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Assessment of specific human antibodies against SARS-CoV-2 receptor binding domain by rapid in-house ELISA

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Abstract.
BACKGROUND: The recently emerged SARS-CoV-2 caused a global pandemic since the last two years. The urgent need to control the spread of the virus and rapid application of the suitable health measures raised the importance of available, rapid, and accurate diagnostic approaches.
OBJECTIVE: The purpose of this study is to describe a rapid in-house optimized ELISA based on the expression of the receptor binding domain (RBD) of the SARS-CoV-2 spike protein in a prokaryotic system.
METHODS: We show the expression of the 30 kDa recombinant SARS-CoV-2 RBD-6×His in four different *E. coli* strains (at 28°C using 0.25mM IPTG) including the expression strain *E. coli* BL21 (DE3) Rosetta Gami. SARS-CoV-2 rRBD-6×His protein was purified, refolded, and used as an antigen coat to assess antibody response in human sera against SARS-CoV-2 infection.
RESULTS: The assessment was carried out using a total of 155 human sero-positive and negative SARS-CoV-2 antibodies. The ELISA showed 69.5% sensitivity, 88% specificity, 78.5% agreement, a positive predictive value (PPV) of 92.3%, and a negative predictive value of 56.5%. Moreover, the optical density (OD) values of positive samples significantly correlated with the commercial kit titers.
CONCLUSIONS: Specific human antibodies against SARS-CoV-2 spike protein were detected by rapid in-house ELISA in sera of human COVID-19-infected patients. The availability of this in-house ELISA protocol would be valuable for various diagnostic and epidemiological applications, particularly in developing countries. Future studies are planned for the use of the generated SARS-CoV-2 rRBD-6×His protein in vaccine development and other diagnostic applications.

Keywords: SARS-CoV-2, RBD, ELISA, diagnosis, human sera, COVID-19

1. Introduction

Since December 2019, the world witnessed a rapidly escalating outbreak of SARS-CoV-2 virus, the causative agent of COVID-19 disease. SARS-CoV-2 virus is a positive strand RNA enveloped virus. It causes sudden acute respiratory syndrome and has spread over the world with high speed and contagious potential. The SARS-CoV-2 virus epidemic outperformed the previous outbreaks caused by either Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) during the last twenty years, both also members of human coronaviruses (HCoVs) family.

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The rapid assault of SARS-CoV-2 in December 2019 has left healthcare institutions throughout the world baffled. To limit the rapid impact on health, several health measures and methods have been implemented, starting with the quarantine of clinically suspected persons, cancellation of social and professional meetings, application of WHO recommendations, and finally, country lock down. Within a few weeks, the virus had wrecked devastation on social, economic, and even political levels on a daily basis [1].

Since the declaration of SARS-CoV-2 outbreak a pandemic on March 11th, 2020, by the World Health Organization (WHO), there became an urgent need for a large number of testing toolkit for detection and surveillance of SARS-CoV-2 infections among both asymptomatic individuals and patients presenting with mild to severe respiratory symptoms.

Reverse transcriptase polymerase chain reaction (RT-PCR) with primer/probe combinations is the gold standard approach for diagnosis of SARS-CoV-2 infection, allowing detection of the viral nucleocapsid (N), RNA-dependent RNA polymerase (RdRP), and envelope (E) genes [2]. To avoid false negative results, consider factors such as viral load, sample collection and transportation, RNA extraction protocol, type of enzyme inhibitors, and RT-PCR method when performing RT-PCR [3,4]. Traditional viral culture is useful, but it takes time and necessitates biosafety level-3 facilities, which are not readily available [5]. Novel technologies such as clustered regularly interspersed short palindromic repeats (CRISPR) and loop-mediated isothermal amplification (LAMP) were also used to build rapid diagnostic tests [6], however their use is still limited [7].

Serology testing is a reliable, straightforward, and cost-effective method for detecting infections both directly and indirectly. It has been successfully used in epidemiological surveillance studies in the past. Several serology-based rapid point-of-care assays were also developed to shorten assay turnaround times [8]. Within the context of the SARS-CoV-2 pandemic, assessing antibody response with a fast, reliable and easy to handle assay is a corner stone in determining previous infections among unvaccinated groups. In addition, SARS-CoV-2 serological assay can quantitatively and qualitatively monitor immune response and identify convalescent individuals who showed strong anti SARS-CoV-2 response thus potentially serving as therapeutic plasma donors.

