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Original Article

AuNP Coupled Rapid Flow-Through Dot-Blot Immuno-Assay for COVID-19 (SARS-CoV-2) Explicit IgG Antibody Revelation

Short Title: Dot-Blot Immuno-Assay for COVID-19.

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Abstract

Background: Flow-through dot-blot assay (FT-DBA) for SARS-CoV-2 specific IgG detection will provide a reliable and affordable immunoassay for the rapid serosurveillance against COVID-19.

Method: SARS-CoV-2 antigens were immobilized on nitrocellulose membrane to capture IgG immunoglobulins, which were then detected with AuNP anti-human IgG. A total of 181 samples were characterized with in-house and commercial immunoassay. The positive panel consisted of RT-PCR positive samples from patients with both <14 days and >14 days from the onset of symptoms, while the negative panel contained samples collected either from the pre-pandemic era dengue patients from healthy donors during the pandemic period.

Results: In-house ELISA selected a total of 79 true seropositive and 100 seronegative samples. The sensitivity of samples with <14 days using FT-DBA was 94.7% which increased to 100% for samples >14 days. The overall detection sensitivity and specificity were 98.8% and 98%, respectively, whereas the overall PPV and NPV were 97.6% and 99%. Moreover, comparative analysis between ELISA and FT-DBA revealed clinical agreement of Cohen's Kappa value of 0.944.

Conclusion: The assay can confirm past SARS-CoV-2 infection with high accuracy within 2 minutes compared to ELISA. It can help track SARS-CoV-2 disease progression, population screening, and vaccination response.

Keywords: COVID-19, SARS-CoV-2, Serological test, Sensitivity, Specificity, Serosurveillance.

Introduction

A newly discovered coronavirus named SARS-CoV-2 triggered the pneumonia outbreak in China's Hubei province in December 2019. [1] WHO termed the infection COVID-19 (coronavirus disease 2019), which has now spread beyond China and has become a full-blown pandemic. [2] To combat the virus's spread, affected countries have adopted numerous public health measures such as isolation, quarantine, regional lockdown, social distancing, restriction on public movement, and local and international traveling. Despite these preventive measures, the disease is surging across countries, with more than 113 million confirmed cases with 2.76 M deaths to date (26th March 2021). [3]

SARS-CoV-2 has a higher transmission rate than the previous two coronaviruses: SARS-CoV and MERS-CoV. [4] Research is going on therapeutics and vaccination, but no specific treatment option is available yet. [5] Early diagnosis is critical for successfully containing the disease. [6] Thus, diagnostic tests had become essential for combating COVID-19. FDA is issuing emergency use authorization (EUA) for different categories of *in vitro* diagnostic tests to increase testing capacity. [7] While nucleic acid and antigen-based tests can detect active infection, low viral load and variation in test sensitivity

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issues increase the threat of false-negative results, limiting their use. [8, 9] Many COVID-19 victims are asymptomatic with a viral load lower than symptomatic individuals. [10] This hinders the usefulness of these tests and makes epidemiological evaluation of the disease complex. [11] Moreover, amidst mass COVID-19 vaccination, it is essential to understand the antibody dynamics for COVID-19 eradication. [12, 13]

WHO recommends systemic serosurveys to understand the whole disease spectrum and many countries have already taken the initiative. [12, 14-18] Other than assessing risk and prevalence, serology testing is vital for contact tracing**,** the understanding immune response against the virus, identifying potential plasma donor, in some instances to be used in adjunct with a molecular diagnosis, and last but not the least to check the success of vaccination program in place. [13, 19-22]

The widely used tests for serology-based diagnosis of SARS-CoV-2 include enzyme-linked immunosorbent assay (ELISA), automated chemiluminescence assay (CLIA), neutralization assay, and rapid immunoassay. [19] Except for rapid tests, all assay systems require sophisticated instruments, longer testing time, and qualified staff to conduct the tests and interpret the results, which renders these assays inadequate when a large number of testing are required immediately. [23, 24] This calls for an urgent need for easy and affordable rapid point-of-care testing (POCT). The present study reports developing such a rapid POCT antibody test based on membrane immune-concentration flow-through principle for SARS-CoV-2 specific IgG detection. There are numerous reports on rapid lateral flow immunochromatographic assay systems for SARS-CoV-2 antibody detection. [7, 25-28] However, a rapid test based on immune-concentration procedure has not been reported yet. Flow-through system offers enhanced sensitivity and faster assay time than LFIA. Moreover, LFIA systems have the possibility of the false-negative signal depending on the immobilized analyte concentration, which is absent in the flow-through system. [29, 30] Among numerous reporter molecules, colloidal gold nanoparticles (AuNP) have a wide range of applications due to their remarkable biocompatibility. [31-33] AuNP-based probes are exceptional for their simplicity and high contrast visualization when coupled with a rapid immunoassay system. [34-36]

This study reports developing and validating a rapid dot-blot serological assay to detect SARS-CoV-2 specific IgG in human serum using characterized seropositive and seronegative samples. Moreover, when compared with nucleocapsid (NCP) or receptor-binding domain (RBD) ELISA specific for SARS-CoV-2, an additive sensitivity was observed in our assay system.

