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Original Article

A serological assay to detect human SARS-CoV-2 antibodies

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الكلمات المفتاحية: الفحص المناعي المرتبط بالإنزيم؛ فيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة؛ الأجسام المضادة؛ التماثل للشفاء؛ الانتشار المصلي

الملخص

أهداف البحث: يعتبر فيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة، هو العامل المسبب لمرض فيروس كورونا-٢٠١٩، يمكن أن يؤدي إلى مرض تنفسي حاد. المرضى الذين يعانون من أمراض مصاحبة أساسية لديهم مخاطر عالية للإصابة بمرض فيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة. الفحوصات المصلية مطلوبة بشكل عاجل لتشخيص الحاملين للمرض من غير أعراض ظاهرة، وتقدير انتشار العدوى والوقاية من المرض ومكافحته. لذلك كان الهدف من هذه الدراسة هو تطوير الفحص المناعي المرتبط بالإنزيم (إليزا) للكشف عن وجود الأجسام المضادة الناتجة عن الإصابة بفيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة عند البشر.

طرق البحث: تم تصميم وتأسيس اختبار مصلي بطريق الإليزا للكشف عن الأجسام المضادة ضد فيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة حسكة البروتين من عينات المصل المأخوذة لمرضى فيروس كورونا-٢٠١٩ منومين تأكدت إصابتهم بالفيروس بواسطة تفاعل البلمرة المتسلسل. أُستخدم ٤٢ مصلا من مرضى تماثلوا للشفاء كعينات تحكم إيجابية، بينما استخدمت ١١٧عينة مصل لما قبل الجائحة كعينات تحكم سلبية.

النتائج: أظهرت مقارنة بين بروتينات مختلفة لفيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة أن الطول الكامل لحسكة البروتين كان لها أقوى تفاعل للأجسام المضادة لفيروس كورونا-٢ المسبب للمتلازمة التنفسية الحادة الوخيمة في عينات مصل المرضى بالمقارنة بالوحدات الفرعية ١ والوحدة ٢. بالإضافة لذلك، كانت المقايسات حساسة بحيث يمكن اكتشاف الأجسام المضادة بتركيزات منخفضة للغاية.

الاستنتاجات: كان الفحص الحالي محددا ولم يتفاعل مع فيروس كورونا سارس الآخر أو فيروسات الجهاز التنفسي الأخرى كفيروس الإنفلونزا. بالإضافة لذلك، كان لديه حساسية عالية وكان الاختبار له قدرة لتحديد الأجسام المضادة بتركيزات منخفضة للغاية. ويعتبر هذا الاختبار كطريقة فرز واعدة على مستوى السكان ولإجراء دراسات وبائية مصلية مستقبلية.

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Abstract

Objectives: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), can lead to severe respiratory illness. Patients with underlying comorbidities have a high risk of contracting COVID-19. Therefore, serological assays are urgently needed to diagnose asymptomatic carriers of SARS-CoV-2, to estimate the prevalence of infection, and for disease prevention and control. This study aimed to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-SARS-CoV-2 antibodies in humans.

Methods: An ELISA test was designed and established to detect antibodies against the SARS-CoV-2 spike protein in serum samples from 41 quantitative reverse transcription polymerase chain reaction (qRT-PCR) - positive hospitalised COVID-19 patients. Forty-two convalescent patients' sera served as positive controls, while 117 prepandemic serum samples were used as negative controls.

Results: A comparison between different SARS-CoV-2 proteins was performed, which included the full-length spike (S) protein and the S1 and S2 subunits. The fulllength S protein showed the strongest reactivity for anti-SARS-CoV-2 IgG antibodies in patients' serum samples. Additionally, since antibodies could be detected at very low concentrations, the assay was found to be sensitive.

Conclusion: The current assay was specific, since crossreactions with other SARS coronaviruses and respiratory viruses such as influenza were not found.

