A method to prevent SARS-CoV-2 IgM false positives in gold immunochromatography and enzyme-linked immunosorbent assays

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Abstract

Objective: To investigate the interference factors that lead to false-positive novel coronavirus (SARS-CoV-2) IgM detected using gold immunochromatography assay (GICA) and enzyme-linked immunosorbent assay (ELISA) and the corresponding solutions.

Methods: GICA and ELISA were used to detect SARS-CoV-2 IgM in 86 serum samples, including 5 influenza A virus (Flu A) IgM-positive sera, 5 influenza B virus (Flu B) IgM-positive sera, 5 *Mycoplasma pneumoniae* IgM-positive sera, 5 *Legionella pneumophila* IgM-positive sera, 6 sera of HIV infection patients, 36 rheumatoid factor IgM (RF-IgM)-positive sera, 5 sera from hypertensive patients, 5 sera from diabetes mellitus patients, and 14 sera from novel coronavirus infection disease (COVID-19) patients. The interference factors causing false-positive reactivity in the two methods were analyzed, and the urea dissociation test was employed to dissociate the SARS-CoV-2 IgM-positive serum using the best dissociation concentration.

Results: Two methods detected positive SARS-CoV-2 IgM in 22 middle-high level RF-IgM-positive sera and 14 sera from COVID-19 patients; the other 50 sera were negative. When urea dissociation concentration was 6 mol/L, SARS-CoV-2 IgM were positive in 1 middle-high level RF-IgM-positive sera and in 14 COVID-19 patient sera detected using GICA. When urea dissociation concentration was 4 mol/L and the avidity index (AI) lower than 0.371 was set to negative, SARS-CoV-2 IgM were positive in 3 middle-high level RF-IgM-positive sera and in 14 COVID-19 patient sera detected using ELISA.

Conclusion: Middle-high level of RF-IgM could lead to false-positive reactivity of SARS-CoV-2 IgM detected using GICA and ELISA, and urea dissociation tests would be helpful in reducing false-positive results of SARS-CoV-2 IgM.

Keywords: novel coronavirus; gold immunochromatography assay; enzyme-linked immunosorbent assay; false-positive; urea

Introduction

The outbreak of novel coronavirus (SARS-CoV-2) in Wuhan, China, has spread rapidly throughout the country, as well as to other countries around the world. The outlook of prevention and control of novel coronavirus infection disease (COVID-19) is still grim. Up to February 20, 2020, the number of confirmed COVID-19 cases exceed 70,000, and this number is rising steadily, placing enormous emphasis on the timely and accurate diagnosis and treatment of the disease. At present, the diagnosis of COVID-19 is

mainly based on epidemiological history inquiry, laboratory testing, and chest radiology examination. Among these examinations, the detection of nucleic acid from SARS-CoV-2 is the direct evidence for COVID-19 diagnosis [1-3]. Detection of SARS-CoV-2 nucleic acid should be performed in special laboratories by professional technicians, and has the disadvantages of insufficient supply of detection kits in a public health emergency, low throughput, and time-consuming procedures. Moreover, the swabs taken from the throat may not always reveal the infection of SARS-CoV-2 for patients; additional sampling is always performed for the accurate diagnosis. Therefore, nucleic acid detection may not the best choice for screening large-scale populations infected with SARS-CoV-2 [4].

The detection of serum-specific IgM and IgG, especially the former, is routinely used in clinical laboratories to evaluate the acute phase infection of pathogens in the serum [5,6]. In many infections, IgM can be detected as early as one week after infection. When the level of IgM reaches the detection limit of the assay kit, the detection of IgM can avoid false-negative results owing to sampling. At present, the main methods for the detection of specific antibodies in clinical laboratories are gold immunochromatography assay (GICA) and enzyme-linked immunosorbent assay (ELISA) [7-11], both of which have the advantages of mature methodology, high flux detection, simple operation, rapid detection, no special equipment, and they are low cost. Using these two methods to detect SARS-CoV-2 IgM can identify or screen SARS-CoV-2 infection in suspicious and close contact populations earlier, more quickly and effectively, and improve the accuracy of epidemiological monitoring, which is very important for patient management and epidemic prevention and control. However, in the process of using GICA and ELISA to detect SARS-CoV-2 IgM, we found that there was interference from rheumatoid factor IgM (RF-IgM) in the two methods.

