

**A Peptide-based Magnetic Chemiluminescence Enzyme
Immunoassay for Serological Diagnosis of Coronavirus Disease 2019
(COVID-19)**

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Main point: A Peptide-based Magnetic Chemiluminescence Enzyme

Immunoassay for the detection of SARS-CoV-2 antibodies was developed; 71.4% (197/276) and 57.2% (158/276) of the COVID-19 inpatients were positive for IgG and IgM against SARS-CoV-2.

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Abstract

SARS-CoV-2, a novel β -coronavirus, cause severe pneumonia and has spread throughout the globe rapidly. The disease associated with SARS-CoV-2 infection is named COVID-19. To date, real-time RT-PCR is the only test able to confirm this infection. However, the accuracy of RT-PCR depends on several factors; variations in these factors might significantly lower the sensitivity of detection. Here, we developed a peptide-based luminescent immunoassay that detected immunoglobulin G (IgG) and IgM. The assay cut-off value was determined by evaluating the sera from healthy and infected patients for pathogens other than SARS-CoV-2. To evaluate assay performance, we detected IgG and IgM in the sera from confirmed patients. The positive rate of IgG and IgM was 71.4% and 57.2%, respectively. Therefore, combining our immunoassay with real-time RT-PCR might enhance the diagnostic accuracy of COVID-19.

Keywords. SARS-CoV-2; COVID-19; Serological Test; chemiluminescence immunoassay.

Introduction

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

Clinical manifestations related to SARS-CoV-2 infection range from no symptomatology to fatal pneumonia. However, early case detection is one of the most critical public health interventions for controlling the spread of COVID-19. COVID-19 cases can be identified based on exposure status, symptomatology, and chest imaging. However, positive confirmation of the infection requires nucleic acid testing by either nasal, pharyngeal, or anal swab. To date, real-time reverse transcription-PCR (RT-PCR)-based viral RNA detection has demonstrated clinical utility in being a sensitive and accurate method for diagnosing SARS-CoV-2 infection. Nevertheless, real-time RT-PCR failed to positively confirm several suspected cases of patients presenting with clinical symptoms. Furthermore, real-time RT-PCR may lead to false-negative results due to variations in several possible factors, such as the quality of the specimen collected, the source of the PCR reagents, the multi-steps in RNA preparation, and fluctuations in the viral load during different phases of

SARS-CoV-2 infection. Due to the limitations of RT-PCR, serum-specific antibody detection for COVID-19 has gained attention as an attractive assay. 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 severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV), SARS-CoV-2 are enveloped positive-sense single-stranded RNA viruses [1, 2]. Genomic analysis of SARS-CoV-2 revealed four 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 G (IgG) and IgM against SARS-CoV-2.

Methods

Human sera

A total of 276 sera samples were collected from 276 patients from three 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. Additionally, 99 patients reported recent

exposure to persons with a confirmed infection. The 200 normal human sera were collected from healthy people more than one year before the SARS-CoV-2 outbreak. In total, one hundred and sixty-seven 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 *Saccharomycopsis* (3).

All sera samples were inactivated at 56 °C for 30 min. 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). Briefly, two target genes, open reading frame 1ab (*ORF1ab*) and nucleocapsid protein (*N*), were

simultaneously amplified and investigated. PCR step were from the manufacturer's protocol. A cycle threshold value (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 2019-nCov 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 (BSA) by amino or carboxyl terminal cysteine. Twenty peptides deduced from the genomic sequence from GenBank (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 μ L/each sample) were mixed with the beads carrying corresponding peptides for 10 min 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 cut-off value of the test was determined as the mean luminescence value of the 200 normal sera standard deviation (SD) 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 MCLIA for SARS-CoV-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, one from the S protein showed the best performance. We used the assay based on this peptide for the following study. To determine the cut-off 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 S/co values for IgG and IgM were 0.152 ± 0.109 and 0.151 ± 0.107 , respectively (Fig. 1). The cut-off 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 (Fig. 1A-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 (Fig. 2A-D). The coefficient of variation (CV) of IgG and IgM detection in different concentration samples were all below 6% (Fig. 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 the S/co value ranged from 1 to 200 linear reflected serum antibody concentration (IgG, $R^2 = -0.902$, $p < 0.001$, Fig. 3A; IgM, $R^2 = -0.946$, $p < 0.001$, Fig. 3B), which assured the rationality for further quantitative comparison based on S/co values.

