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ORIGINAL ARTICLE

Rapid Detection of COVID-19 by Serological Methods and the Evaluation of Diagnostic Efficacy of IgM and IgG

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SUMMARY

Background: **In December 2019, a novel coronavirus (SARS-CoV-2) causing symptomatic illness (COVID-19) occurred in Wuhan, China. Travel-associated cases were reported in many other countries leading to epidemic transmission. The number of cases has increased rapidly but laboratory diagnosis is limited.**
Methods: **We collected samples from two groups of patients diagnosed with COVID-19 for experiments. In one group, 63 serum samples were analyzed IgG and IgM antibodies by enzyme-linked immunosorbent assay (ELISA) and 35 healthy serum samples were served as controls. In the other group, 91 plasma samples were analyzed by colloidal gold-immunochromatographic assay (GICA) for IgG and IgM antibodies and 35 healthy plasma samples were served as controls. Throat swab samples for nucleic acids retest were collected from 81/91 of these participant.**
Results: **The sensitivity of the combined ELISA IgM and IgG detection was 55/63 (87.3%). Sensitivity of the combined GICA IgM and IgG detection was 75/91 (82.4%). Both methods were negative for healthy controls and had a specificity of 100%. In 81 cases, the follow up throat swab samples were retested by RT-PCR, showing that 42 cases were positive. The sensitivity was 51.9% (42/81). The area under the receiver operating characteristic (ROC) curve for IgG ($AUC_{(IgG)}$) was 0.934. The area under the ROC curve for IgM ($AUC_{(IgM)}$) was 0.812. The area under the ROC curve for IgG + IgM ($AUC_{(IgG+IgM)}$) was 0.983.**
Conclusions: **The serological test of SARS-CoV-2 can be used as an important supplement to the existing RT-PCR test for the specific and rapid diagnosis of COVID-19. $AUC_{(IgG)} > AUC_{(IgM)}$ indicates that IgG has better classification performance than IgM. $AUC_{(IgG + IgM)} > AUC_{(IgG)}$ indicates that the combination of IgG and IgM has better classification performance than IgG alone.**
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KEY WORDS

2019-nCoV, SARS-CoV-2, COVID-19, enzyme-linked immunoassay, colloidal gold-immunochromatographic assay

LIST OF ABBREVIATIONS

ELISA - enzyme-linked immunosorbent assay
GICA- gold-immunochromatographic assay
COVID-19 - novel coronavirus-infected pneumonia
ICTV - International Committee on Taxonomy of Viruses
SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2
WHO - World Health Organization
ARDS - acute respiratory distress syndrome
RT-PCR - real-time reverse transcription polymerase chain reaction
ROC - receiver operating characteristic
NP - SARS-CoV-2 nucleocapsid protein
EP - SARS-CoV-2 envelope proteins
rN - recombinant nucleocapsid protein
RDB - receptor-binding domain

INTRODUCTION

In December 2019, a cluster of acute respiratory illness, now called novel coronavirus disease (COVID-19), occurred in Wuhan, Hubei Province, China [1-3]. The disease has rapidly spread from Wuhan to other areas. According to the most recent statistics of the World Health Organization (WHO), COVID-19 has already been diagnosed in as many as 1,568,275 people from 211 countries worldwide, causing nearly 92,814 deaths [4]. The global infection fatality rates are 3.48% and the outbreaks in health care workers indicate human-to-human transmission. The International Committee on Taxonomy of Viruses (ICTV) announced that the virus causing this disease was officially classified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [5] and the WHO announced that the official name of the disease caused by this virus is Coronavirus disease 2019 (COVID-19) [6]. The type of pneumonia caused by SARS-CoV-2 is a highly infectious disease and the ongoing outbreak has been declared by WHO as a global public health emergency.
Huang first reported 41 cases of COVID-19 in which most patients had a history of exposure to Huanan Seafood Wholesale Market [2]. From January 10 to 24, 2020, the number of people diagnosed with the SARS-CoV-2 infection in China increased by 31.4 times. The number of infected patients doubled every 7.4 days and the transmission rate of individual infected patients was 2.2 [2]. The ratio of male to female deaths was 3.25:1, the median time from the first symptom to death was 14 days [8].
Zhu [1] confirmed that SARS-CoV-2 was a new β-coronavirus belonging to the subgenus botulinum of Coronaviridae. Full-genome sequencing and phylogenic analysis indicate that SARS-CoV-2 is genetically distinct from SARS-CoV but both viruses are technically in the same clade and they are classified as sarbecoronaviruses [1]. SARS-CoV-2 gene encodes multiple structural proteins, such as nucleocapsid protein (NP) and envelope protein (EP). They include multiple antigen epitopes, which can be used as immunogens. By the specific binding of antigen and antibody, the presence of antigen can be detected by antibody, thus indirectly verifying whether the samples contain SARS-CoV-2 [9]. The detection of SARS-CoV-2 antibody is divided into IgM and IgG. In general, IgM is produced early and can be used as an indicator of early infection, while IgG is produced late and can be used as an indicator of previous infection [9].
Diagnosis is based on clinical history, laboratory and chest radiographic findings, but case confirmation currently relies on nucleic acid-based assays. Some patients have false negative nucleic acid test results and miss cases confirmation. We have verified two detection tests (ELISA and GICA) can be used to supplement existing RT-PCR nucleic acid detection [9].