The affinity of cross reaction between specific antigens and antibodies was lower than that of specific reaction [12]. Urea can be used as a dissociating substance between antigen-antibody reactions to evaluate the affinity of IgG, such as the evaluation of the affinity of *Toxoplasma gondii* IgG in different detection systems [13,14]. Therefore, we hypothesize that the use of the urea dissociation test will help to eliminate or reduce the influence of RF-IgM on the detection of SARS-CoV-2 IgM antibodies. Meanwhile, IgM-positive sera of other pathogens were collected to evaluate the detection performance of GICA and ELISA for SARS-CoV-2 IgM.

Materials and methods

Study setting and patients

Serum from a total of 86 patients with different pathogen infections and related chronic diseases were collected from the Affiliated Hospital of North Sichuan Medical College and Nanchong Central Hospital from January 25, 2020 to February 15, 2020. According to the Notice on the Issuance of Strategic Guidelines for Diagnosis and Treatment of Novel Coronavirus (SARS-CoV-2) Infected Pneumonia (Fifth Edition Version) [15], the 5 patients with Flu A IgM-positive sera, 5 patients with Flu B IgM-positive sera,

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5 patients with *Mycoplasma pneumoniae* IgM-positive sera, 5 patients with *Legionella pneumophila* IgM-positive sera, 6 patients with HIV infection, 36 patients with RF-IgM-positive sera, 5 hypertensive patients, and 5 diabetes mellitus patients had no clinical symptoms or imaging evidence of COVID-19. The other 14 COVID-19 patients met the diagnostic criteria, and serum were collected within 3-7 days after the beginning of the clinical symptoms. In addition to 36 RF-IgM-positive serum samples, detection levels of RF-IgM in the remaining 50 serum samples were lower than 20.00 IU/mL.

Assay

The IgM against Flu A and B, *M. pneumoniae*, and *L. pneumophila* were detected by indirect immunofluorescence assay (Respiratory tract 8 joint detection kit, EUROIMMUN, Inc., Germany). RF-IgM was detected by rate nephelometry assay (IMMAGE800, Beckman Coulter, Inc., America). HIV combi PT was detected by electrochemiluminescence assay (Cobas E602, Roche, Inc., Germany). HIV infection was confirmed by immunoblotting assay (the confirmed information was fed back by CDC). SARS-CoV-2 nucleic acid was detected using real-time polymerase chain reaction (kit provided by Shanghai Zhijiang Biotechnology Co., Shanghai, China; detection instrument provided by Shanghai Hongshi Biotechnology Co., Shanghai, China). GICA and ELISA were used for SARS-CoV-2 IgM detection (kit provided by Beijing Hotgen Biotechnology Co., Beijing, China: lot number 20200208 and 20200229 for GICA, 20200101 and 20200201 for ELISA). Optical density in ELISA plates was measured using a Microplate Reader (PHOMO, Autobio Diagnostics Co., Zhengzhou, China).

Urea dissociation test of GICA

Sera (100 μL) were added into 1 mL sample diluents (phosphate-buffered saline, PBS, NaCl and Tween 20), mixed, and then 100 μL of the diluted sample was put into the sample hole of the test card. The liquid was chromatographed upward under the capillary effect; when the liquid was about to reach the upper absorbent paper, 100 μL PBS solution containing 6 mol/L urea was added into the sample hole of the test card and the results were observed after 20 to 25 min. At first, the SARS-CoV-2 IgM in the sample bound with the anti-human-IgM labelled by colloidal gold, and then bound with the SARS-CoV-2 recombinant antigen at test line (T) position to form a complex of SARS-CoV-2 antigen, SARS-CoV-2 IgM, and anti-human-IgM labelled by colloidal gold. A complex of goat polyclonal IgG and anti-human-IgM labelled by colloidal gold was formed at the control line (C) position. Positive standard: colloidal gold color reaction occurs at both T-line and C-line positions; negative standard: only colloidal gold color reaction occurs at the C-line position.