SARS-CoV-2 spike protein receptor binding domain (RBD) is a promising candidate for the development of SARS-CoV-2 serological tests. Formed of 224 amino acids residues, it is the key entry point of SARS-CoV-2 to the host cells through binding with angiotensin converting enzyme (ACE-2). It harbors the unique mutations differentiating SARS-CoV-2 from other members of HCoVs [9]. Most studies attempting the use of SARS-CoV-2 RBD in serological assays relied on the expression of RBD from mammalian expression systems. Albeit its usefulness and numerous advantages, mammalian expression is time consuming, expensive, and requires sophisticated laboratory settings. Only a few studies reported RBD expression in prokaryotic expression systems for investigating its structural and binding characteristics [10,11]. Less than a handful of studies reported the reactivity of RBD produced in *E. coli* with human sera of infected SARS-CoV-2 [12].

Here, we report the expression and purification of SARS-CoV-2 spike RBD from *E. coli* suitable for reactivity with human anti-SARS-CoV-2 IgG in human sera. Our study provides an improved immunoassay format for assessment of human anti-SARS-CoV-2 spike protein RBD through rapid in-house ELISA protocol.

2. Materials and methods

2.1. Plasmids, bacterial strains and chemicals

The expression vector pET-30b(+) and bacterial strains used as host cells; *E. coli* DH5α, *E. coli* BL21(DE3) pLysS, *E. coli* BL21(DE3) Arctic RIL, *E. coli* BL21(DE3) C43 and *E. coli* BL21(DE3) Rosetta Gami, strains were obtained from Novagen (USA). Ni-NTA affinity matrix was from Invitrogen (USA), DNA and protein markers were from Thermo-Fisher, and plasmid miniprep kits were from Thermo-Scientific. The chemiluminescence SARS-CoV-2 IgG (CoV-2 IgG kit) was from Abbott (USA).

2.2. Human immune sera

A total of 155 preserved human serum samples (105 seropositive and 50 seronegative for SARS-CoV-2 antibody determined by commercial SARS-COV-2 IgG kit) (Abbott, Ireland), were tested. These sera were previously collected from patients in the first five months of the pandemic and before emerging of SARS-CoV-2 vaccines. The use of the serum samples was approved by the NRC ethical committee (Reference # NRC-20192).

2.2.1. Construction of SARS-CoV-2 RBD expression vector

The nucleotide sequence encoding RBD domain retrieved from NCBI database (NC_045512.2) of se-

vere acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, was used to construct an expression system. The region sandwiched between nucleotides 955 and 1623 in spike gene ORF, was codon optimized for expression in *E. coli* BL21(DE3) using IDT (Integrated DNA Technologies) codon optimization tools. The codon-optimized RBD sequence was cloned in frame with an N-terminal histidine tag in pET30b(+) vector (Novagen) between the restriction sites KpnI and BamH1 at the 5' and 3' ends, respectively. The resulting peptide corresponds to the region of R319-F541 designated by [13,14] as the RBD of SARS-CoV-2 spike protein. The recombinant plasmid was first transformed into chemically competent *E. coli* DH5α using the heat chock method [15] and then purified using a plasmid miniprep purification kit (Thermo scientific). The presence of the insert in frame was verified by Sanger sequencing (Macrogen Sanger sequencing facility, Korea).

Then, the recombinant expression vector was transformed into four different chemically competent *E. coli* expression strains: *E. coli* BL21(DE3) pLysS, *E. coli* BL21(DE3) Arctic RIL, *E. coli* BL21(DE3) C43 and *E. coli* BL21(DE3) Rosetta Gami.