Materials and Method

Design Concept

Rapid flow-through dot-blot immunoassay (FT-DBA) is a qualitative immunoassay to detect the presence of SARS-CoV-2 specific IgG antibodies in human serum. It utilizes the solid phase capture technique in a membrane immune-concentration flow-through system (Figure 1). The test device is a plastic cassette that contains a combo made up of an absorbent pad with a nitrocellulose (NC) membrane with its active side on top, visible through a circular window. The test media (active side of the NC membrane) has

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two adjacent dots, designated T (test) and C (control) position to indicate test result and test kit validity, respectively. A mixture of SARS-CoV-2 antigens is immobilized on the test dot, and the control dot contains immobilized mouse IgG. The SARS-CoV-2 specific antibody, if present in the serum, is captured on the NC test dot, which becomes visible after the addition of AuNP conjugate.

Reagents and Material

SARS-CoV and SARS-CoV-2 specific nucleocapsid (NCP), envelope (E), spike S1, spike S2, and receptor-binding domain (RBD) recombinant proteins were purchased from The Native Antigen (UK, Kidlington), MP Biomedicals (California, USA), Sino Biological (China), Fapon Biotech Inc. (China), and Creative Diagnostics (USA). Gold colloids (particle size: 10nm and 40nm) were purchased from Bhat Biotech Ltd. (India) and BBI Solutions (UK). PBS (Phosphate-buffered saline) tablets (pH 7.4), Tris-Buffered Saline (TBS) pH 7.2, glycerol, and sodium chloride (NaCl) were purchased from Thermo Fisher Scientific (USA). Cold-water gelatin was purchased from Sigma-Aldrich. Gold dilution and stabilization buffer (Bhat Biotech Ltd.), mouse IgG antibody (Fapon Biotech), goat-anti-mouse IgG (Fapon Biotech) and, goat-anti-human IgG (Fapon Biotech) were also purchased. Nitrocellulose membrane**s** were purchased from Ken Biotech (China), Bhat Biotech Ltd. (India), and Sartorius (France) to determine the optimal support matrix for the immunoassay. Other materials (such as plastic cassettes) were purchased from Bhat Biotech (India) and Changzhou Sengfeng (China).

Optimization of AuNP Conjugate

Gold nanoparticles have already been accepted as a remarkable diagnostic tool worldwide. [37] Therefore, the research team employed AuNP conjugated with anti-human IgG to detect SARS-CoV-2 specific IgG in human serum. Anti-human IgG-AuNP and anti-mouse IgG-AuNP conjugates were prepared according to the protocol described by Oliver C. (2010). [38] Briefly, two different sized (10 nm and 40 nm) gold colloids were evaluated and to determine the optimal concentration of both proteins for conjugation, aliquots of the anti-human IgG/anti-mouse IgG solutions (5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml) in phosphate-buffered saline (PBS) were prepared. 1 ml of gold colloid solution was added to each aliquot, and the tubes were incubated for 15 mins at room temperature. The minimum amount of anti-human IgG/anti-mouse IgG required to stabilize the conjugates was determined by assessing color change and agglomeration. The conjugates were then stabilized using 1% cold-water gelatin. Excess antibodies were removed using glycerol gradient, and the conjugates were dialyzed against Tris-buffered saline (TBS) for 1 hr at room temperature. The final preparation was diluted with TBS and 1% cold-water gelatin and stored at 4 °C.

Selection of Control Samples for Assay Development

In this assay's development and optimization, two SARS-CoV-2 positives and two negative control sera were utilized. Clinical symptoms, RT-PCR confirmation, serostatus verification with in-house and commercial chemiluminescence assay (ROCHE, Elecsys Anti SARS-CoV-2) were considered control selection criteria. [39] Besides, the antibody kinetics of positive control individuals were studied longitudinally to avoid spectrum bias. [40]

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Specimen for Clinical Validation

The clinical performance of rapid FT-DBA has been evaluated with three panels of serum samples (n=181). Two sera panels comprise single and multiple collections of SARS-CoV-2 positive serum samples (n=81) from forty-five RT-PCR confirmed individuals with clinical signs and symptoms of COVID-19. Panel 1 consists of twenty RT-PCR positive samples that have been collected within two weeks from the onset of symptoms. Panel 2 (n=61) samples were also from RT-PCR positive individuals with symptom onset of >14 days. Panel 3 samples were negative samples (n=100) collected during i) pre-pandemic era from healthy donors, ii) April to June 2020 from RT-PCR negative individual, and iii) before the outbreak from positive dengue patients (n=24). The panels were characterized with SARS-CoV-2 IgG ELISA described by Sil *et al.* against SARS-CoV-2 antigens: NCP and RBD [39, 41]. Comparative analyses were carried out with these samples, between in-house ELISA assays and kit developed in this work. Moreover, according to FDA guidelines, the seropositive and seronegative samples based on in-house ELISA results were tested with the developed assay. All the samples were stored at -80 °C until further use.

Assay Development

At the development phase of the assay, each component and steps were optimized and screened at first. SARS-CoV-2 recombinant antigens: NCP, E, S1, S2, and RBD proteins were utilized as the potential capturing agent. 6 different cocktail preparations (antigen dilution ranged from 1:10 to 1:800) from 16 antigens were analyzed. The combination generating the highest signal without cross-reaction was immobilized as a test dot. To avoid blocking of nitrocellulose membrane, sample processing steps were optimized by diluting samples to 1:2, 1:4, and 1:8 in the commercial buffer (Bhat Biotech Ltd., India).

Assay Procedure

The untreated serum sample was thawed at 37 °C before starting the test procedure. During testing, the sample was diluted with 2-3 drops (50-75 µl) of dilution buffer. 50 µl of diluted serum was then added to test media following two drops of (50µl) of wash buffer. The addition of one drop of AuNP conjugate mixture, followed by two drops of (50µl) of wash buffer, completed the test. Development of control dot attests to the fact that the device is working correctly and presence or absence of test dot specifies positive or negative result. Results were interpreted as shown in Figure 2.