Urea dissociation test of ELISA

Sera (8 μL) were added into 800 μL sample diluents (0.02M PBS), mixed, and then 100 μL of the diluted sample, negative control, positive control were added to the wells of the plates coated with

SARS-CoV-2 recombinant antigen, and the plates were incubated at 37 ℃ for 30 min. The plates were washed five times and 100 μL of PBS solution (containing 0 mol/l, 1 mol/L, 2 mol/L, 4 mol/L, 6 mol/L, and 8 mol/L urea in different wells) was added and incubated at 37 ℃ for 10 min. After three more washes, anti-human-IgM horseradish peroxidase (HRP) labelled antibody was added into the reaction system to form an indirect immune complex. Following five washes to remove unbound substances, the substrate was added for the color reaction. The results were interpreted by the ratios of the sample optical density value and the cut-off optical density value (S/CO), as follows: positive, S/CO equal to 1.00 or greater; negative, S/CO less than 1.00. The results of affinity index (AI) were the ratio of S/CO value of different dissociated urea concentration to that of PBS with 0 mol/L urea. The AI threshold value was set as the middle value between the highest AI value of false-positive sample results with the outliers removed and the lowest AI value of all SARS-CoV-2 infection samples. The results were interpreted as follows: positive, AI value of sera ≥ AI threshold; negative, AI value of sera < AI threshold.

Statistical analysis

Statistical analyses were performed by SPSS, version 19.0 (SPSS Inc., USA). Fisher's exact test was used for the specific comparison between before and after urea dissociation of GICA and ELISA for the detection of SARS-CoV-2 IgM in serum with positive RF-IgM. The specific comparison between before and after urea dissociation of GICA and ELISA for the detection of SARS-CoV-2 IgM in all control serum was made using Pearson's chi-square test. The statistical significance of all tests was defined as $P<0.05$ by two-tailed tests.

Results

The results of 2019 nCoV IgM in different serum samples

The results of SARS-CoV-2 IgM were negative in both GICA and ELISA of the 5 Flu A IgM-positive sera, 5 Flu B IgM-positive sera, 5 *M. pneumoniae* IgM-positive sera, 5 *L. pneumophila* IgM-positive sera, 6 sera from HIV infection patients, 5 sera from hypertensive patients, and 5 sera from diabetes mellitus patients (Table 1). A total of 22 of the 36 RF-IgM-positive samples were positive, and 14 COVID-19 patient samples were positive for SARS-CoV-2 IgM in both GICA and ELISA (Tables 1 and 2).

Comparison of SARS-CoV-2 IgM results and detection performance before and after urea dissociation test of GICA

When the dissociation concentration of urea was 6 mol/L, the dissociation test of GICA was carried out for 22 sera with RF-IgM positive and 14 samples from COVID-19 patients that were positive for SARS-CoV-2 IgM in GICA before urea dissociation. The results of SARS-CoV-2 IgM for 21 serum samples with positive RF-IgM turned negative (Figure 1), whereas those for the 14 samples from the COVID-19 patients remained positive. In the urea dissociation test, the specificity of GICA after

dissociation was significantly higher than before dissociation (P<0.001), and the sensitivity was not affected (Table 3).

Comparison of SARS-CoV-2 IgM results and detection performance before and after urea dissociation test of ELISA

The urea dissociation test of ELISA was carried out with PBS containing 0 mol/L, 1 mol/L, 2 mol/L, 4 mol/L, 6 mol/L, and 8 mol/L urea in 22 RF-IgM-positive serum samples and serum from 14 COVID-19 patients that were positive for SARS-CoV-2 IgM in ELISA before urea dissociation. When the dissociation concentration of urea was 4 mol/L, and according to the AI calculation method set to be 0.371, the results of SARS-CoV-2 IgM in 19 serum samples with positive RF-IgM turned negative, whereas those of SARS-CoV-2 IgM in 14 serum samples from COVID-19 patients remained positive (Figure 2). Through the urea dissociation test, the specificity of ELISA after dissociation was significantly higher than that before dissociation (P<0.001), and the sensitivity was unaffected (Table 4).