Additionally, it was highly sensitive, since the test was able to identify antibodies even at very low concentrations. Therefore, this assay has promise as a screening method at the population level and may be used for in future seroepidemiological studies.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can lead to serious illness, mostly in those with underlying health conditions.[1] This novel coronavirus, which was first identified in Wuhan, China at the end of 2019, rapidly spread worldwide, and on 11 March 2020 it was declared a pandemic by the WHO.[2] In KSA, the total confirmed number of COVID-19 cases was 344,785, with 331,330 recoveries and 5,296 deaths (https://covid19.moh.gov.sa/ accessed 25 Oct 2020). Almadinah Almunawwarah has recorded a total of 26,586 of these cases, with 25,572 recoveries, 874 active cases, and 140 deaths. These numbers suggest a recovery rate of more than 96% and a case fatality rate of approximately 0.53.

At present, the confirmatory diagnostic assay for SARS-CoV-2, which is an RNA virus, involves the amplification of viral RNA using reverse transcription-polymerase chain reaction (RT-PCR), which transcribes complementary DNA (cDNA) from the viral RNA template. The processing of samples from nasopharyngeal swabs requires a series of steps to purify the viral RNA,[3] which is then converted to cDNA using reverse transcriptase. Quantification of the amplified transcripts by quantitative RT-PCR (qRT-PCR) is performed using precise primer and probe groups for the amplified viral sequence. All these steps are time-consuming, laborious, and expensive.[4,5] Moreover, high false-negative rates have been reported due to colonization of several respiratory viruses in the lower respiratory tract.[6] There is also a high level of uncertainty regarding virus transmissibility and virulence.[2]

Serological assays are needed to complement nucleic acid-based molecular diagnostic tests. Several commercial rapid immunoassays are available but have high false-positive rates and consequently cannot be utilized to precisely estimate seroprevalence in populations with low levels of exposure to the virus.[7,8] The enzyme-linked immunosorbent assay (ELISA) is widely used in a broad spectrum of fields, including experimental research, diagnostics, and serologic surveillance.[9] The high sensitivity of this test permits the examination of a very small sample volume without a pre-treatment step.[10] The reliability of any immunological assay depends on the stable standardization of all reagents and measures used.[11] Since the principle mechanism of immunoassays relies on specific antigen—antibody reactions, the assays have been used globally for pharmacokinetic studies during drug monitoring.[12] The advantages of the ELISA as a screening assay are the simple process, high specificity and sensitivity, high productivity, rapidity (as the test can be run without sample pre-treatment), safety, and cost-effectiveness (as it uses low-cost reagents).[12,13] During the past few years, ELISA-based methods for the detection of antibodies to a variety of viruses have improved. Advances have been made as a result of recent progress in recombinant DNA technology, leading to growth in the choice of antibodies and probes as well as systems.[14]

Indirect ELISA for the serological detection of SARS-CoV-2 antibodies has been performed using a host of antigens such as nucleocapsids (N), receptor-binding domains, S1 subunits, and ectodomain of spike (S) glycoprotein[15,16] as well as the full-length spike protein.[17] In this study, an in-house ELISA was designed, developed, and extensively tested to detect human antibodies specific to SARS-CoV-2 in the Almadinah Almunawwarah region of KSA. The specificity of the ELISA was evident from the absence of cross-reactive antibodies to other SARS coronaviruses and other respiratory viruses such as influenza. Additionally, the assay using S protein as the capture antigen showed a high sensitivity for detecting low concentrations of anti-SARS-CoV-2 antibodies in human serum samples.

Materials and Methods

De-identified serum samples were obtained from the MOH hospital in the Almadinah Almunawwarah region. All participants provided written informed consent before the study. All the COVID-19 patients (aged 1—80 years) whose samples were used were collected between 24 April 2020 and 23 May 2020. The patients' sera were selected based on the onset of COVID-19 symptoms (8 to 20 d). A total of two hundred samples were used: forty-one RT-PCR confirmed COVID-19 patients and one hundred and seventeen pre-pandemic serum samples used as negative controls (NCs). Forty-two convalescent patients' sera were used as positive controls since commercial positive controls were not available at the time the assays were performed.

SARS-CoV-2 recombinant proteins

Recombinant SARS-CoV-2-S full length (cat no. 40589-V08B1), SARS-CoV-2-S1 (cat no. 40591-V08H), and SARS-CoV-2-S2 (cat no. 40590-V08B) proteins were all purchased from Sino Biological, USA.

