for nasal or pharyngeal swab specimens, according to the WHO guidelines. Nasal and pharyngeal swab samples were collected for extracting SARS-CoV-2 RNA. The National Medical Products Administration approved the commercial use of real-time RT-PCR assay for SARS-CoV-2, which was provided by DAAN Gene Co., Ltd. (Guangzhou, China; approval no. 20203400063). In brief, 2 target genes, ORF1ab (ORF1ab) and nucleocapsid protein (N), were simultaneously amplified and investigated. The PCR steps were from the manufacturer’s protocol. A cycle threshold (Ct) value less than 37 was defined as a positive test result, and a Ct value of 40 or more was defined as a negative test. A medium load, defined as a Ct value of 37 to less than 40, required confirmation by retesting. These diagnostic criteria were based on the recommendation by the National Institute for Viral Disease Control and Prevention (Beijing, China).

Synthetic Peptide-Based Luminescent Immunoassay
We developed a chemiluminescent immunoassay for the detection of the 2019 novel CoV antibody using synthetic peptide antigens as the immunosorbent. Epitopes of antigens of SARS-CoV-2, including the orf1a/b, S, and N proteins, were analyzed by online servers (http://www.cbs.dtu.dk/services/BepiPred/, http://www.epitope-informatics.com/Links.htm). These predicted epitope peptides were then synthesized (Sangon Biotech Co., Ltd., Shanghai, China) and conjugated with bovine serum albumin by amino or carboxyl terminal cysteine. Twenty peptides deduced from the genomic sequence from GenBank (accession no. NC_045512.1) were synthesized as candidate antigens from the orf1a/b, S, and N proteins. Each kind of peptide was labeled with biotin, and the biotinylated peptide was purified and subsequently bound to streptavidin-coated magnetic beads. For the antibody assay, serum samples (100 μL/each sample) were mixed with the beads carrying corresponding peptides for 10 minutes at 37°C. Then, the beads were washed 5 times, subjected to antibody conjugation, again washed 5 times, and finally allowed to react with the substrate. The assay was performed on a luminescence reader (Peteck 96-I; Bioscience Diagnostics, Tianjin, China).

Evaluation of the Luminescent Immunoassay
The cutoff value of the test was determined as the mean luminescence value of the 200 normal sera standard deviation plus 5-fold cross-validation. Sera from 276 patients with COVID-19 and 167 patients with foreign pathogens were used to evaluate the performance of the assay.

RESULTS

Evaluation of Synthetic Peptide-Based Magnetic Chemiluminescence Enzyme Immunoassay for Severe Acute Respiratory Syndrome Coronavirus 2
Twenty synthetic peptides derived from the amino acid sequence of ORF1a/b, S, and N proteins were used to develop the MCLIA for detecting IgG and IgM antibodies against SARS-CoV-2. To screen these peptides, 5 sera from confirmed patients with COVID-19 and 10 normal sera were used to react with these peptides, respectively. Among the tested peptide, 1 from the S protein showed the best performance. We used the assay based on this peptide for the following study. To determine
the cutoff value of this assay, serum samples from 200 healthy blood donors who donated blood 1–2 years before the COVID-19 outbreak were tested first. The mean signal-to-cutoff (S/co) values for IgG and IgM were 0.152 ± 0.109 and 0.151 ± 0.107, respectively (Figure 1). The cutoff value for IgG and IgM detection were determined as 0.7 and 0.7, respectively.

To test the specificity of the assays, the serum samples from 167 patients infected with other respiratory pathogens, such as influenza A virus, influenza B virus, parainfluenza virus, adenovirus, respiratory syncytial virus, mycoplasma, *S. pneumonia*, *K. pneumonia*, *A. baumannii*, *C. albicans*, and *S. aureus* were tested. The mean CL values for IgG and IgM in non-SARS-CoV-2-infected patients were 0.121 ± 0.062 and 0.120 ± 0.065, respectively (Figure 1A and B). These results showed that no cross-reactivity was observed for these 20 pathogens, indicating a high specificity.

To test the stability of this MCLIA-based serological diagnosis method, serum samples with different concentrations were measured 10 times (Figure 2A–D). The coefficient of variation of IgG and IgM detection in different concentration samples were all below 6% (Figure 2), implying successful assay stability for IgG/IgM detection. Furthermore, series dilutions for 6 serum samples (3 for IgG, 3 for IgM) were performed, and S/co values were collected. Regression analysis revealed that the S/co value that ranged from 1 to 200 linear reflected serum antibody concentration (IgG, $R^2 = -0.902$, $P < .01$ [Figure 3A]; IgM, $R^2 = -0.946$, $P < .01$ [Figure 3B]), which assured the rationality for further quantitative comparison based on S/co values.