2.3. Expression of SARS-CoV-2 rRBD-6×His

E. coli BL21(DE3) pLysS, *E. coli* BL21(DE3) Arctic RIL, *E. coli* BL21(DE3) C43 and *E. coli* BL21(DE3) Rosetta Gami transformed with recombinant plasmid pET30b(+) rRBD were grown in LB liquid medium containing 25 μg/ml kanamycin till the optical density at 600 nm reached 0.6–0.8. Then the incubation temperature was reduced to either 20°C, 28°C or left at 37°C to test the optimal thermal condition for RBD-6×His expression. After reaching the desired temperature, 0.25 or 0.5 mM Isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added, and expression was continued for the desired period of time (4 hours or overnight). To test the expression of RBD-6× His, total proteins of 1 ml of induced bacterial cultures were precipitated using 60% trichloroacetic acid, washed with acetone, and then resuspended in 1× Laemmeli loading buffer.

2.3.1. Testing solubility of SARS-CoV-2 rRBD-6×His

Induced bacterial pellets of different *E. coli* expression hosts described above were resuspended in phosphate-buffered saline and subjected to lysis by 5 repeated cycles of freezing (in frozen alcohol bath) and thawing (at 42°C). The lysed cells were then centrifuged, and the supernatant and pellet were separately mixed with Laemmli buffer, boiled at 95°C, and analyzed by 15% SDS-PAGE.

2.3.2. Purification and renaturation of SARS-CoV-2 rRBD-6×His

For purification of SARS-CoV-2 rRBD-6×His, 100 ml of induced pET30b(+)-RBD/*E.coli* BL21 (DE3) Rosetta Gami were centrifuged, resuspended 10 ml lysis/washing buffer 1 (25 mM Tris-HCl pH8, 500 mM NaCl, 10 mM Imidazole, 8 M urea), incubated for 2 hours at room temperature with gentle agitation, and then sonicated (10 seconds with a 10-second pause for 5–8 cycles). After centrifugation, the supernatant was incubated with Ni-nitriloacetic acid agarose (Invitrogen) equilibrated with the same buffer. The column was then washed with 10 volumes of washing buffer 1 and 10 volumes of washing buffer 2 (25 mM Tris pH8, 500 mM NaCl, 50 mM Imidazole, 8 M urea). rRBD-6× His was then eluted in elution buffer (25 mM Tris pH8, 500 mM NaCl, 750 mM Imidazole, 8M urea). The elution fractions were then checked on 15% SDS-PAGE.

Fractions containing highest concentration of purified rRBD-6× His were pooled and their buffer exchanged in six successive dialysis steps in 1× phosphate-buffered saline containing gradual decreasing concentrations of urea (8M, 6M, 4M, 2M, 1M, and 0M). The dialysis steps were carried out in a temperature controlled incubator at 25°C, using a dialysis bag of molecular weight cutoff of 1 kDa with gentle agitation. After dialysis, protein concentration was determined using the Bradford method [16] and the dialyzed sample was checked on 15% SDS-PAGE.

2.4. Development and optimization of SARS-CoV-2 RBD human IgG ELISA

The in-house generated SARS-CoV-2 rRBD-6×His protein was utilized for establishing a rapid ELISA using the method of Alandijany et al. [17] with some modifications. Briefly, Flat Bottom Micro titer ELISA plates were coated for 1 hr. at room temperature with 100μl of a range of concentration (typically 6.25 ng to 100 ng per well) of purified SARS-CoV-2 rRBD-6×His in 50 mM carbonate buffer pH 9.6. After blocking with 3% gelatin for 1 hr. at room temperature, and washing with 0.01 M TBS containing 0.05% tween 20, pH 7.4, serial dilutions from human sera (typically from 1:10 to 1:320) in 0.01 M TBS containing 0.5% gelatin pH 7.4 were added and incubated for 30 min at room temperature. The plates were washed and incubated with Protein A peroxidase conjugate, at 1:10000 dilution in the same buffer for 30 min at room temperature. The wells were then washed and 100 μl of an O-phenylenediamine solution (0.33 mg/ml in 0.1 M citrate buffer, pH 5.2, in the

presence of 0.04% hydrogen peroxide) were added and the absorbance values were determined at 450 nm with an ELISA reader after 15 min. All measurements were made in duplicates and the results were expressed as the median of three results. The highest signal-to-noise ratio for positive controls with the minimal background was determined in order to identify the optimized conditions. To determine the optimum conjugate dilution, plates were coated with 100 ng/well of SARS-CoV-2 rRBD-6×His, positive and negative human sera were used at 1:40 dilution, and conjugate dilutions ranged from 1:2000 to 1:64,000. The protocol was performed as described above. Testing of human sera for the presence of IgG antibody directed to SARS-CoV-2 RBD protein was performed using the optimized ELISA conditions.