Detection of antibodies against SARS-CoV-2 in COVID-19 patients

IgG 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 (IQR, 37-56; range, 0.66-84 years), and 151/276 (54.71%) were men (Table S1). The mean CL values for IgG were 18.62 ± 32.87 , ranging from 0.05-194.56; IgM were 5.50 ± 22.60 , ranging from 0.04-318.16. The majority (197/276; 71.4%) of patients were positive for IgG antibodies against SARS-CoV-2, while 57.2% (158/276) 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% (225/276) (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 IgG- or IgM-positive group and IgG- or IgM-negative group and compared the intervals between fever onset and antibody testing between the groups. As shown in Fig. 4, both IgG positive and IgM positive groups had longer intervals than that of IgG negative and IgM negative groups, with median intervals 13 days (IQR,10-17) vs. 10 days (IQR, 5-12) for IgG and 13 days (IQR, 10-17) vs. 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 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.

Till 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. IgG was detected in 71.4% (197/276) of sera samples

and was higher than the detection rate of IgM (57.2%, 158/276). A combination of the two antibodies enhanced the detection rate to 81.5% (225/276). 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. One reason 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 (Fig. 4).

IgG can be detected as early as 2 days after the onset of fever. IgM 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, while detecting antibodies in a serum sample may avoid these issues.

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Figure Legends

Fig. 1. Evaluation of the synthetic peptide-based MCLIA for the detection of IgG and IgM against SARS-CoV-2. Serum samples were obtained from 200 healthy blood donors, 167 people infected with other respiratory pathogens and 276 patients with confirmed SARS-CoV-2 infection. The serum IgG (A) and IgM (B) were analyzed by MCLIA.

Fig. 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).

Fig. 3. The correlation between the serial dilution ratio and calculated S/co values. A. correlation between serial dilution ratio and S/co values in IgG detection in 3 serum samples ($n=3$ 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).

Fig. 4. Difference in the time since fever onset between groups with positive or negative results in virus specific antibodies. Boxplots indicate medians (middle line), third and first quartile (box), and whiskers show $1.5 \times$ the interquartile range above and below the box. Numbers of patients (N) are depicted underneath boxplot. p value:

unpaired, two-sided Mann-Whitney U test.