**Detection of Antibodies Against Severe Acute Respiratory Syndrome Coronavirus 2 in Coronavirus Disease 2019 Patients**

Immunoglobulin G and IgM were further examined by MCLIA in serum samples from 276 patients with SARS-CoV-2 infection confirmed by RT-PCR. The median age of these patients was 48 years (interquartile range [IQR], 37–56; range, 0.66–84 years), and 151 of 276 (54.71%) were men (Supplementary Table S1). The mean confidence level values for IgG were 18.62 ± 32.87, ranging from 0.05 to 194.56; IgM were 5.50 ± 22.60, ranging from 0.04 to 318.16. The majority (197 of 276; 71.4%) of patients were positive for IgG antibodies against SARS-CoV-2, whereas 57.2% (158 of 276) of patients were positive for IgM antibodies against SARS-CoV-2. Overall, 225 patients showed positive assays for IgM or IgG, and the total positive rate reached 81.52% (225 of 276) (Table 1). There was a small cohort of patients that showed negative results for IgG or IgM. We classified these patients with a clear record of fever onset into the IgG- or IgM-positive group and the IgG- or IgM-negative group and compared the intervals between fever onset and antibody testing between the groups. As shown in Figure 4, both IgG-positive and IgM-positive groups had longer intervals than that of IgG-negative and IgM-negative groups, with median intervals of 13 days (IQR, 10–17) versus 10 days (IQR, 5–12) for IgG and 13 days (IQR, 10–17) versus 11 days (IQR, 7–14) for IgM, respectively.

**DISCUSSION**

We developed a luminescent immunoassay for IgG and IgM against SARS-CoV-2, which, to our knowledge, was the first such assay allowing us to study the antibody response to the newly identified coronavirus. This assay was based on a peptide from the S protein, which was screened out from 20 candidate peptides deduced from the genomic sequence. Using a synthetic peptide as an antigen enhanced the stability and repeatability of the assay, and theoretically this would be more specific than using a virus as an antigen. Moreover, this peptide showed high specificity in our assay; for example, none of the 167 sera from patients infected with pathogens other than SARS-CoV-2 reacted with this peptide.
Until now, real-time RT-PCR was the only test able to confirm SARS-CoV-2 infection. In this study, we detected both IgM and IgG in the same sera from the 276 infection-confirmed patients. Immunoglobulin G was detected in 71.4% (197 of 276) of sera samples and was higher than the detection rate of IgM (57.2%, 158 of 276). A combination of the 2 antibodies enhanced the detection rate to 81.5% (225 of 276). Previous research has demonstrated a different sensitivity for detecting IgG and IgM in SARS [3]. There was a small portion of patients that showed negative results for a virus specific to IgG and IgM.

Figure 2. Reproducibility of the assay. (A) Repeated detection of serum samples with high antibody titer (10 repeats). (B) Repeated detection of serum samples with middle antibody titer (10 repeats). (C) Repeated detection of serum samples with low antibody titer (10 repeats). (D) Repeated detection of samples from a healthy control (10 repeats). CV, coefficient of variation; Ig, immunoglobulin; NC, Negative Control; S/co, signal-to-cutoff.

Figure 3. The correlation between the serial dilution ratio and calculated signal-to-cutoff (S/co) values. (A) Correlation between serial dilution ratio and S/co values in immunoglobulin (Ig)G detection in 3 serum samples (n = replicates in each dilution for each sample). (B) Correlation between serial dilution ratio and S/co values in IgM detection in 3 serum samples (n = 3 replicates in each dilution for each sample).
for this might be that the period of virus infection was still not long enough for the humoral immune system of these patients to mount an adequate response to the virus. Indeed, those patients with positive results for the antibodies had a longer time since symptoms onset than those with negative results (Figure 4).

CONCLUSIONS

Immunoglobulin G can be detected as early as 2 days after the onset of fever. Immunoglobulin M was not detected earlier than IgG, similar to the situation in MERS [4], which limits its diagnostic utility. It was reported that 20%–50% of patients with SARS could not be confirmed by RT-PCR [5], and this elicited speculation that there might be a comparable aspect of SARS-CoV-2 infection that also cannot be detected by real-time RT-PCR. Failure of detection by real-time RT-PCR can be caused by issues from sampling, RNA extraction, and PCR amplification, whereas detecting antibodies in a serum sample may avoid these issues.

Supplementary Data

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

Note

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Table 1. Positive Rates of Serum IgG or IgM in 276 Patients With Confirmed SARS-CoV-2 Infection

| Antibody | Positive No. | Negative No. | Positive Rate (%) |
|----------|--------------|--------------|------------------|
| IgG      | 197          | 79           | 71.37            |
| IgM      | 158          | 118          | 57.24            |
| IgG and/or IgM | 225 | 51 | 81.52 |

Abbreviations: Ig, immunoglobulin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Figure 4. Difference in the time since fever onset between groups with positive or negative results in virus-specific antibodies. Boxplots indicate medians (middle line) and third and first quartile (box), and whiskers show 1.5× the interquartile range above and below the box. Numbers of patients (N) are depicted underneath boxplot. P value: unpaired, 2-sided Mann-Whitney U test.