2.5. Statistical analysis

Graphs were generated using Graphpad prism software (version 8). Statistical analysis was carried out using the SPSS program and the calculations were as follows:

The cut-off value of the developed assay was determined as follow:

$$\textit{cut off value} = \textit{mean values of negative samples} + (3 \times \textit{standard deviation})$$

The sensitivity, specificity, agreement, PPV and NPV were calculated as follow:

$$\textit{Sensitivity} = \left(\frac{\textit{the number of true positive}}{\textit{the total number of true positive} + \textit{false negative samples}} \right) \times 100$$

$$\textit{Specificity} = \left(\frac{\textit{the number of true negative}}{\textit{the total number of true negative} + \textit{false positive samples}} \right) \times 100$$

$$\textit{Agreement} = \left(\frac{\textit{the total number of true positive} + \textit{true negative samples}}{\textit{the total number of samples}} \right) \times 100$$

$$\textit{Positive Predicted value (PPV)} = \left(\frac{\textit{the number of true positive}}{\textit{the total number of true positive} + \textit{false positive samples}} \right) \times 100$$

$$\textit{Negative Predicted value (NPV)} = \left(\frac{\textit{the number of true negative}}{\textit{the total number of true negative} + \textit{false negative samples}} \right) \times 100$$

3. Results

3.1. Establishment of SARS-CoV-2 recombinant RBD construct in pET-30b

The receptor binding domain of SARS-CoV-2 spike protein is known to play a pivotal role in binding to ACE2 in the host cell [18]. Anti-SARS-CoV-2 spike protein RBD IgG is known to be present following SARS-CoV-2 infection. Accordingly, we aimed to express SARS-CoV-2 spike protein RBD in prokaryotic expression system to ensure its low cost, rapid and efficient production for detection of anti-SARS-CoV-2 IgG in human sera. We retrieved the nucleotide sequence of the full-length SARS-CoV-2 spike ORF from the NCBI database (NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome) [19]. The region sandwiched between nucleotides 955 and 1623 was codon optimized for expression in *E. coli* BL21(DE3) (corresponding to R319 to F541 of SARS-CoV-2 spike protein). The nucleotide sequence was cloned in frame with an N-terminal histidine tag in pET-30b(+) vector (Novagen) between the restriction sites KpnI and BamH1 at the 5' and 3' ends, respectively. Fused to the N-terminal histidine tag, the 6×His-RBD was formed of 789 nucleotides. After transformation in *E. coli* cloning host (*E. coli* DH5α), colony PCR was carried out to check the presence of the RBD fragment in the vector (Fig. 1).

3.2. Expression and purification of recombinant SARS-CoV-2 RBD-6×His (SARS-CoV-2 rRBD-6×His)

SARS-CoV-2 rRBD-6×His is predicted to be formed of 262 amino acids with estimated molecular mass of $\sim$ 30 kDa. To determine the expression host and induction conditions giving the highest yield of SARS-CoV-2