Indirect enzyme-linked immunosorbent assay (ELISA)

The ELISA protocol was adapted from a previously established protocol with slight modifications.[18,19] To establish, develop, and assess in-house ELISA with selected SARS-CoV-2 proteins, the assay was performed using serial dilutions of different serum samples (n = 200), categorised as follows: 1) qRT-PCR-confirmed hospitalised COVID-19 patients (n = 41); 2) convalescent COVID-19 patients

(n = 42); and 3) archived samples from healthy volunteers taken 1 year prior to the pandemic (n = 117). The serum samples used to check the reactivity to the viral proteins were obtained from patients with a wide range of ages (1−80 years).

An indirect ELISA was designed and established to detect antibodies present in serum samples that were directed against SARS-CoV-2. A 96-well ELISA plate (Costar; Corning) was coated with SARS-CoV-2-S (full length), SARS-CoV-2-S1, and SARS-CoV-2-S2 recombinant proteins. All SARS recombinant proteins were reconstituted in phosphate-buffered saline (PBS, pH 7.2), then the plates were coated with 100 μl/well using different protein concentrations (0.5−4 μg/mL). The SARS-CoV-2 proteins were tested and adjusted to determine the optimal coating concentration. Plates were then covered with an adhesive seal and incubated overnight at 4 °C. Plates were washed 5 times with washing buffer (PBS containing 0.05% Tween-20, Sigma−Aldrich) using an automated microplate washer (Elx 50, Bio Tek).

The plates were then blocked with 150 μL/well of blocking buffer (PBS containing 0.05% heat-inactivated at 56 °C for 60 min foetal bovine serum (FBS); Sigma−Aldrich) for an hour at room temperature. Serum samples were then serially diluted to 1:100 using blocking buffer and added at 100 μL/well in triplicate. Plates were incubated for 30 min at room temperature and then washed 5 times with washing buffer.

Specific alkaline phosphatase-conjugated secondary antibodies, goat anti-human IgG (1:1000 in blocking buffer), and IgM (1:2000) (Sigma−Aldrich), were then added at 100 μl/well and incubated at room temperature for 30 min. Plates were then washed 5 times with washing buffer. Finally, 100 μL/well of the ready substrate p-nitrophenyl phosphate (p-NPP) (Sigma−Aldrich) was added. The plates were kept in the dark, away from direct light until the colour developed (30 min), and then the reaction was terminated by adding 100 μL/well of stopping solution (1.2 N sodium hydroxide, Reagecon, UK). Optical density (OD) at 405 nm was measured using an absorbance microplate reader (ELX800, BioTek). SARS-CoV-2 recombinant S proteins (S1 and S2 subunits) were used to compare the reactivity of the assay.

Cut-off calculation for ELISA

The sample was defined as ELISA antibody-positive if the OD_{405} value was three standard deviations (SD) above the mean of the NCs. The calculated cut-off OD_{405} value (mean NCs + 3 SD) was $0.19 + (3 \times 0.033) = 0.29$.

Background values were calculated while performing the assay to ensure that the ODs represented the actual antibody concentrations of the samples. The background value was deducted from all the sample readings' OD_{405} values before calculating the cut-off.

Statistical analysis

For the comparison between full-length S, S1, and S2 proteins, results from the serum samples' ELISA titers were displayed as a single point. A paired t-test was used, and the differences were considered significant at $p < 0.05$. The assay

endpoint was determined at different dilutions of patient sera. Data were analysed using GraphPad Prism 8 (GraphPad, San Diego, CA).

Results

Comparison of different SARS-CoV-2 recombinant proteins as capture antigens

The differences in the reactivity of the recombinant viral S proteins (S, S1, and S2) of SARS-CoV-2 to RT-PCR-confirmed COVID-19 patient sera were compared. The recombinant proteins were used at 2 μg/mL and the serum samples were serially diluted from 1:25 to 1:6400. As shown in Figure 1, the full-length S protein had the strongest
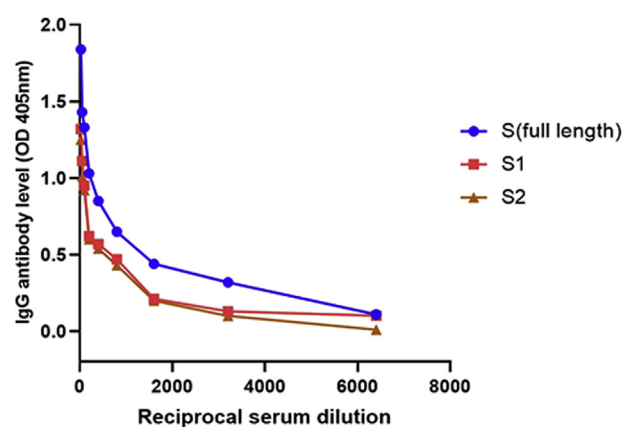