Discussion

SARS-CoV-2 infection patients have many clinical symptoms similar to those of common respiratory tract pathogens such as Flu A, Flu B, *M. pneumoniae*, and *L. pneumophila*, including fever, fatigue, and cough. Moreover, the majority of COVID-19 patients have pre-existing diseases such as diabetes, hypertension, and other endocrine and metabolic diseases [16,17]. Therefore, this study fully considered the above situation when selecting the control population. According to some reports, the bloodwork of SARS-CoV-2 infection patients mainly showed decreased lymphocyte counts. Therefore, this study also included HIV infection patients with similar phenomena into the control group [1,2,17]. The patients enrolled in each group are limited owing to insufficient amounts of diagnostic reagents and available time. At the same time, before the completion of the trial, only 14 cases of COVID-19 patients were recruited in our study. Although the relatively small sample size inevitably shows some bias in these two methods, the improvement in specificity was clear.

RF is an autoantibody against the FC segment of denatured IgG, the main type of which is IgM. It is the main factor that causes the interference of immune responses [18-20]. When the SARS-CoV-2 IgM test was carried out for all control serum and serum from COVID-19 patients by GICA and ELISA, the results showed that false-positive interference only occurred in RF-IgM-positive serum, and the serum from COVID-19 patients were all positive, indicating that the two methods had high sensitivity but specificity needed to be improved. The results of this study showed that when the RF-IgM concentration was lower than 70 IU/ml, there was no interference between the two methods in detecting SARS-CoV-2 IgM. In the other 29 sera with RF-IgM concentration above 70 IU/ml, 22 cases showed positive results from the two methods, suggesting that a middle-high level of RF-IgM greatly influenced the detection of SARS-CoV-2 IgM. However, the results of SARS-CoV-2 IgM in the other seven sera with high RF-IgM level were

negative, which may be related to the blocking of the cross-reaction site of RF-IgM and needs further investigation. The mechanism that causes RF-IgM false-positive results in the two methods may be that RF-IgM reacts with SARS-CoV-2 recombinant antigen, and RF-IgM combines with gold labelled anti-human IgM or HRP labelled anti-human-IgM, resulting in false-positive results. This cross reactivity can be reduced by urea dissociation.

The middle-high level of RF-IgM could cause false-positive results of SARS-CoV-2 IgM detected by GICA and ELISA. Therefore, when serum is RF-IgM-positive, it is difficult to evaluate the real SARS-CoV-2 IgM status. This study attempts to eliminate or reduce the interference by urea dissociation. The main basis for selecting the time point of adding urea solution in the GICA method was as follows: first, after a certain reaction time, the specific antigen antibody may bond more firmly and it may be more challenging to be dissociated by the urea; second, at this time point, the liquid content in the sample well was small, with little change in urea concentration in the dissociation solution added later, thereby ensuring the dissociation effect. According to our previous study [21], the urea dissociation concentration in GICA was 6 mol/L, resulting in 21 out of the 22 RF-IgM-positive sera that were false positive for SARS-CoV-2 IgM results turned negative, whereas the 14 serum samples from COVID-19 patients were not affected. In addition, when the urea dissociation concentration in ELISA was 4 mol/L and the dissociation time was 10 min, 19 out of the 22 with RF-IgM-positive sera that were false-positive for SARS-CoV-2 IgM results turned negative, whereas the 14 sera from COVID-19 patients were not affected. Therefore, the improved GICA and ELISA not only ensured detection sensitivity, but also improved the corresponding specificity and reliability. Urea dissociation test of ELISA showed a similar detection performance to that of the urea dissociation test of GICA, and this may result from the use of the same recombinant antigen and the fact that both methods are based on the same detection principle.

In conclusion, when GICA and ELISA are used to detect SARS-CoV-2 IgM, the level of RF-IgM in the serum should be evaluated, and the urea dissociation test should be carried out to avoid the risk of false-positive results. At the same time, the results of this study suggest that the urea dissociation test cannot completely eliminate the interference of RF-IgM. Therefore, when SARS-CoV-2 IgM results are still positive after urea dissociation, RT-PCR should be used for nucleic acid diagnosis. In addition, it should be emphasized that serological tests are a complementary method to nucleic acid detection. The preferred method for detection of acute disease is via molecular testing, rather than testing for IgM, precisely because of the possibility of inaccurate results. Based on our research results, we suggest that all of the above-mentioned methods should be used to eliminate or reduce the impact of cross reaction when using GICA and ELISA methods to detect SARS-CoV-2 IgM, which will help in the preliminary screening of suspected and high-risk groups, as well as in the assessment, prevention, and control of the SARS-CoV-2 epidemic and the formulation of appropriate prevention systems.