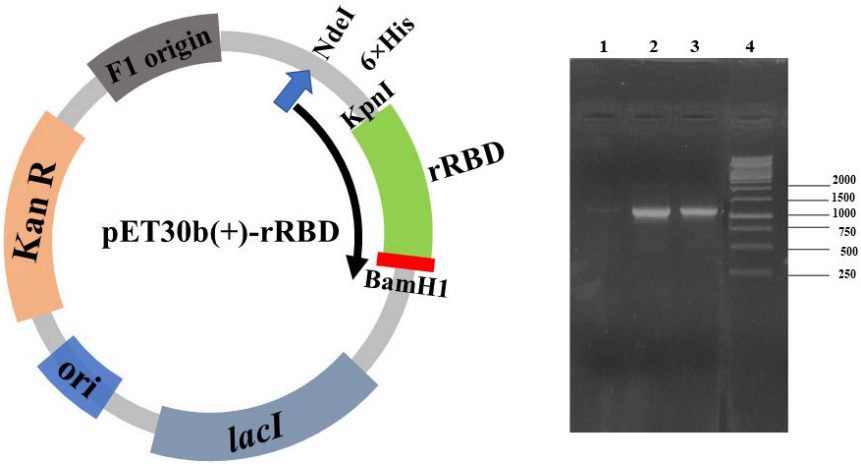

Fig. 1. Confirmation of the presence of SARS-CoV-2 rRBD insert in pET-30b(+) by colony PCR from *E. coli* DH5α transformed with pET-30b(+)-rRBD and selected on 25mg/ml kanamycin. Left panel: Design of pET30b(+)-rRBD. The codon optimized SARS-CoV-2 RBD sequence was cloned between KpnI and BamH1 sites. The translation starts at ATG codon (NdeI site, blue arrow) and stops before BamH1 site (red bar). Right panel: colony PCR from three different colonies, lane 4: 1 kb DNA ladder (Thermo). The PCR was carried out using T7-promoter and T7-terminator universal primers. The specific band (around 1002 bp) corresponds to the size of SARS-CoV-2 RBD fused to the N-terminal 6×His tag (between NdeI and KpnI restriction sites) with the upstream region from pET30b(+).

RBD-6×His, we tested four *E. coli* expression hosts (*E. coli* BL21(DE3) pLys, *E.coli* BL21(DE3) Arctic RIL, *E. coli* BL21(DE3) C43, and *E. coli* BL21(DE3) Rosetta Gami. In addition, we tested the expression of SARS-CoV-2 rRBD-6×His at different induction temperatures (20°C and 37°C). We observed a higher level of expression of a ∼30 kDa band, corresponding to the approximate predicted molecular mass of SARS-CoV-2 RBD-6×His when the cultures were induced by 0.25 mM IPTG and incubated overnight at 20°C (Supplementary figure 1a). However, we could not observe any detectable difference between the expression levels of SARS-CoV-2 rRBD-6×His in different expression hosts (Fig. 2). Accordingly, we used *E. coli* BL21(DE3) Rosetta Gami as the expression host for the following steps.

Next, we investigated the solubility of SARS-CoV-2 rRBD-6×His by detecting its cellular distribution between the soluble and insoluble fractions. However, under all tested conditions, SARS-CoV-2 rRBD-6×His localized exclusively in the insoluble fraction (Supplementary figure 2a). In an attempt to reduce inclusion bodies formation, we changed the expression condition by adding 0.25 mM IPTG at $OD_{600} = 0.8$ and incubating at 28°C for 4 hours only. However, SARS-CoV2 rRBD-6×His still localized in the insoluble fraction (Supplementary figure 2b). Hence, we carried out the purification procedure under denaturing conditions (in presence of 8 M urea in lysis, washing and elution buffers) to allow the solubilization of SARS-CoV-2

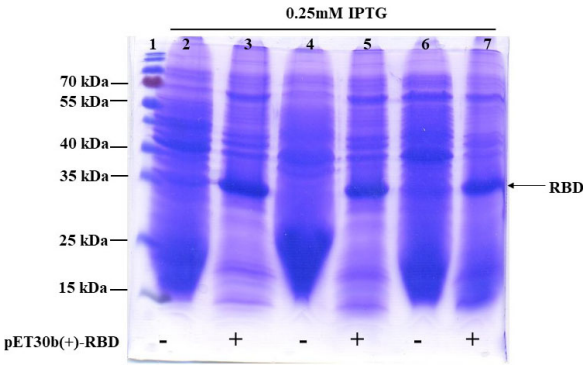

Fig. 2. Expression of SARS-CoV-2 rRBD-6×His in different *E. coli* expression hosts. The cultures were induced using 0.25 mM IPTG and incubated overnight at 20°C. Samples from induced cultures were treated with TCA, dissolved in 1× Laemmli buffer and loaded on 15% SDS-APGE. 1- Prestained protein marker (Thermo), lanes 2, 4 and 6 total protein of non-transformed *E. coli* expression hosts: *E. coli* BL21(DE3) pLys or *E. coli* BL21(DE3) Arctic RIL or *E. coli* BL21(DE3) Rosetta Gami, respectively, lanes 3, 5 and 7- total protein of *E. coli* BL21(DE3) pLys or *E. coli* BL21(DE3) Arctic RIL or *E.coli* BL21(DE3) Rosetta Gami transformed with pET30b(+)-RBD, respectively. Black arrow points to the overexpressed 30 kD protein corresponding to the predicted molecular mass of rRBD-6xHis.

rRBD-6×His. We successfully purified SARS-CoV-2 rRBD-6×His to homogeneity (Fig. 3A). To renature SARS-CoV-2 rRBD-6×His prior to downstream applications, we carried out successive dialysis steps of the purification fractions in gradual decreasing concentration of urea and successfully recovered SARS-CoV-2 rRBD-6×His (Fig. 3B).