Figure 1: Optimization of the SARS-CoV-2-S antigen concentration. The sera from four COVID-19 patients and negative controls (NCs) at 1:100 dilution were tested against decreasing concentrations of the SARS-CoV-2 spike protein (4 μg/ml to 0.5 μg/ml) for IgG and IgM reactivity.
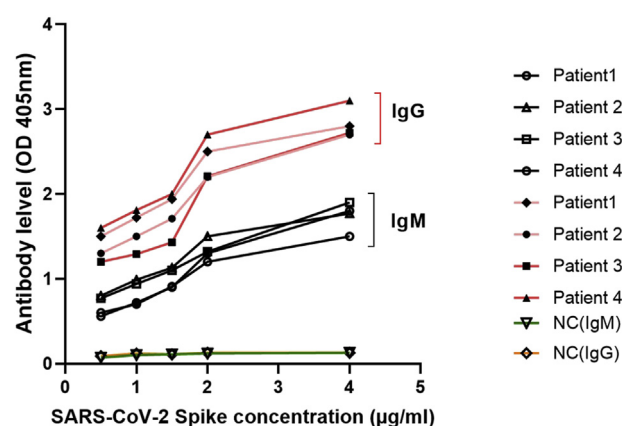

Figure 2: Differences in reactivity between the SARS-CoV-2 proteins as capture antigens. Microtiter plates were coated overnight with 2 μg/ml of viral antigens (S [full length], S1, and S2), and patient serum samples were used at dilutions ranging from 1:100 to 1:6400. Indirect ELISA was performed as described in the Methods. The full-length S protein showed significantly higher reactivity than S1 and S2 ($p < 0.01$).
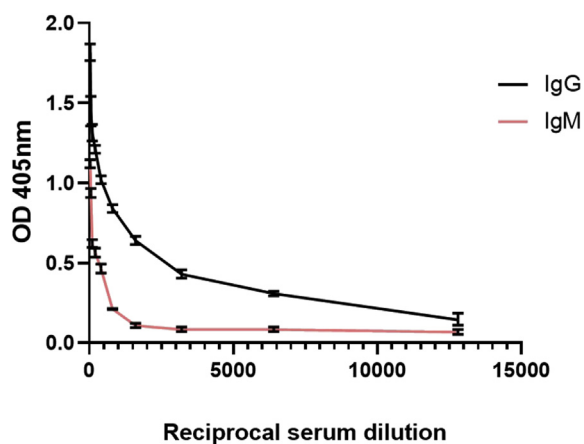
Figure 3: Identification of the endpoint for sensitivity of the in-house ELISA. The endpoint was determined using patient sera at different dilutions and occurred at 1:6400 and 1:800 for IgG and IgM, respectively.

reactivity and showed significantly higher ($p < 0.01$) levels of anti-SARS-CoV-2 IgG than S1 and S2 (median OD_{405}: S = 0.85, S1 = 0.57, S2 = 0.54).