Intensity Scale Generation

An intensity scale was developed for the semi-quantitative determination of the detection limit (LOD) of the rapid immunoassay. One positive control serum with a high antibody titer was selected based on reference ELISA value. [39] Two-fold serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32) of the selected serum were prepared in standard negative serum and run through the assay. A five-point gradient scale was generated for semi-quantitative detection of IgG in the sample.

Internal validation

Rapid dot-blot assay performance validation was designed to determine its clinical efficiency. The rapid assay's performance was analyzed with the selected samples (n=181) and characterized seropositive and seronegative samples. The selected specimens' serostatus was first characterized with an established in-house ELISA test against SARS-CoV-2 recombinant NCP and RBD antigens. [39, 42] All the samples were then tested with the rapid dot-blot assay to evaluate their performance.

Co-efficient of variation

The coefficient of variation (CV) demonstrates test reproducibility and precision. The intra-assay and inter-assay variations were tested, with five replicates of two positive serum samples on the same day and in 15 different days for later. The coefficient of variation was determined using the following formula.

$$Coefficient\ of\ variation\ (CV) = \left(\frac{Standard\ Deviation}{Mean}\right) X100\%$$

Data Analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and area under the curve (AOC) with 95% confidence interval were estimated to see the effectiveness of this rapid dot blot assay. The calculation was done using a 2×2 table format with the formula shown in Table 1. The analysis was performed with STATA 13 (StataCorp, LP, College Station, Texas, USA), and GraphPad Prism 8.3 was used for graphical presentation.

Justification of Intensity Scaling with ELISA

To justify the intensity scale, the mean difference of the Ratio (OD/cut-off IgG) with the different intensity scale was assessed by an independent sample t-test.

Results

Screening and optimization of test components

Test performance of rapid dot-blot mainly depends upon the appropriate combination of four major factors: coating agent concentration, nitrocellulose membrane blocking effect, sample processing, and sample volume. Different combinations of SARS-CoV and SARS-CoV-2 antigens were evaluated as capture antigens. Our results showed that the combo which contained *E. coli* derived envelope and S2 proteins cross-reacted with negative controls (Data not shown). Nevertheless, the combination containing human cell line derived NCP and RBD showed the best results with good sensitivity and specificity with the controls. Henceforth, a combo containing 1:10 dilution of NCP and RBD proteins (Sino Biologicals) was selected to be used as test dot immobilized on NCM.

Moreover, a well-characterized mouse IgG (Fapon) was immobilized as a control dot on NCM. Direct use of sample without any processing blocked the NCM, which interfered with result interpretation. Sample processing steps were optimized to avoid blocking NCM. Two-fold dilution was finalized among three dilution dilutions, and 50µl of diluted samples were used for testing. Moreover, AuNP conjugate prepared from 10

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nm gold colloid gave a better resolving background than 40 nm. Thus, further evaluation conjugates of 10 nm gold colloids were chosen.

Semi-Quantitative LOD Determination

A two-fold serial diluted sample (P-1) was evaluated, and a scale was generated (Figure 3). Based on the results we found in our experiment, a range of 0.5 to 3 plus scale was considered positive for semi-quantitative differentiation of the dot-blot result, whereas negative was considered the absence of antibody.

Sample selection and characterization through ELISA

As a combination of NCP and RBD was used as capture antigen in the test platform, the serum samples of SARS-CoV-2 RT-PCR confirmed patients (n=81), dengue positive patients (n=24), and healthy donors (n=76) were first characterized using IgG ELISA test against these two SARS-CoV-2 immunogens on previously developed in-house ELISA [39, 42]. Data analysis showed that the serum of COVID-19 infected individuals had a different antibody titer range (Figure 4). Three patterns of antibody response were observed among positive patients with blood collection in the first two weeks of infection: i) 16 patients mounted IgG response against both SARS-CoV-2 proteins and two were entirely negative, ii) two patients achieved only anti-NCP IgG, and iii) two patients mounted an only anti-RBD response. In the second group, all the patients are seropositive for both SARS-CoV-2 antigens except for two patients; one did not develop NCP-IgG, but RBD-IgG for other vice-versa result was observed (Figure 4). In the negative serum group, only one sample from the dengue panel showed cross-reactivity to SARS-CoV-2 RBD protein (Figure 4A), but no reaction against NCP (Figure 4B); all the other serums were negative for both SARS-CoV-2 antigens. Both the OD/cut-off of RBD (Figure 4A) and NCP (Figure 4B) of the positive cases at <14 and >14 days showed highly significant ($p<0.001$) with the participants who were negative against SARS-CoV-2. Besides this, the OD/cut-off between <14 and >14 days of RBD and NCP also showed a significant difference.

Detection of Sensitivity and Specificity of the Rapid Dot-blot Kit

The rapid dot blot assay's performance efficiency was evaluated with the three chosen sample panels of serum (n=181). The assay detected SARS-CoV-2 infection in patients with symptoms less than 14 days with 85.0% (62.1%, 96.8%) sensitivity which increased to 100% (94.1%, 100.0%) after 14 days and the Cohen's Kappa test agreement was 81% (Kappa=0.814; $p<0.001$) and 95% (Kappa=0.948; $p<0.001$), respectively (Table 2). Detection specificity was 98.0% (93.0%, 99.8%). The positive predictive value was 89.5% (66.9%, 98.7%) and 96.8% (89.0%, 100%) for <14 days and >14 days, respectively. Negative predictive agreements of the assay were 97.0% (91.6%, 99.4%) and 100% (96.3%, 100%) for both panels (<14 and >14 days) (Table 3). To check the specificity and cross-reactivity we have run 76 and 24 sera from healthy donors and dengue positive samples, respectively. Among them only 2 samples were misdiagnosed and the overall specificity was found 98.0% (95% CI: 93.0%, 99.8%) (Table 2). The overall sensitivity was noted 96.3% with a PPV and NPV of 97.5% and 97.0%, respectively. The overall test agreement was 94.4% (Kappa=0.944; $p<0.001$).