MATERIALS AND METHODS

Study design and participants

This research was approved by the institutional ethics board of Wuhan Jinyintan Hospital. All patients with confirmed COVID-19 admitted to Jinyintan Hospital from January 18 to February 4, 2020, were enrolled. Oral consent was obtained from patients. All cases with COVID-19 enrolled in this study were confirmed according to the SARS-CoV-2 Pneumonia Prevention and Control Program (4th edition) published by the National Health Commission of China [10]. The samples for clinical testing were collected from two groups of patients. In one group, 63 serum samples were collected on February 2, 2020 for ELISA IgG and IgM antibodies. In the other group, 91 plasma samples were collected on February 3 and 4, 2020, for GICA, of 81 cases throat swab samples were taken for nucleic acids retest. 35 healthy individuals served as controls in both groups.

ELISA

The SARS-CoV-2 IgG/IgM antibody ELISA kit was manufactured by Zhu Hai Liv Zon Diagnostics Inc. The samples were tested according to the manufacturer’s instructions. The nucleocapsid protein (NP) of SARS-CoV-2 is the most abundant protein in coronavirus, with strong immunogenicity and conserved sequence [11]. Thus, the SARS-CoV-2 antibody detection kit uses recombinant nucleocapsid protein (rN) as the diagnostic antigen to capture viral antibodies IgM or IgG in blood samples.
IgM capture ELISA: Mouse anti-human IgM monoclo-

nal antibody (μ chain) was coated on ELISA plates. Serum sample (100 μL, 1:100 dilution) was added to the pre-coated plates. For each test, 100 μL of corresponding reference substance was added to three wells of negative control and two wells of positive control, and one well as blank control without sample. Then plates were incubated at 37°C for 1 hour. After washing, 100 μL HRP-labeled rN protein of SARS-CoV-2 was added and the plate was incubated at 37°C for 30 minutes followed by washing. Substrate buffer (50 μL) and TMB substrate solution (50 μL) were added to each well and then incubated at 37°C for 15 minutes after gently mixing. To each well, 50 μL 2M H_2SO_4 was added to terminate the color reaction. Finally, the OD450 was measured and recorded immediately using an Infinite 200 PRO microplate reader. Cutoff value calculation: IgM cutoff value = 0.100 + average OD value of negative control. The test was considered negative when OD450 was below the cutoff value and positive when OD450 was greater than or equal to the cutoff value.

IgG indirect ELISA: rN protein of SARS-CoV-2 was coated on ELISA plates. Serum sample (5 μL) was diluted in 100 μL dilution buffer and then added to the plates. After incubation and washing, 100 μL HRP-labeled monoclonal mouse anti-human IgG was added to the plates for detection. The other operation steps were conducted as above. Cutoff value calculation: IgG cutoff value = 0.130 + average OD450 value of negative control. The test was considered negative when OD450 was below the cutoff value and positive when OD450 was greater than or equal to the cutoff value.