Optimization of SARS-CoV-2 spike protein coating concentration

IgG reactivity was tested to decrease the concentrations of the SARS-CoV-2-S protein (4 μg/mL to 0.5 μg/mL) against 1:100 diluted serum samples from four COVID-19 patients. As is evident from Figure 2, the highest OD obtained was at 4 μg/mL. The results also indicated a higher level of IgG antibodies against the SARS-CoV-2 spike protein than IgM at all serial serum dilutions (Figure 2). The samples taken from the pre-pandemic NCs showed sustained baseline levels of both IgG and IgM. The conjugate concentration was also varied and tested (data not shown).
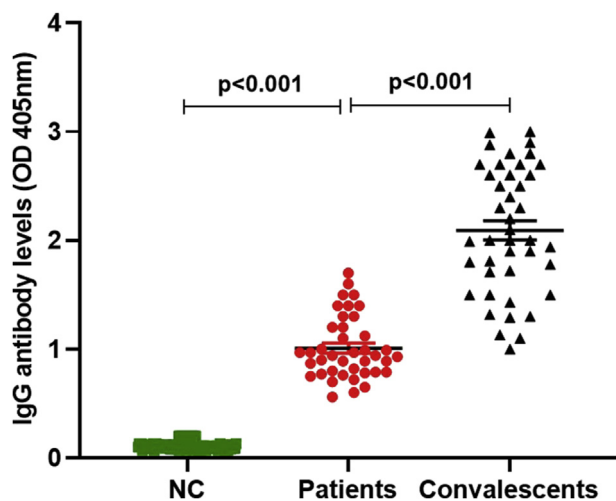

Figure 4: Comparison between the serum samples of the COVID-19 patients (n = 41), convalescents (n = 42), and pre-pandemic NCs (n = 117). Convalescents sera showed significantly higher antibody levels compared with patients' sera (mean ± SEM, $p < 0.001$).

Calculation of the test endpoint

The background reactivity was assigned, and the background OD_{405} value was set to 0.12. Additionally, to ascertain the sensitivity of the in-house ELISA, the endpoint titers were calculated as the last dilution before which the reactivity decreased below an OD_{405} of 0.12. Hence, the endpoint was obtained when the serum dilution reached 1:6400 for IgG and 1:800 for IgM (Figure 3).

Additionally, all seropositive samples were confirmed using a chemiluminescent microparticle immunoassay with a sensitivity of 99.99%, which showed similar results at more than 98% (data not shown). The validity of the assay was also confirmed by the distinct OD values obtained for the patients (n = 41) and NC (n = 117), while the convalescent sera (n = 42) showed higher IgG reactivity and significant antibody levels when compared with patients' sera ($p < 0.001$) (Figure 4).

Discussion

Serological analyses are useful for identifying any previous exposure to SARS-CoV-2 in individuals with undetectable levels of the virus on qRT-PCR and for distinguishing asymptomatic individuals. The current ELISA was standardised by measuring the background reading and using pooled convalescent sera from the recovered patients as positive controls. The assay background was defined as the reading acquired from the detection system in the absence of any test sera and had to be lower than the reading from any serum ODs. Additionally, to confirm that the antigen was working, serial dilutions of a positive control had to be used for each assay.[20] The positive control was also used to monitor plate-to-plate variation in OD. The current assay showed background readings that were close to zero. As expected, all sample readings were lower than the positive control. A titration curve was established to assess the reproducibility of the protein coating and to exclude nonspecific reactions.

The results were considered positive if the cut-off was equal to or above the mean + 3 SDs of the NC sera. All the OD readings of the patients' sera for both IgM and IgG isotypes were above the calculated cut-off.

Several pre-pandemic samples that had been previously confirmed by RT-PCR and ELISA to have viral RNA and IgG antibodies for influenza A and B, respectively, were used to provide increased reassurance of specificity. Hence, the pre-pandemic sera were used as NCs and as a check for specificity, and no reactivity was observed (data not shown).

Several coronaviruses, such as NL63, 229E, and NL63, circulate within the population and are responsible for a high percentage of common colds.[21] Therefore, the potential for these antibodies to be present is high; however, none of the control sera showed any reactivity (data not shown). This result is supported by the results of another study that showed the same trend using convalescent sera from recovered patients who were positive for several coronaviruses.[22]

The current assay was developed to detect seroconversion resulting from SARS-CoV-2 exposure. It was designed to produce reactivity to the dominant and virulent spike protein of

the virus. It is straightforward and rapid and can be performed at any biosafety level 1 laboratory. The use of samples from individuals with a wide range of ages (1–80 years) was intentional. Older individuals are likely to have had previous human coronavirus infections, which is used to verify the specificity of the assay against common coronaviruses. The results in this study are similar to those of recent studies, which found that sera from subjects reacted well to spike proteins from human coronaviruses but not to SARS-CoV-2 directly.[23,24]