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Moreover, when the Dot-blot assay kit was evaluated with in-house ELISA characterized seropositive and seronegative sera, the sensitivity and specificity in less than 14 days samples were 94.7% (95% CI; 94.7%, 99.9%) and 98.0% (95% CI; 93.0%, 99.8%), respectively, with 94.4% PPV and 98.8% NPV. As expected, the values increased for the samples collected more than 14 days of symptom onset, and sensitivity and specificity were 100% (95% CI; 94.1%, 100.0%) And 98.0%, respectively. The PPV and NPV for this phase were 96.8% and 100%, respectively. The overall sensitivity and specificity were calculated it was found to be 98.8%(95% CI; 93.3%, 100%)and 98%, respectively, with 97.6% PPV and 99.0% NPV (Table 4, and 5)

Dot-blot Assay Can Semi-quantitatively Reveal Antibody Titer

When compared with reference ELISA values, it was revealed that the FT-DBA could detect SARS-CoV-2 specific IgG antibodies in human serum even when OD/Cut-off ratio was meager. Linear regression model between the intensity scale and IgG cut-off of RBD and NCP showed significant difference.

The highest intensity 3+ had the highest IgG (Cut off) for RBD (7.37±2.18) and NCP (5.78±2.44), which showed significant differences with the intensity scale of 2+ (p= 0.001 and 0.003), respectively. Similarly, the intensity scale of 2+ showed significant differences with the intensity scale of 1+ of RBD and NCP (p=0.003 and 0.029), respectively. Whereas a significant difference (p=0.030) was noted in RBD between 1+ and 0.5+ scales. No such difference was found in NCP (Figure 5). Mean ELISA values in RBD and NCP corresponding to each intensity scale are listed in the supplementary Table 1 and 2.

Co-Efficient Variation

Our analysis showed no intra assay variation in the assay, but a 9.98% coefficient of variance was found in the inter-assay for the serum samples used.

Discussion

Rapid dot-blot has been a valuable tool in diagnosing and epidemiological study of viral diseases. [41, 43, 44] The current demand for SARS-CoV-2 antibody detection rapid testing systems primarily consists of lateral flow immunoassay (LFIA) with various output ranges, but the use of flow-through dot-blot based approach is not yet widely available. [26, 27] Present research includes developing and evaluating a rapid flow-through dot-blot assay (FT-DBA) to detect SARS-CoV-2 specific IgG immunoglobulins in human serum. The Dot-blot principle provides a more reliable field testing framework than LFIA, where the later has usual limitations, including mass transport limitation, binding kinetics of immunogen, and competitive inhibition of target analyte (Figure 6). [30]

Nucleocapsid and spike, for their respective role in viral pathogenesis and entry into a host cell, are considered the two most crucial target immunogens of SARS-CoV-2, and the receptor-binding domain (RBD) of the spike is more advantageous for having the potential to induce neutralizing antibodies. [45] Typically, an immune reaction to NCP evolves earlier than RBD, but exceptions have also been reported. [42] In that context,

for increased sensitivity in the acute phase of infection, the developed assay was designed with dual immune capture property instead of one, which might fail to identify a seroconverted person. [46, 47] Another issue considered was the possibility of cross-reaction with other alpha and beta-coronaviruses surrounding the use of the SARS-CoV-2 serological test. The two human coronaviruses SARS-CoV and MERS-CoV, tend to pose the greatest likelihood of cross-reaction. Due to the high degree of sequence identity, SARS-CoV antigens are known to cross-neutralize SARS-CoV-2. [1, 48] However, it has been seventeen long years since the SARS-CoV epidemic, and specific antibody response against the virus has been reported to be diminished (90%). [49] A false-positive reaction at this point is an unlikely event. MERS-CoV is still active in the population along with the four endemic low pathogenic human CoVs (229E-CoV, NL63-CoV, OC43-CoV, and HKU1-CoV), so most humans might bear antibody against them. [50, 51] Nevertheless, their cross-reactivity against SARS-CoV-2 nucleoprotein and RBD protein is very low. Except for SARS-CoV, other human coronaviruses do not have any sequence resemblance to RBD and S1 domain of spike protein of SARS-CoV-2. [52]

Moreover, the assay was designed to take into account the dengue-endemic situation of Bangladesh. Since both diseases have common characteristics in the early phase and there has been a report of antigenic cross-reactivity between SARS-CoV-2 and dengue virus (DENV), there is a chance of misdiagnosis. [53] To avoid the risk, 24 pre-pandemic dengue positive serum was incorporated into the evaluation panel. Another strengthening point of the assay is that it was developed using SARS-CoV-2 positive serums studied longitudinally for antibody response in one of our previous studies, thus eliminating any chance of spectrum bias. [40, 54]

Laboratory evaluation revealed that when challenged with RT-PCR positive or RT-PCR positive and seropositive samples, the developed assay's overall sensitivity was 96.3% (Table-2) for the former but increased to 98.8% (Table 4) for the latter group. The difference was that two RT-PCR positive samples were never seroconverted in our study, reported by others. [55] A comparative study conducted between conventional ELISA and developed assay revealed a high correlation, which others can find. [56] Dot intensity seemed to increase with corresponding ELISA value upon disease progression (Figure 5, Supplementary Tables 1 and 2). Statistical analysis revealed equivalent clinical agreement between the two techniques as well as with the gold-standard method of RT-PCR, with a Cohen's Kappa value of 0.84 (strong agreement) and 0.94 (robust agreement), respectively, in between < 14 and >14 days (Table 2). The similar significant difference was observed between the results of two-time points in both assay techniques (Table 2). Moreover, a slightly increased sensitivity was observed in the dot assay compared to the two ELISA, which might have an additive effect of using two proteins instead of one (Table 2).