GICA

The SARS-CoV-2 IgG/IgM antibody GICA kit was manufactured by Zhu Hai Liv Zon Diagnostics Inc. SARS-CoV-2 NP is used as the diagnostic antigen in the kit. The detection principle is as follows: a colloidal gold-labeled murine anti-human IgM or IgG monoclonal antibody was coated on the reagent binding pad, a purified rN of SARS-CoV-2 was coated on the test area of the nitrocellulose membrane and a goat anti-mouse antibody was coated on the control area.

The samples were tested according to the manufacturer's instructions. For each test, 10 μL of plasma sample and 100 μL of sample diluent were added vertically onto the sample pad of the test strip. The strip was then placed flat to allow the solution to migrate up the membrane, through the gold-labeled pad, the testing area, quality control area, and finally to adsorption zone. After 10 minutes, the result was judged by the color of the test and control lines. The interpretation of GICA test results is shown in Figure 1.

RT-PCR assay for SARS-CoV-2

Throat swab samples were collected for extracting SARS-CoV-2 RNA from patients. After collection, throat swabs were placed into a sterile test tube with 1 mL sterile saline. Viral RNA purification kit (QIAamp Viral RNA Mini Kit, Qiagen) was used as instructed by the manufacturer. For all RNA extractions, 40 μL elution was used for RT-PCR assay of SARS-CoV-2 RNA. Then, n*19 μL mixed reagent of fluorescence PCR detection for SARS-CoV-2 nucleic acid and n*1 μL RT-PCR enzyme (n was the number of reaction tubes) were mixed and vortexed for a few seconds. The above mixture of 20 μL was put into the PCR reaction tube, and 5 μL of the extracted sample was added. RT-PCR analysis was conducted using the ABI 7500 Real-Time PCR System. The PCR parameters were 45°C for 10 minutes, 95°C for 3 minutes, followed by 45 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and a single fluorescence detection point at 58°C. Two target genes, including open reading frame 1ab (ORF1ab) and nucleocapsid protein, were simultaneously amplified and tested during the RT-PCR assay. The RT-PCR assay was performed using a SARS-CoV-2 nucleic acid detection kit according to the manufacturer's protocol (Shanghai ZJ Bio-Tech Co., Ltd). A cycle threshold value (Ct-value) less than 37 was defined as a positive test result, a Ct-value of 40 or more was defined as a negative test, and a Ct-value between 37 - 40 was defined as doubtful, requiring confirmation by retesting. These diagnostic criteria were based on the recommendations of the National Institute for Viral Disease Control and Prevention (China).

Statistical analysis

All analyses were performed using SPSS 22.0. Categorical variables were expressed as frequencies (percentages) and performed using the chi-square test with Yates's correction or Fisher's exact test, as appropriate. $p < 0.05$ was considered statistically significant. For multiple statistical comparisons, chi-square test was corrected by Bonferroni's correction (0.05/test numbers).

RESULTS

ELISA results

63 serum samples from COVID-19 patients were tested by ELISA. The results showed that 28 IgM antibodies were positive. The sensitivity was 44.4% (28/63) and the specificity was 100% (35/35). 52 IgG antibodies were positive with a sensitivity of 82.5% (52/63) and specificity of 100% (35/35). The sensitivity of the combined IgM and IgG detection was 55/63 (87.3%) (Table 1). The results of 63 cases and 35 healthy controls by ELISA IgM and IgG were shown in Figure 2 (A and B).

GICA results

91 plasma samples of COVID-19 patients were tested by GICA, the results showed that 52 samples were positive for IgM antibodies. The sensitivity was 57.1% (52/91) and the specificity was 100% (35/35). 74 samples were positive for IgG antibodies with a sensitivity of 81.3% (74/91) and specificity of 100% (35/35). The sensitivity of the combined IgM and IgG detection was

Table 1. Sensitivity and specificity of IgM and IgG by ELISA, GICA, and RT-PCR.

Methods	Total	Groups	Positive	Negative	Sensitivity (%)	Specificity (%)
ELISA	63	IgM	28	35	28/63 (44.4)	35/35 (100.0)
		IgG	52	11	52/63 (82.5)	35/35 (100.0)
		IgM + IgG [a]	55	8	55/63 (87.3)	35/35 (100.0)
GICA	91	IgM	52	39	52/91 (57.1)	35/35 (100.0)
		IgG	74	17	74/91 (81.3)	35/35 (100.0)
		IgM + IgG [a]	75	16	75/91 (82.4)	35/35 (100.0)
RT-PCR	81	-	42	39	42/81 (51.9)	-

[a] positive if either of the two markers is positive.

