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ARTICLE

Development of serological assay for detection of antibodies in the N protein of SARS-CoV-2 in human sera in Mongolia

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Abstract: *The capability to detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and identify immune responses among the population is crucial for managing the outbreak of the COVID-19 pandemic.*

Although PCR-based nucleic acid detection techniques are utilized to detect viral infection in people, alternative tests capable of distinguishing between exposure and infection are urgently needed beyond this restricted window of detectable viral replication. Antibodies are produced in human sera within a few days after viral infection, providing longer period for performing tests to acquire reliable database.

Herewith, we provide the results of our in-house developed ELISA (Enzyme-Linked Immunosorbent Assay) that displays all of the properties necessary for high-throughput of human sera sample analysis. This test does not involve the handling of live viruses, although it detects a variety of antibody types in serum and plasma of human after exposure to the virus.

For in-house development of the kit, the nucleocapsid (N) gene of SARS-CoV-2 virus was cloned in the prokaryotic expression vector pGEX-6P-1, and purified N protein was used to detect IgG antibodies in human sera samples. In total 76 human serum samples that were collected before novel coronavirus registry in Mongolia in March 2020, as well as 200 serum samples from patients who had been infected by SARS-CoV-2 virus, were used.

Among 200 serum samples, 188 were positive and 12 were false negative, while in non-infected cases 69 were negative and 7 were false positive, suggesting 94 per cent sensitivity and 90.7 per cent specificity of the kit, with p-values of 0.02.

Keywords: *SARS-CoV-2 diagnosis; ELISA; nucleocapsid protein; IgG;*

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INTRODUCTION

The first case of atypical human pneumonia and its causative virus named as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which causes Coronavirus Disease 2019 (COVID-19), was reported in Wuhan, People's Republic of China in December 2019 [1]. At present, more than 313 million people worldwide are infected with SARS-CoV-2 and 5.5 million deaths have been reported globally [2]. According to the Mongolian authorities, more than 395 thousand cases of the virus infections were registered as of 12 January 2022 [3].

SARS-CoV-2 is a betacoronavirus and the seventh member of the Coronaviridae family of viruses [1]. It is an enveloped virus with positive stranded RNA which contains ORF1a, ORF1b and genes of major structural proteins, such as envelope (E), spike (S), membrane (M) and nucleocapsid (N) [4]. Two proteins (M and E) have vital roles for virus assembly [5] [6]. The S protein located on the surface of the viral particle is required for virus attachment to host cells, mediating the interaction with the angiotensin-converting enzyme 2 (ACE2) with the receptor binding domain of S protein [7]. As for N protein, it is the crucial structural protein of the virus and is functionally served in the expression of the virus RNA, packaging of virions [8] [9] as well as intervening with the host cell cycle processes [10]. Both S and N proteins are highly immunogenic and are profusely expressed during infection [11].

As per WHO recommendation, diagnostics of SARS-CoV-2 is based on genomic nucleic acid detection of the virus in human respiratory samples. Due to this reason and the rapid outgrowth of SARS-CoV-2 worldwide, high demand for rapid and highly-efficient diagnostic tests for the whole population in a short period of time need to be employed in order to identify infected people that include asymptomatic carriers, who became immune to, or prevent the further viral spread that is critical for controlling the pandemic.

ELISA is a commonly used assay to screen the presence of specific antibody responses in a qualitative and quantitative manner in people. After 7 days of infection, specific antibodies IgM and IgG against SARS-CoV-2 can be detected in the serum of around 40 per cent of COVID-19 patients, and after 14 days, seroconversion rates were elevated to more than 90 per cent [12]. Because the virus has been cleared out in the later stages, antibody testing is reported to be more sensitive [13]. In order to ensure reliable results both nucleic RT-PCR tests and antibody tests should be performed depending on virus infection and its clearance time.

Therefore, at the populational level, ELISA can be automated to test thousands of samples for providing accurate results: 1. for those who want diagnosis after a few days from onset of symptoms and require further monitoring, 2. for verifying whether vaccines are working or not, if ELISA is using the same antigens that are utilized for that particular vaccine production, 3. for contact tracing, 4. for determining population's herd immunity, and. 5. for risk of subsequent re-infection.