Considering all these, the assay described in the present study can be considered a more feasible option for serosurveillance study than conventional ELISA, especially for low- and middle-income countries (LMICs) like Bangladesh. Moreover, with the starting of current vaccination programs, the serological test will be essential in addressing two fundamental issues: vaccine prioritization and monitoring of protective immunity development post-vaccination.

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Recommendations

1. The implementation of rapid antibody tests in low and middle-income countries (LMICs) would facilitate serostatus assessment after natural infection.
2. The scarcity of vaccines in many LMICs, the antibody tests would provide a way for better implementation of the vaccination to those who have not been exposed or the ones that have low to no antibodies, thereby breaking the chain of SARS-CoV-2 transmission.
3. We have observed that flow-through immunoassay presented higher specificity with comparable sensitivity.
4. Implementation of RBD-specific antibody assay is necessary for observing the efficacy of vaccination.
5. Implementation of NCP-specific antibody assay will provide an insight into the previous infections. Simultaneously, the absence of NCP-specific antibody and the presence of RBD-specific antibody would correlate with the vaccination program's efficacy in providing protection.

Article Highlights

- Dot blot assay provided comparable sensitivity and specificity to SARS-CoV-2 NCP-IgG and SARS-CoV-2 RBD-IgG ELISA in both <14 days and >14 days.
- The Dot-blot assay intensity directly correlated with the SARS-CoV-2 NCP-IgG and SARS-CoV-2 RBD-IgG ELISA intensity.
- The dot-blot assay's high specificity indicates that it can distinguish between SARS-CoV-2 antigen and other common coronavirus antigens.

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Consent for Publication

All authors reviewed and approved the final version and have agreed to be accountable for all aspects of the work, including any issues related to accuracy or integrity

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

Mr. Md. Ahsanul Haq reports a patent 10202006327W pending; Prof. Dr. Bijon Kumar Sil reports a patent 10202006327W pending; Dr. Mohd Raeed Jamiruddin reports a

patent 10202006327W pending to Intellectual Property Office of Singapore; Dr. Nihad Adnan reports a patent 10202006327W pending; The authors report no other conflicts interest in this work. This research has the potential possibility for applying patent rights. Mainul Haque will not be part of the patent.

Table 1: Formula For Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) Calculation.

Table 2: Comparison of AUC, sensitivity, specificity, and Kappa of Dot blot with RBD-IgG and S1-IgG at different time points with RT-PCR positive and negative samples.

Table 3: Positive and negative predicted value and test agreement of the assay procedure of rapid dot blot at different time points.

Table 4: Comparison of AUC, sensitivity, specificity, and Kappa of Dot-blot with seropositive or seronegative samples at different times.

Table 5: Positive and negative predicted value and test agreement of the dot-blot assay with characterized seropositive and seronegative samples.

Supplementary Table 1: Difference in mean OD/cut-off in NCP-IgG ELISA compare to dot-blot intensity scale.

Supplementary Table 2: Difference in mean OD/cut-off in RBD-IgG ELISA compare to dot-blot intensity scale.

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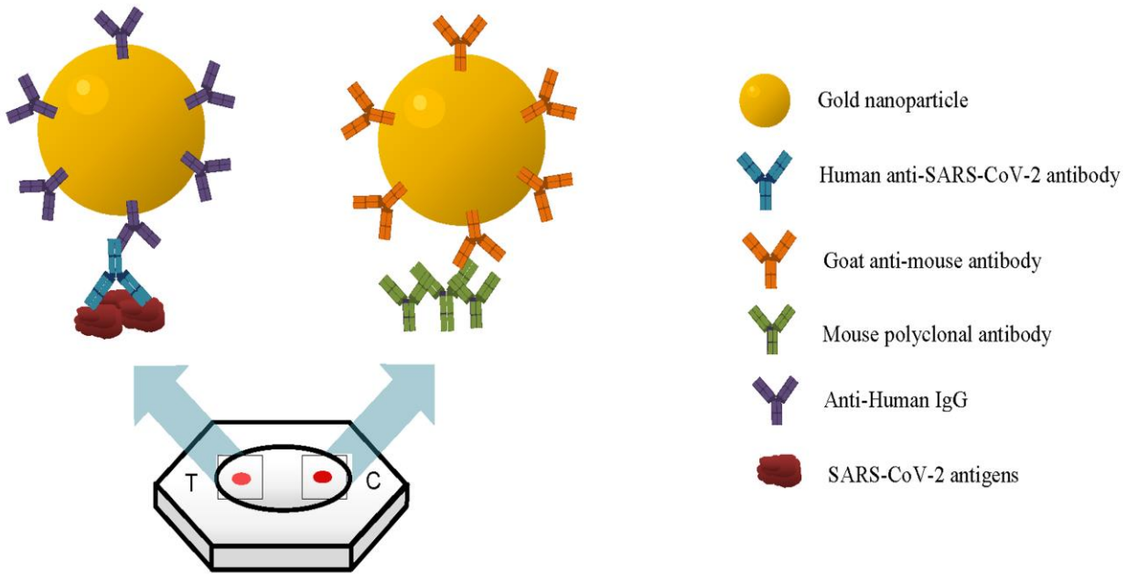
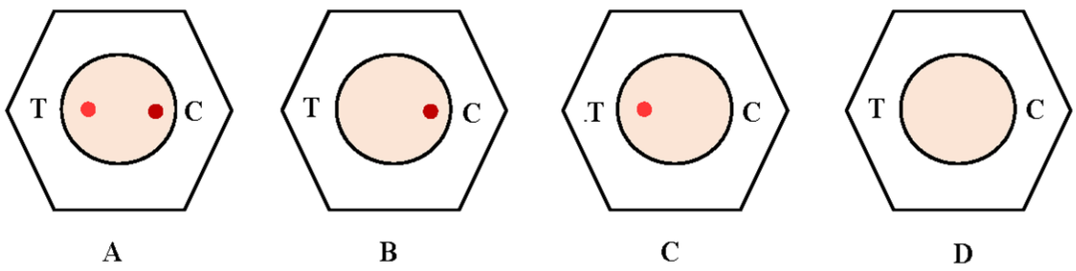
595 **Figures:**