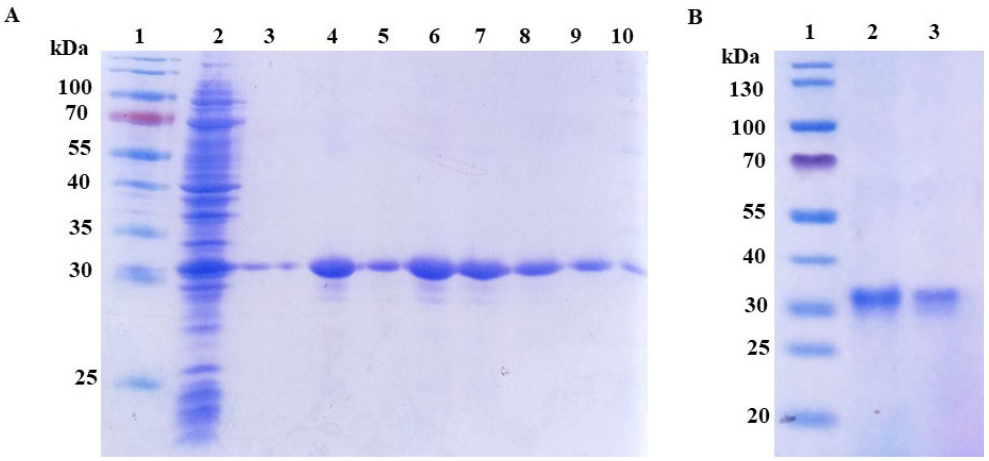

Fig. 3. Purification and renaturation of SARS-CoV-2 rRBD-6×His. A: Purification of SARS-CoV-2 rRBD-6×His under denaturing conditions: Induced *E. coli* BL21(DE3) Rosetta Gami transformed with pET30b(+)-rRBD were lysed and purified with Ni-NTA agarose matrix as described before. Lane 1- Prestained protein marker (Thermo), lane 2- induced *E. coli* BL21(DE3) Rosetta Gami transformed with pET30b(+)-rRBD lysate, lanes 3 to 10- elution fractions of SARS-CoV-2 rRBD-6×His. B: Recovery of renatured SARS-CoV-2 rRBD-6×His. Elution fractions 4 to 10 (in 3A) were pooled and dialyzed in decreasing urea concentration. Lane 1- Prestained protein marker (Thermo), lane 2- denatured SARS-CoV-2 rRBD-6×His, lanes 3- renatured SARS-CoV-2 rRBD-6×His after dialysis.

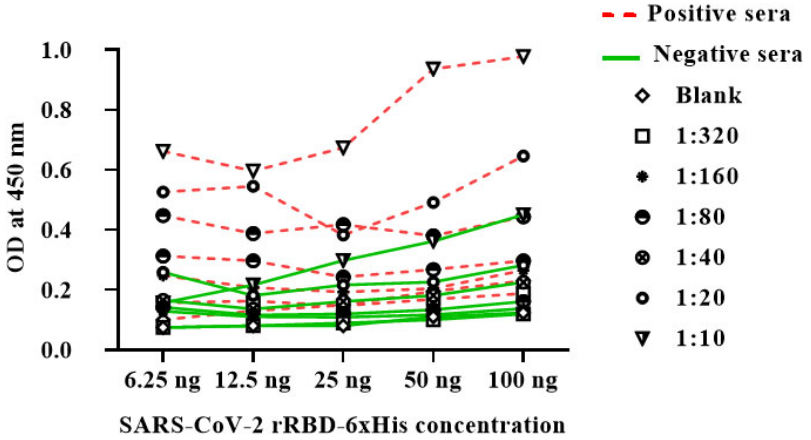

Fig. 4. Optimization of indirect ELISA utilizing different concentrations of purified SARS-CoV-2 rRBD-6×His for positive and negative human sera.