The aim of the present study was to develop SARS-CoV-2 nucleocapsid (N) protein-based ELISA kit, as well as to utilize it to detect specific antibodies against SARS-CoV-2 in human serum samples.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected from a total of 200 patients admitted to the laboratory of Virology of the National Center for Communicable Diseases in Ulaanbaatar. SARS-CoV-2 infection was tested by RT-PCR using specimens taken from respiratory tract and by immunological assays in blood samples after recovery. Samples serving as controls were sera of 76 healthy individuals that were collected in 2017, and stored in -80°C, which were intended to be used for other purposes, thus having no history of SARS-CoV-2 virus infection.

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Gene cloning, expression and purification of nucleocapsid (N) protein of SARS-CoV-2

Based on the reference sequence of N gene from SARS-CoV-2 dataset on NCBI, forward primer, including coding sequence of BamHI site and reverse primer that contains NotI site, were designed and PCR was performed using those primers. The sequence coding in respect to N protein, which has BamHI and NotI sites, was cloned in the prokaryotic expression vector pGEX-6P-1, fused to C-terminus of Glutathione S-transferase (GST).

The constructing vector was transformed into *E.coli* BL21 strain, grown in LB medium, and cells were induced by IPTG for 3 hours resulting in GST-N fusion protein. Purification was accomplished using affinity chromatography that involves glutathione coupled to a Sepharose matrix. It is in place to note that N protein was not separated from Glutathione S-transferase, since we did not observe any distinction in the absorbance of ELISA results between fusion protein and N protein after cleavage. Lastly, the concentration and purification of N protein was determined by the BCA protein assay and SDS-PAGE gel bands. The purified recombinant N protein was detected and analyzed by Western blotting and adsorbed on ELISA plate as an antigen.

N-based ELISA binding assay

The recombinant N protein based on ELISA kit was used to detect specific IgG antibody against SARS-CoV-2. First of all, 96 ELISA plates were coated with 100µl/well SARS-CoV-2 N protein using a coating buffer at 4°C overnight. Plates were then washed twice with 200µl wash solution. Afterwards, the wells were blocked by 200µl/well 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) at 37°C for 2 hours. The wells were washed 3 times with a wash solution. Three replicates of 100µl of diluted human sera were included on every plate following 1 hour of incubation at RT. The wells were washed and incubated for 1 hour at RT with 100µl of horseradish peroxidase (HRP) conjugated anti-human IgG diluted in blocking solution. After the final wash, 100µl of tetramethylbenzidine (TMB) solution was added to each well and were incubated for 15 min at RT. The reaction was then terminated by adding 50µl of stop solution and the absorbance was detected at 450nm.

Statistical analysis

GraphPad Prism was used for standard statistical analyses using data obtained from the ELISA results. In addition, an unpaired two-sample T-Test was performed to verify if there was any significant difference between the infected and the healthy groups.

RESULTS AND DISCUSSION

Cloning, expression and purification of SARS-CoV-2 nucleocapsid protein

N gene was amplified utilizing primers containing BamHI and NotI sites, and cloned and fused to the C-terminus of Glutathione S-transferase in the prokaryotic expression vector pGEX-6P-1. Total expressed proteins were obtained from BL21 expression host. During the purification of the recombinant protein, supernatant and pellet that were formed after sonication and centrifugation, were loaded to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separately. Following up on the result, it is concluded that the recombinant N proteins were soluble instead of forming inclusion bodies. This provided an easy way to isolate our desired protein within the supernatants; as expected, the soluble GST-N fusion protein was effectively isolated through a glutathione-based affinity chromatography. Next, SDS-PAGE was used to confirm whether N protein was adequately expressed after being induced with IPTG for 0 hour, 2 hours, and 3 hours respectively. Upon staining with Coomassie blue, our target protein, which was expected at 55 kDa, was overexpressed with varied setting times, showing that N protein induction was successful (Figure 1).

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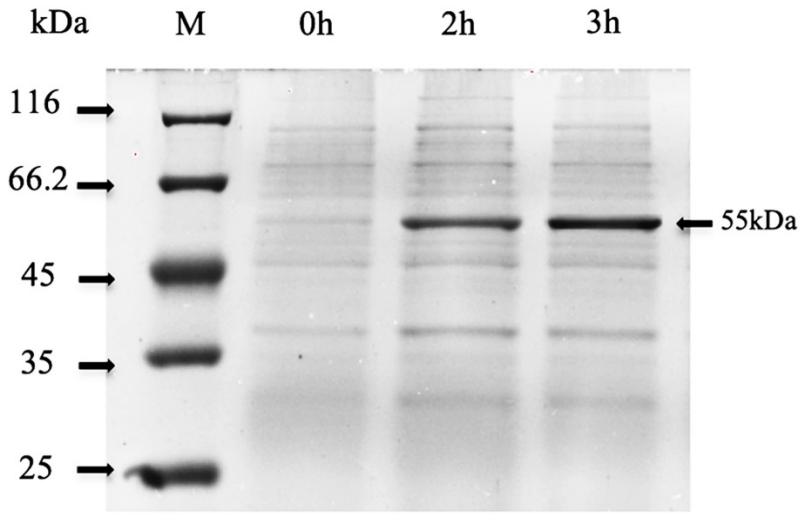