Table 2. Test results of SARS-CoV-2 IgM + IgG for different lengths of stay.

Groups	Positive	Negative	Positivity rate (%)	p
< 4 days	24	7	77.4	0.032 [b]
4 - 8 days	7	1	87.5	0.250 [c]
> 8 days	24	0	100	

[b] - There is a significant difference in the positivity rate among the three groups.
[c] - There is no significant difference between the positivity rate of 4 - 8 days group and > 8 days group.

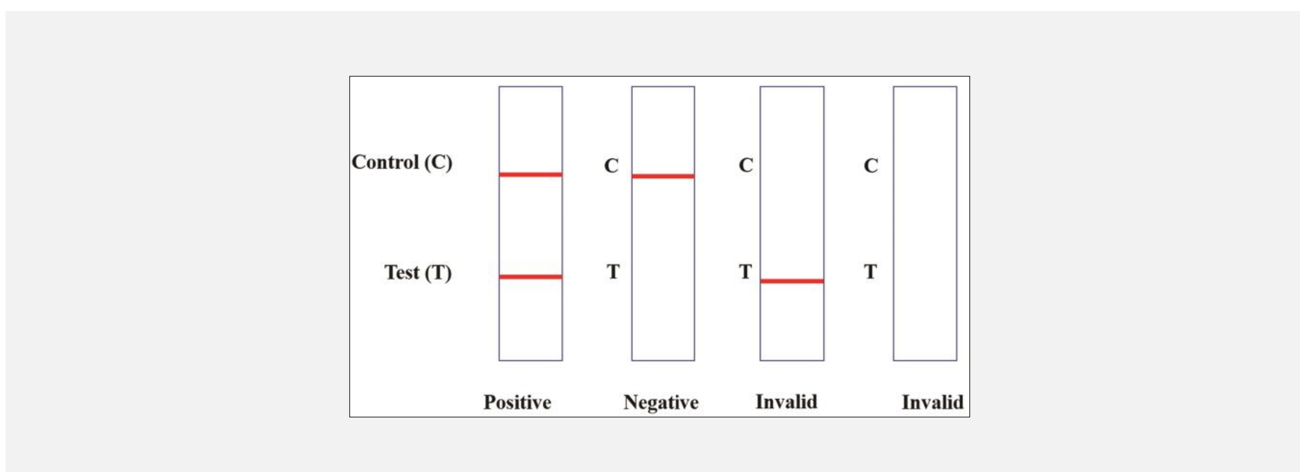

Figure 1. The interpretation of GICA test results.

Positive result - control line (C) and test line (T) appear. Negative result - Only one quality control line (C) appears and no test line (T) appears. Invalid result - No line or only one test line (T) indicates that the test is invalid and the sample needs to be tested again.

75/91 (82.4%) (Table 1).

RT-PCR results

81 samples of known COVID-19 patients were tested by RT-PCR, the results showed that 42 cases were positive, the sensitivity was 51.9% (42/81) (Table 1).

Effects of hospitalization duration on serum SARS-CoV-2 IgM + IgG antibody detection results

The hospitalization duration here is equal to the test date minus the admission date. Since the positive rate of ELISA is higher than that of GICA (87.3% vs. 82.4%), we only discuss the ELISA method here. Serum from