596 **Figure 1:** Design concept of the rapid flow-through dot-blot immunoassay (FT-DBA).



597

598 **Figure 2:** Interpretation of the test result-(a) positive; (b) negative; (c) and (d) invalid
599 test.



600

601 **Figure 3:** Dot intensity and scale of measurement.

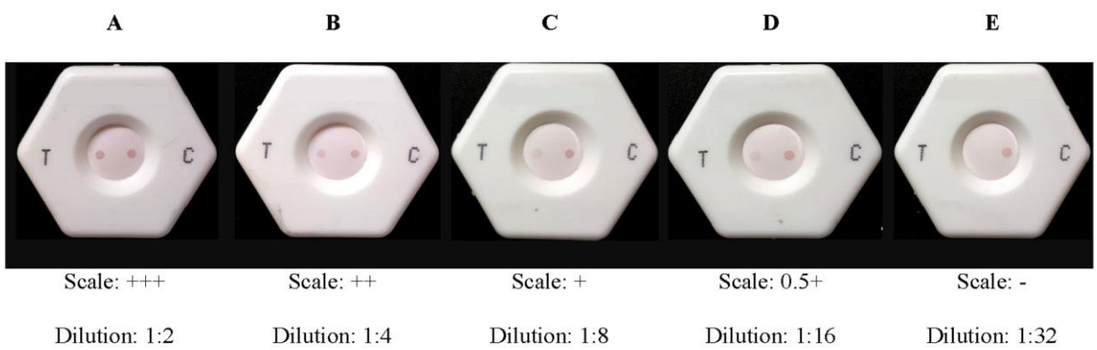


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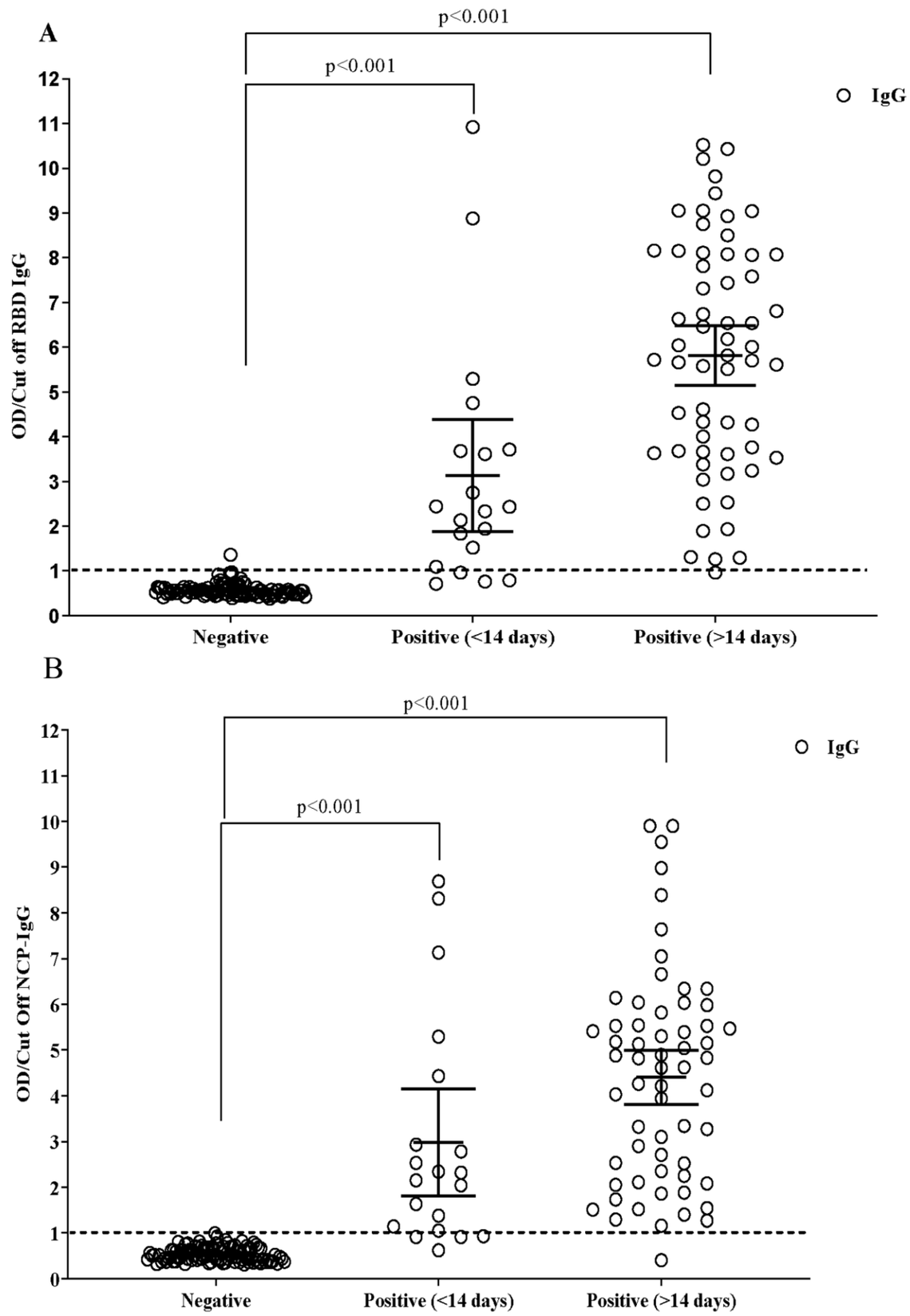
Figure 4: Mean difference in the positive (<14 and > 14 days) and negative samples in in-house ELISA to detect RBD (A) and NCP (B) specific IgG against SARS-CoV-2. The linear regression model was used to estimate the p-value, and the data were represented as mean with standard deviation (SD).