Figure 1. SDS-PAGE gel illustrating the expression of N protein after inducing with Isopropyl β- d-1-thiogalactopyranoside (IPTG). Lane 1: protein marker, lane 2: without IPTG induction, lane 3: after 2 hour induction, lane 4: after 3 hour induction

Affinity of N protein

In order to determine suitable antigen concentration to achieve precise result on ELISA, a purified protein in different concentrations was run using SDS-PAGE along with known concentration of standard bovine serum albumin (BSA) (Figure 2A). Moreover, bicinchoninic acid (BCA) assay was carried out, providing greater concentration accuracy. (Figure 2B). Based on the standard curve and SDS-PAGE, 6 mg/ml of protein concentration was achieved. Based on this calculation, the plates have been coated at concentration 25 µg/ml.

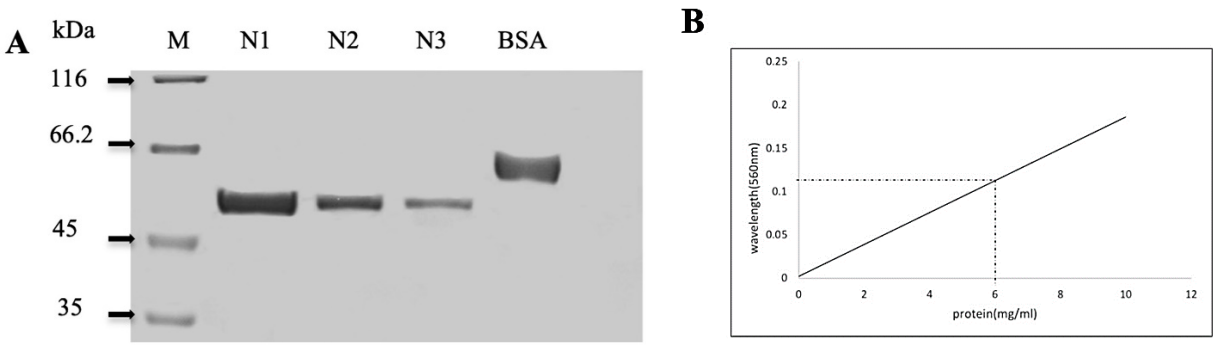

Figure 2. Analysis of protein pattern by SDS-PAGE and BCA standard. (A) The purified protein was subjected to 10 per cent SDS gel electrophoresis. Lane 1: protein marker, lane 2: 1µl of N protein, lane 3: 0.5µl of N protein, lane 4: 0.25µl of N protein and lane 5: 4mg/ml standard bovine serum albumin. (B) BCA assay produces a linear standard curve 0 to 10mg/ml concentration. The dotted line indicates our purified protein concentration

SDS-PAGE was performed with N protein concentrations, ranging from 0.25µl to 2µl, for western blot analysis to further validate the recombinant N protein antigenicity against healthy and SARS-CoV-2 positive sera. As shown on western blot results, an elevated signal proportional to the quantity of N protein interacting with convalescent sera as a primary antibody was identified with anti-human IgG conjugated with HRP and enhanced chemiluminescence ECL substrate, while there were no signals for healthy individuals' sera (Figure 3).