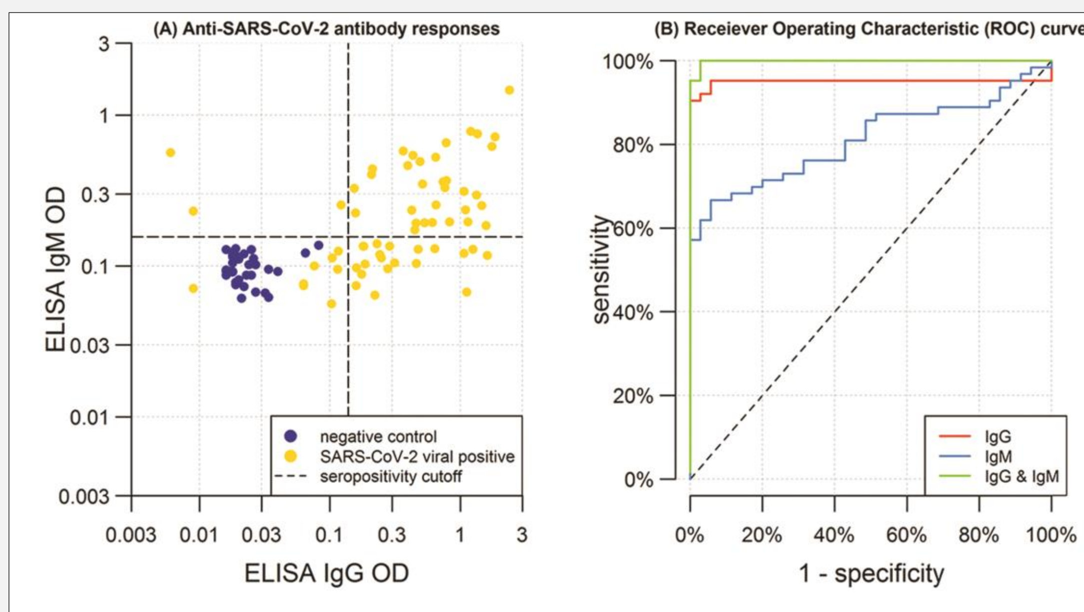

Figure 2. The results of 63 COVID-19 cases and 35 healthy control by ELISA.

(A) Anti-SARS-CoV-2 antibody response. According to negative control, IgM and IgG cut-off was set up as 0.15. (B) Receiver operating characteristic (ROC) curve. The greater the area under curve (AUC), the better the classification performance. $AUC_{(IgG \& IgM)} = 0.983$, $AUC_{(IgG)} = 0.934$, $AUC_{(IgM)} = 0.812$.

63 confirmed COVID 19 patients were divided into < 4 days, 4 - 8 days and > 8 days, according to the hospitalization duration. The positivity rate of each group was shown in Table 2. Fisher's exact test showed a difference in the positivity rate among the three groups ($p = 0.032$). The positivity rate of < 4 days group was significantly lower than that of > 8 days group ($p = 0.015$), but there was no significant difference between the positive rate of 4 - 8 days group and > 8 days group ($p = 0.25$). The median hospitalization duration was 4 days (interquartile range: 2 - 11).

Receiver Operating Characteristic (ROC) Curve evaluation of sensitivity and specificity

The cutoff ELISA OD levels for classifying sero-positive samples were 0.15 for IgG and IgM. In Figure 2 (A and B), we use receiver operating characteristic (ROC) curve analysis to evaluate the trade-off between sensitiviity and specificity achieved by varying the cutoff level. The area under the ROC curve for IgG ($AUC_{(IgG)}$) was 0.934. The area under the ROC curve for IgM ($AUC_{(IgM)}$) was 0.812. When the cutoff ELISA levels for IgG and IgM were fixed to be equal to each other, the area under the ROC curve for IgG + IgM ($AUC_{(IgG + IgM)}$) was 0.983. $AUC_{(IgG)} > AUC_{(IgM)}$ indicates that IgG has better classification performance than IgM. $AUC_{(IgG + IgM)} > AUC_{(IgG)}$ indicates that the combination of IgG and IgM has better classification performance than IgG alone.

DISCUSSION

Because SARS-CoV-2 is highly contagious, early detection of COVID-19 patients is important for quarantine and treatment. SARS-CoV-2 nucleic acid test false negatives have occurred in many clinical institutions, which has caused test results to be inconsistent with clinical symptoms and imaging examinations. This involves many factors such as sample collection and storage, RNA extraction methods, and the quality of nucleic acid detection kits [12,13]. Due to the high infectivity of SARS-CoV-2, medical staff often disinfect patient samples before testing them, which also increases the risk of occurrence of false negatives [14].