Seroconversion is correlated with the shedding of an infectious virus to undetectable levels. Infectious viruses are very difficult to harvest from naso/oropharyngeal samples when patients have high levels of serum neutralising antibodies. Therefore, serological assays are being trialled to eradicate or reduce infections.[3] Moreover, anti-SARS-CoV-2 IgG antibodies in the serum of convalescent patients could neutralise viral activity in a pseudotype entry assay. Therefore, the presence of neutralising antibodies against the lethal SARS-CoV-2 in recovered individuals is presumed to be protective against re-infection.[25]

These findings provide support for other studies that have reported the detection of SARS-CoV-2-specific IgG and IgM antibodies in RT-PCR-confirmed COVID-19 samples with no cross-reactivity with serum sero-positive to other human coronaviruses. Likewise, all NC samples in this study were below the calculated cut-off values, suggesting high specificity of the assay, as has also been corroborated by others.[15] However, Algaissi et al. used S1 and N proteins as capture antigens, unlike the full-length S protein that was used in the current study. Recombinant N and S proteins have also been evaluated in the serodiagnosis of COVID-19 patients, to complement the RT-PCR based assays.[16,26] In line with the current study, robust IgG response has been reported by Prince et al. (2020) targeting S and N proteins, although rare false-positive results have been observed.[27]

The full-length S protein showed a strong reactivity to IgG antibodies from the sera of COVID-19 patients that was stronger than that observed with S1 and S2. This could indicate the presence of a greater quantity of epitopes on the considerably larger full-length S protein than those in S1 and S2. Therefore, the full-length S protein should be used in screening for seroconversion of a population by detecting antibodies against the virus, as has also been observed previously.[17] Moreover, the detection of antibodies against the full-length S protein is generally linked with previous exposure to the virus, regardless of the patient's status.

However, in another study with four recombinant antigens, including the full-length S protein, some less severe COVID-19 patients remained seronegative despite the high sensitivity of the ELISA.[16]

Although Middle East Respiratory Syndrome coronavirus (MERS-CoV) is of the same genus as SARS-CoV-2, no reactivity was observed when archived patients were used in the current assay, which reflects a lack of cross-reactivity between the two viruses (data not shown). This result is in agreement with a previous study with a similar finding.[22]

One strength of this study was the ELISA format, in which the alkaline phosphatase enzyme is conjugated with the secondary antibody that attaches to the serum immunoglobulins. Furthermore, the very strict cut-off (3 SDs above the mean of the NC) in the current assay reduced the detection of cross-reactive antibodies in the population and decreased false-positive results. While the study's main aim was not to evaluate serology as a diagnostic test, the assay showed early recognition of SARS-CoV-2-specific antibodies in the progression of infection and was consistently positive for several weeks after the start of the infection.

Some limitations of the study were the absence (at this time) of an extended follow-up of these patients to define the length of antibody response. Likewise, while representing serological responses in PCR-confirmed patients, this assay needs to be trialled in asymptomatic infections to determine how frequently such infections produce a detectable antibody response.

Overall, this assay is suitable for performing studies in the population to draw a precise and full picture of seroconversion. Although this assay does not specifically detect neutralising antibodies, it may be useful to estimate the immunity to the virus in the community as well as to measure disease prevalence.

Conclusion

The current study demonstrated the design and development of an inexpensive in-house ELISA using a recombinant SARS-CoV-2 spike protein. The assay may provide insight into the seroprevalence status of COVID-19, which would be a valuable tool for healthcare decision-makers in disease prevention and control at the population level.

Serological assays are key to determining SARS-CoV-2 exposure and the correlates of protection. The current assay was specific, and cross-reaction with other SARS coronaviruses or respiratory viruses such as influenza was not found. Additionally, it had a high sensitivity, detecting very low antibody concentrations. Therefore, this assay shows promise as a screening method at the population level.

Recommendations

The author recommends further studies be conducted to test other body fluids (i.e., saliva) and to measure additional antibody isotypes such as IgA and other IgG subclasses.

Source of funding

Conflict of interest

The author has no conflict of interest to declare.

Ethical approval

I declare that this manuscript is original, it has never been published, and is not under consideration for publication elsewhere (in part or in whole). This original article was conducted after obtaining ethical approval from the General Directorate of Health Affairs in Almadinah Almunawwarah

(MOH) head committee (**IRB no: H-03-M-084**). IRB no: 496 on 11-05-2020.

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