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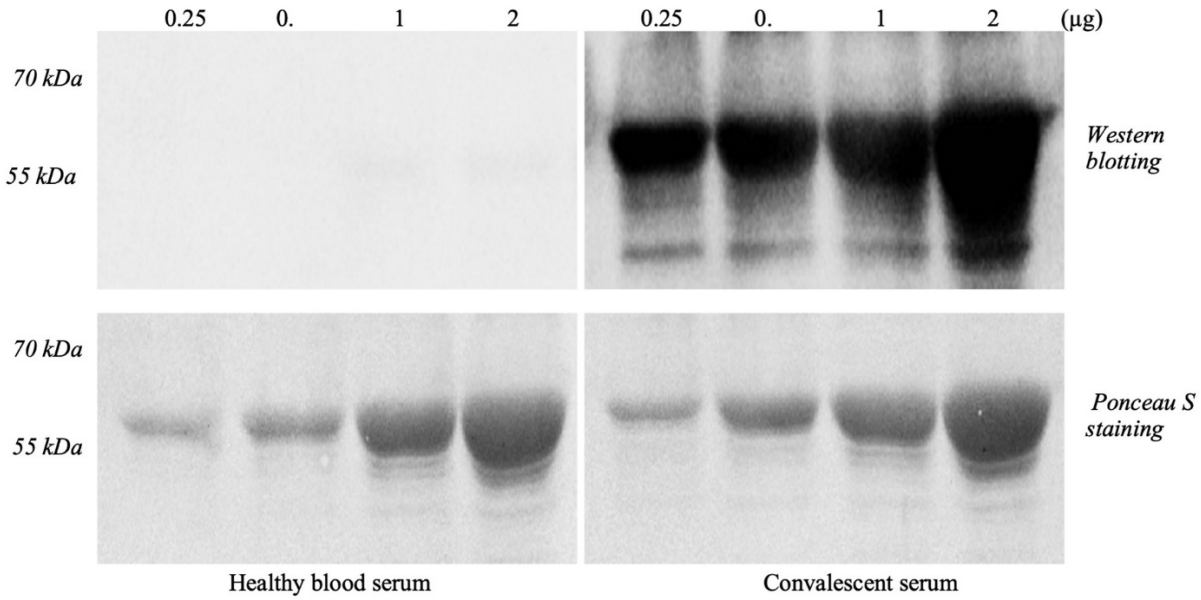

Figure 3. The comparison of the healthy serum and convalescent serum against SARS-CoV-2 nucleocapsid protein using western blot

Despite the distinction of two groups, Ponceau staining was visible on the bottom, where it indicates all proteins according to their concentrations. Therefore, these results clearly showed that the nucleocapsid specific antibody (IgG) binds successfully with the recombinant nucleocapsid protein, as it recognizes convalescent sera.

Performance of serological assays by N based ELISA

In order to validate the capability of our in-house produced ELISA kit to precisely detect SARS-CoV-2 infection, sera from 200 past-Covid-19 patients and 76 healthy individuals were tested using our ELISA kit. Each 96 well plates should be calculated and interpreted separately according to the respective cut-off values in order to obtain accurate results. Cut-off value was calculated by the sum of the mean values from the healthy individuals and two times the standard deviations.

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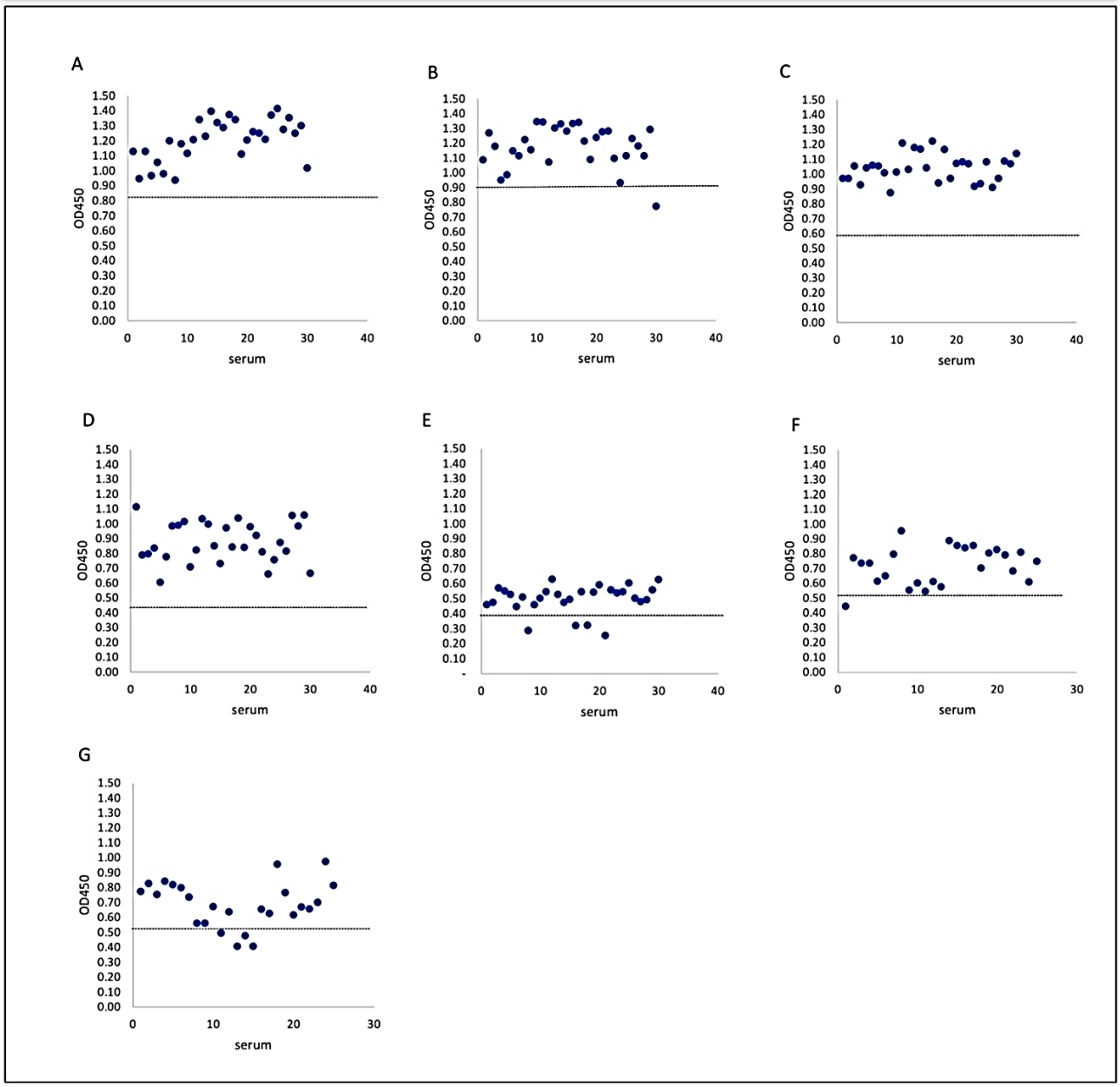