Ethical approval

This study was approved by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College.

Competing interest

The authors confirm that they have no conflict of interest related to the generation of the data published in this manuscript.

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Table 1. SARS-CoV-2 IgM detection in serum using GICA and ELISA

Group	Cases	Positive case（%）

		GICA	ELISA
Flu A IgM	5	0 (0.00%)	0 (0.00%)
Flu B IgM	5	0 (0.00%)	0 (0.00%)
M. pneumoniae IgM	5	0 (0.00%)	0 (0.00%)
L. pneumophila IgM	5	0 (0.00%)	0 (0.00%)
Hypertension	5	0 (0.00%)	0 (0.00%)
Diabetes mellitus	5	0 (0.00%)	0 (0.00%)
HIV infection	6	0 (0.00%)	0 (0.00%)
RF-IgM	36	22 (61.11%)	22 (61.11%)
SARS-CoV-2 infection	14	14 (100.00%)	14 (100.00%)

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308 Table 2. RF-IgM-positive serum results of SARS-CoV-2 IgM detected using GICA and ELISA

Number	RF-IgM (IU/mL)	GICA	ELISA	Number	RF-IgM (IU/mL)	GICA	ELISA
1	34.2	N	N	19	161	P	P
2	35.5	N	N	20	173	P	P
3	39.8	N	N	21	173	P	P
4	56.2	N	N	22	195	N	N
5	56.3	N	N	23	220	N	N
6	66.5	N	N	24	222	P	P
7	69.2	N	N	25	224	P	P
8	73.6	N	N	26	256	P	P
9	74.2	P	P	27	283	P	P
10	76.2	P	P	28	284	N	N
11	82.8	P	P	29	328	P	P
12	84.0	N	N	30	431	P	P
13	98.80	N	N	31	440	P	P
14	118.0	P	P	32	441	N	N
15	119	P	P	33	502	P	P
16	142	P	P	34	1050	P	P
17	145	P	P	35	1680	P	P

18	154	P	P	36	1680	P	P

N: negative; P: positive.

Table 3. Comparison of SARS-CoV-2 IgM detection specificity of GICA before and after urea dissociation

Control	Cases	Specificity	
		Before dissociation	After dissociation
RF-IgM negative	36	100.00% (36/36)	100.00% (36/36)
RF-IgM-positive	36	38.89% (14/36)	97.22% (35/36)*
total	72	69.44% (50/72)	98.61% (71/72)*

*: $P<0.001$ (Compared with before dissociation)

Table 4. Comparison of SARS-CoV-2 IgM detection specificity of ELISA before and after urea dissociation

Control	Cases	Specificity	
		Before dissociation	After dissociation
RF-IgM-negative	36	100.00% (36/36)	100.00% (36/36)
RF-IgM-positive	36	38.89% (14/36)	91.67% (33/36)*
total	72	69.44% (50/72)	95.83% (69/72)*

*: $P<0.001$ (Compared with before dissociation)

Fig. 1 SARS-CoV-2 IgM detected using GICA before and after urea dissociation. N1-N2: SARS-CoV-2 IgM in serum with RF IgM-positive before and after urea dissociation test in GICA; N3-N4: SARS-CoV-2 IgM in serum of SARS-CoV-2 infection patients before and after urea dissociation test in GICA.

Fig. 2 AI of SARS-CoV-2 IgM detected using different urea dissociation concentrations of ELISA. When the dissociation concentration of urea was 4 mol/L, and the AI calculation method set to 0.371, SARS-CoV-2 IgM in 19 sera with RF-IgM positivity turned negative, whereas SARS-CoV-2 IgM in the 14 sera from COVID-19 patients remained positive.