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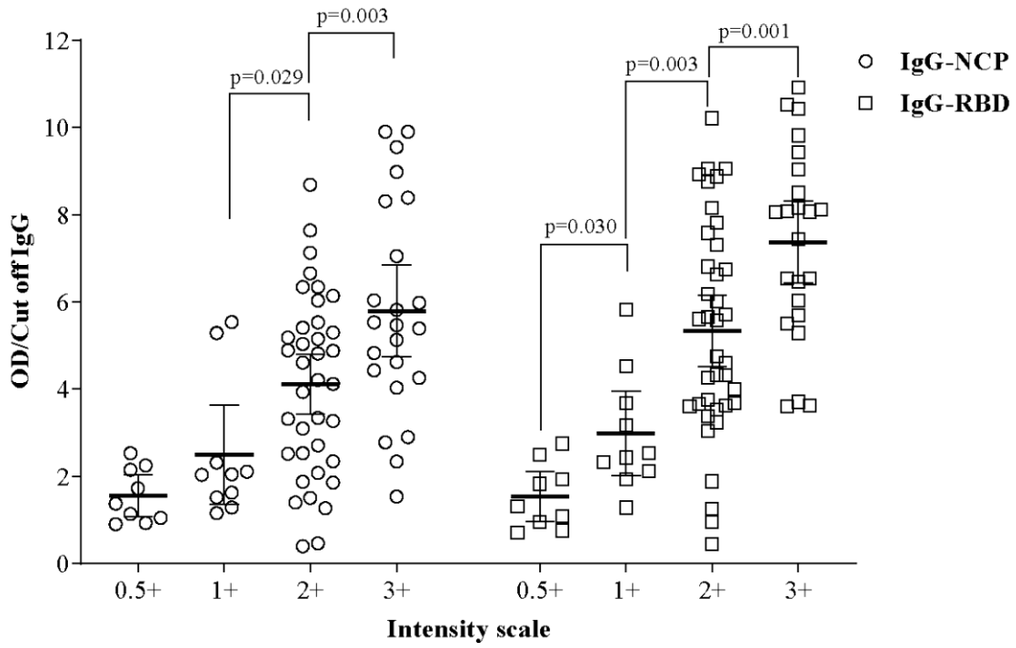
610 **Figure 5:** Mean difference in RBD and NCP specific IgG (Cut off) in contrast with
611 intensity scale. The linear regression model was used to estimate the p-value, and the
612 data were shown as mean with a 95% confidence interval.