Figure 4. The scatter plot of a total 200 patients. In all 200 samples, tests were repeated 3 times using a total of 7 microplates (A, B, C, D, E, F, G). Each plate contained negative sera as a control for determining cut-off value which was expressed as a dashed line. The above line means that antibody was detected

Each sample was assessed in triplicates, using 7 microtiter plates in total. According to the cut-off values, 188 out of 200 patients had IgG antibodies against SARS-CoV-2 (Figure 4), whereas 7 from 76 served as healthy controls, which were falsely positive, ultimately achieving 94 per cent of sensitivity and 90.7 per cent of specificity with p-value of 0.02 (Table 1).

Table 1. Results of IgG against SARS-CoV-2 detection in total 276 samples

Total		200	Total		76
Positive	True positive	188 (94%)	**Negative**	True negative	69 (90.7%)
	False negative	12		False positive	7

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Several measures were taken to control the outbreak of novel coronavirus SARS-CoV-2, which started in Mongolia in early November 2020. A core strategy to facilitate the containment of COVID-19 pandemic is to recognize this disease effectively. Currently, RT-PCR method is mainly used to detect the presence of the virus [14] and the test has to be conducted in biosafety laboratories with skilled professionals.

In this study, we have developed N protein based ELISA kit and utilized it for the detection of specific antibodies against SARS-CoV-2 infection in 200 convalescent sera and 76 healthy individuals' sera. Due to its conservation and its high immunogenicity, N protein was chosen as an antigen. Our in-house developed ELISA kit was used to determine IgG in serum samples from COVID-19 patients. Our results displayed 188 samples present with IgG while in the control group 7 were false positives, giving a 94 per cent sensitivity and 90.7 per cent specificity.

In order to improve the sensitivity and specificity of the in-house developed ELISA kit, further measures were taken to use spike (S) protein as an antigen. Also testing IgM against both antigens in sera taken from a specified time period is crucial for achieving accurate results. Compared with nucleocapsid based ELISAs, mixture of N and S protein together increased the sensitivity of IgM and IgG detection [15]. In addition, N and S proteins are currently being used in vaccines against SARS-CoV-2. Mongolia currently has a vaccination rate exceeding 65.4 per cent [2], which suggests that immunity to SARS-CoV-2 via vaccine or infection must be determined. Therefore, using this sensitive assay natural infection and vaccine induced immunity in the whole population can be determined.

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Ethical Statement: The research was approved by the Ethics Committee of the Ministry of Health of Mongolia on 3 November 2020.

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