In order to reduce the high false negative rate of SARS-CoV-2 detected by RT-PCR, the National Health Commission of China has added IgM and IgG antibodies to confirm COVID-19. If SARS-CoV-2 IgG antibody changes from negative to positive or the recovery period is 4 times or more higher than that in the acute period, suspected cases can be used for case confirmation. A

suspect case can be excluded if the detection of the SARS-CoV-2 RT-PCR is negative for two consecutive times (at least 24 hours apart) and the IgM and IgG antibodies are still negative 7 days after the onset of the diagnosis. In this study, both ELISA and GICA used the rN of SARS-CoV-2 as the diagnostic antigen to detect IgM and IgG antibodies in human serum against SARS-CoV-2. In the results described above, the positivity rate of the combined IgM and IgG detection of ELISA and GICA were 55/63 (87.3%) and 75/91 (82.4%), respectively. While the positive rate of RT-PCR was only 42/81 (51.9%). As mentioned earlier, RT-PCR has a very high false negative rate. Therefore, we urgently need new detection methods to assist the diagnosis of COVID-19.

Studies have reported that bat severe acute respiratory syndrome-related coronavirus (SARSr-CoV) Rp3 NP was used as antigen for IgG and IgM ELISA test. It shares 92% amino acid identity to SARS-CoV-2 NP and showed no cross-reactivity against other human coronaviruses except SARSr-CoV [15]. Additionally, Dr. Du reported that SARS-CoV receptor-binding domain (RBD) specific antibody could cross-react with SARS-CoV-2 RDB protein and the anti-serum induced by SARS-CoV RBD could cross-neutralize SARS-CoV-2 [16]. Qian assessed cross-neutralization of SARS-CoV and SARS-CoV-2 using convalescent serum from SARS and COVID-19 patients and found moderate cross-neutralization activities between the two [17]. The genome of SARS-CoV-2 has the highest similarity with that of a SARS-like bat CoV but it is distant from and less related to the MERS-CoVs [18]. Therefore, cross reaction is theoretically possible in both ELISA and GICA. While our study found high levels of specificity, it is possible that there are too few healthy controls, or that being from the same city they are too similar. Given the fact that there are no more human SARS cases, we think that conventional ELISA and GICA may be used as a quick test for COVID-19.

ELISA and GICA for specific IgM and IgG antibodies are conventional serological assays. They can offer a high-throughput alternative, which allows for uniform tests for all suspected patients and avoid unnecessary cross infection. However, several investigators alluded that antibodies to SARS-CoV-2 appear 7 to 14 days after symptoms, so it is important to choose an appropriate time to collect specimens. During SARS-CoV-2 infection, a classical acute antibody response was initiated. Almost all patients became antibody positive and the titer increased rapidly. The median seroconversion time of IgM and IgG was 10 and 12 days after onset, respectively, and the antibody levels increased rapidly after 6 days [19]. In this study, all the patients were at the peak of COVID-19 outbreak, the number of infected patients is far too large and hospitals are oversaturated, causing many patients not to be treated immediately after infection. Most patients can only be quarantined at home waiting for medical resources. When they were admitted to hospital for treatment, they usually have symptoms for more than seven days or longer and most patients may not remember the exact date of onset of symptoms. Thus, the admission time is later than the onset time. A study on the characteristics of COVID-19 inpatients in Wuhan showed that the median time from symptoms to hospital admission was 7.0 days (interquartile range: 4 - 8) [3]. Our study showed that the median time from admission to antibody testing for COVID-19 patients was 4 days (interquartile range: 2 - 11). Thus, we deduced that the interval between the onset of symptoms and antibody detection in COVID-19 patients was about 11 days, which has exceeded the median seroconversion time of 10 days [19]. So, we believe that patients had already produced detectable antibodies when they were admitted.

The major limitation of serological diagnostics such as this, is the absence of detailed knowledge of viral and antibody kinetics in samples collected longitudinally from patients infected with or recovering from SARS-CoV-2. The exact timing during the infection, will influence the probability that serological tests are classified as positive.

CONCLUSION

Currently, RT-PCR detection is still the gold standard of SARS-CoV-2 and the serological detection cannot completely replace RT-PCR, but it can be used as an auxiliary test to evaluate the safety of recurrence and rework and investigate the epidemiology of infection in the broad Chinese population. Therefore, we believe that serological testing for SARS-CoV-2 is meaningful but there is a need to further increase the number of samples tested to verify it.

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Declaration of Interest:
None.

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