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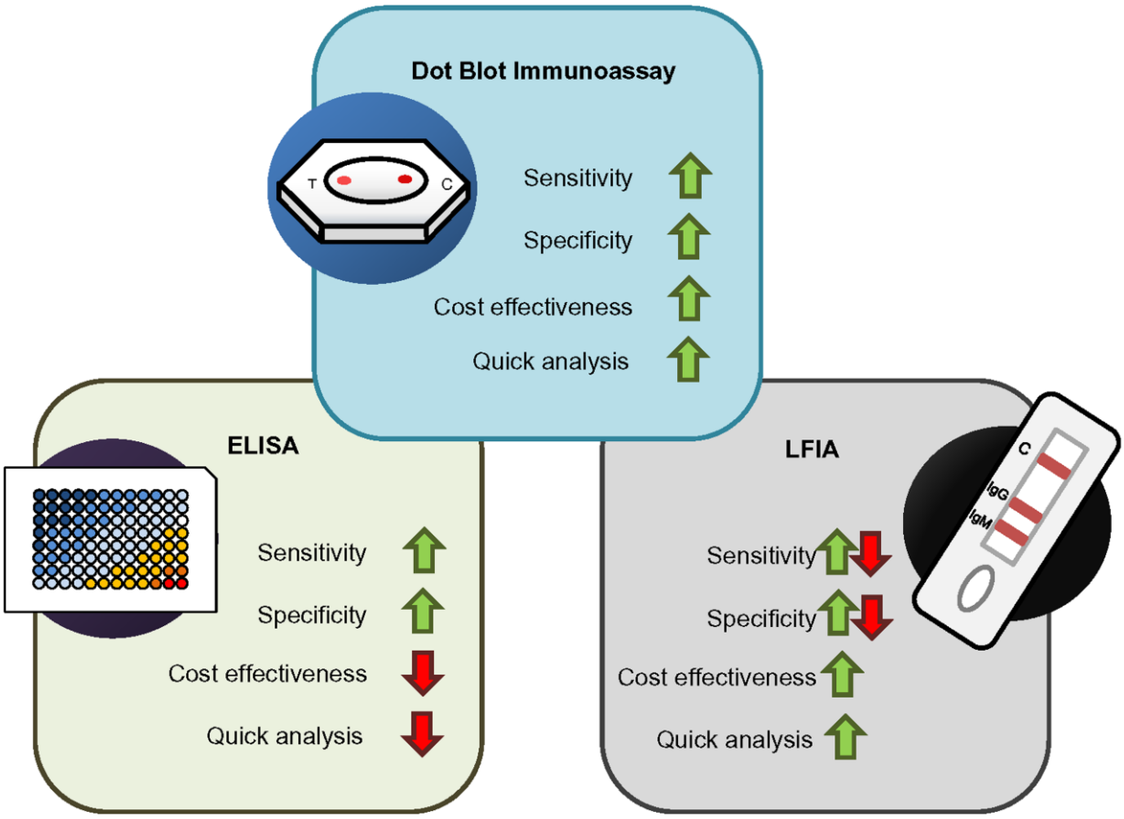
615 **Figure 6:** Comparison between Dot-blot assay with ELISA and LFIA.



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618

Table 1: Formula for Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) Calculation.

Positive test result	True Positive (TP)	False Positive (FP)	**PPV:** TP/(TP+FP)
Negative test result	False Negative (FN)	True Negative (TN)	**NPV:** TN/(TN+FN)
	Sensitivity: TP/(TP+FN)	**Specificity:** TN/(FP+TN)	

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Table 2: Comparison of AUC, sensitivity, specificity, and Kappa of Dot blot with RBD-IgG and S1-IgG at different time points with RT-PCR positive and negative samples.

	AUC (95% CI)	Sensitivity, % (95% CI)	Specificity (95% CI)	Kappa	p-value
<14 Days					
NCP-IgG	0.90(0.81, 0.99)	80.0(56.3, 94.3)	100(96.4, 100)	0.800	<0.001
RBD-IgG	0.90(0.81, 0.99)	80.0(56.3, 94.3)	99.0(94.6, 100)	0.840	<0.001
Dot blot	0.92(0.83, 1.00)	85.0(62.1, 96.8)	98.0(93.0, 99.8)	0.845	<0.001
>14 days					
NCP-IgG	0.99(0.98, 1.00)	98.4(91.2, 100)	100(94.1, 100.0)	0.987	<0.001
RBD-IgG	0.99(0.98, 1.00)	98.4(91.2, 100)	99.0(94.6, 100)	0.974	<0.001
Dot blot	0.99(0.98, 1.00)	100(94.1, 100.0)	98.0(93.0, 99.8)	0.948	<0.001
Overall					
NCP-IgG	0.96(0.93, 0.99)	93.8(86.2, 98.0)	100(96.4, 100)	0.921	<0.001
RBD-IgG	0.96(0.94, 0.99)	93.8(86.2, 98.0)	99.0(94.6, 100)	0.933	<0.001
Dot blot	0.97(0.95, 1.00)	96.3(89.6, 99.2)	98.0(93.0, 99.8)	0.944	<0.001

621 Note: AUC: Area under curve; 95% CI: 95% Confidence interval

622 Cohen's Kappa test was used to evaluate the test agreement

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Table 3: Positive and negative predicted value and test agreement of the assay procedure of rapid dot blot at different time points.

Days	PPV, % (95% CI)	NPV, % (95% CI)
<14 days	89.5(66.9, 98.7)	97.0(91.6, 99.4)
>14 days	96.8(89.0, 100)	100(96.3, 100)
Overall	97.5(91.3, 99.7)	97.0(91.6, 99.4)

625 **Note**: PPV: Positive predictive value; NPV: Negative predictive value

626

Table 4: Comparison of AUC, sensitivity, specificity, and Kappa of Dot-blot with seropositive or seronegative samples at different times.

	AUC (95% CI)	Sensitivity, % (95% CI)	Specificity (95% CI)	Kappa	p-value
<14 days	0.97(0.92, 1.0)	94.7(74, 99.9)	98.0(93.0, 99.8)	0.943	<0.001
>14 days	0.99(0.98, 1.00)	100(94.1, 100.0)	98.0(93.0, 99.8)	0.948	<0.001
Overall	0.98(0.97, 1.0)	98.8(93.3, 100)	98.0(93.0, 99.8)	0.946	<0.001

627 Note: AUC: Area under curve; 95% CI: 95% Confidence interval

628 Cohen's Kappa test was used to evaluate the test agreement

629

Table 5: Positive and negative predicted value and test agreement of the dot-blot assay with characterized seropositive and seronegative samples.

Days	PPV, % (95% CI)	NPV, % (95% CI)
<14 days	94.4(72.7, 99.9)	98.8(93.6, 100)
>14 days	96.8(89.0, 100)	100(96.3, 100)
Overall	97.6(91.5, 99.7)	99.0(94.5, 100)

630 **Note**: PPV: Positive predictive value; NPV: Negative predictive value

631