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Figure 1

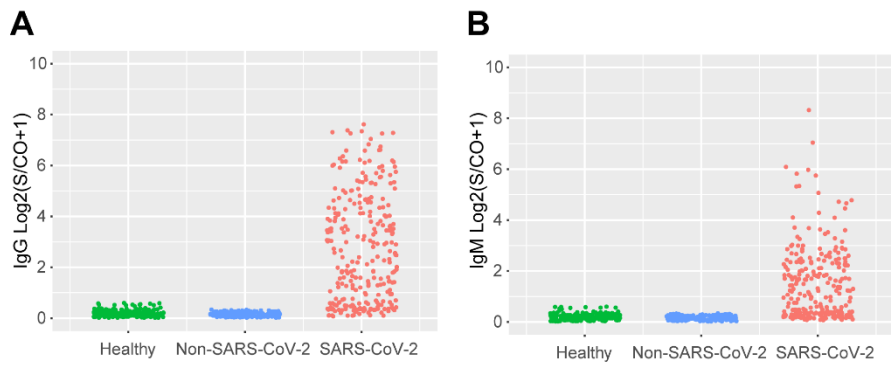


Figure 2

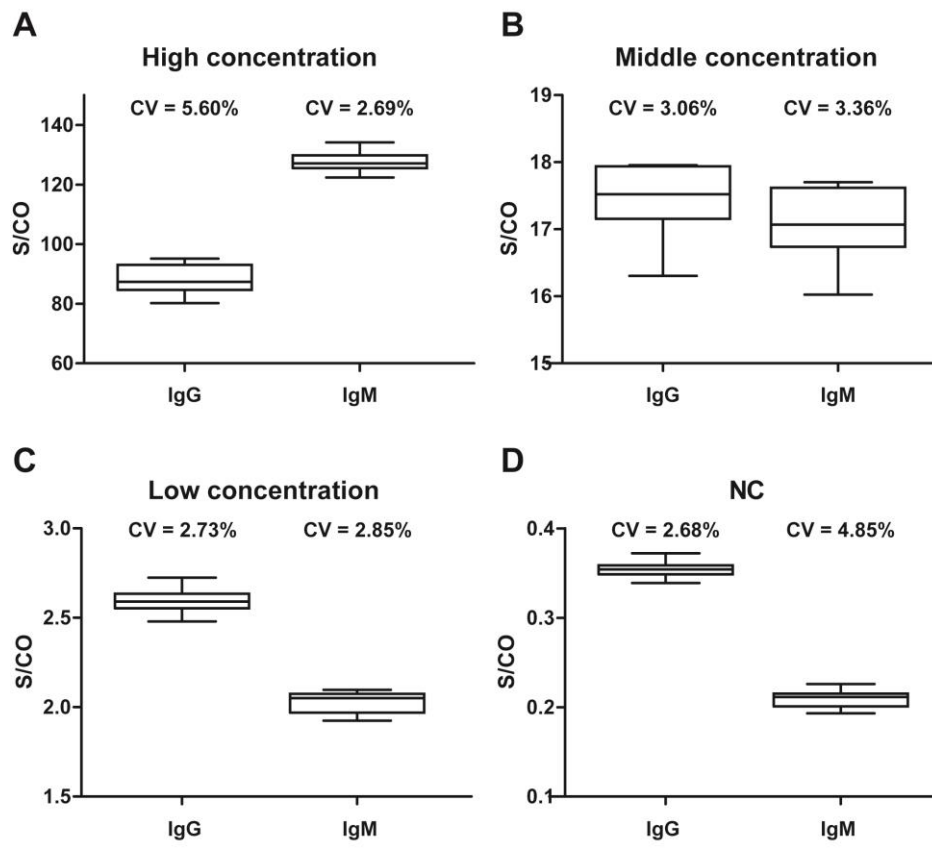


Figure 3

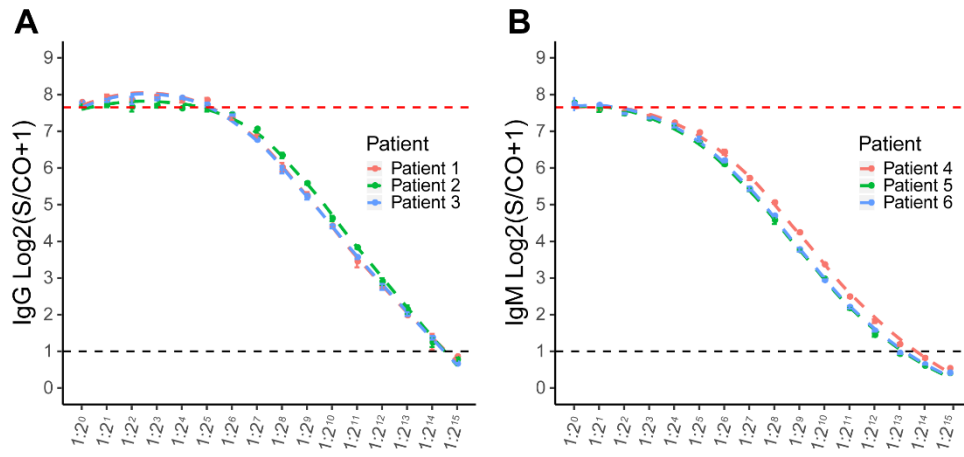


Figure 4