3.3. Assessment of the antigenicity of SARS-CoV-2 rRBD-6×His by detection of specific IgG response in human sera using in house optimized ELISA

In order to optimize the ELISA assay conditions, the protocol should offer enough antigen to bind antibodies, but with low non-specific backgrounds. To determine the optimal antigen-antibody concentration, we began by assaying different SARS-CoV-2 rRBD-6×His concentrations versus various dilutions of human sera with predetermined level of anti SARS-CoV-2 IgG. ELISA plates were initially coated with a concentration range of 6.25 ng to 100 ng/well of renatured SARS-CoV-2 rRBD-6×His (Fig. 4) and allowed to react with positive and negative sera serially diluted in a range of 1:10 to 1:320 (Fig. 5). In parallel, the protein A peroxidase conjugate was assayed at a dilution range of (1:2000 to 1:64000) (Fig. 6). Our ELISA assay optimal conditions were established by two factors: (1) the highest OD450 ratio of positive to negative samples, and (2) the lowest OD450 ratio of negative to blank. The optimized working conditions were as follows: 100 ng of SARS-CoV-2 rRBD-6×His per well for coating, 1:40 of serum dilution, and 1:10,000 of Protein A peroxidase dilution. This protocol was used for all subsequent experiments.

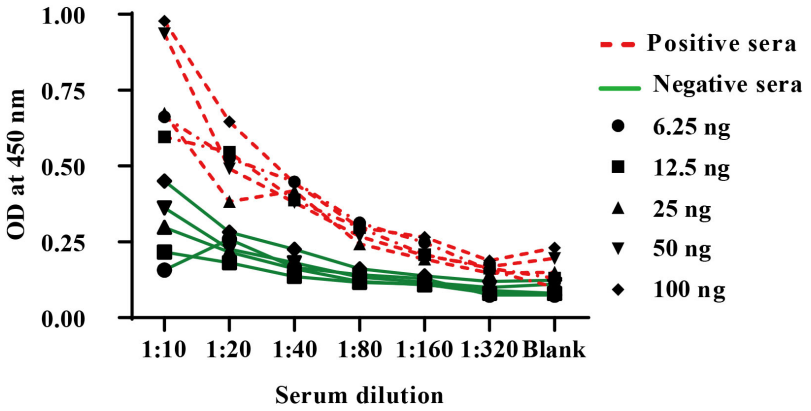

Fig. 5. Optimization of indirect ELISA utilizing different dilutions of positive and negative human sera.

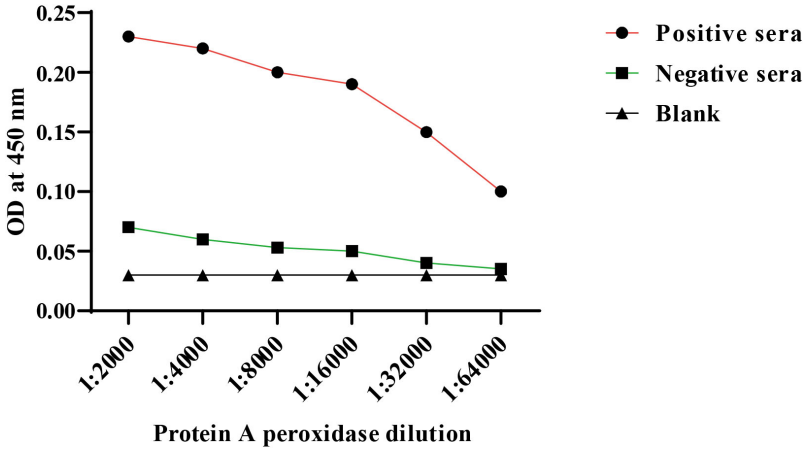

Fig. 6. Optimization of an indirect ELISA utilizing different dilutions of protein A peroxidase conjugate.

3.4. Proof of concepts and in-house validation of anti-SARS-Cov-2 IgG ELISA assay

Negative samples ($n = 50$) based on commercial kit SARS-CoV-2 IgG kit (Abbott, Ireland), were used to calculate the ELISA assay's preliminary cut-off value, which was calculated as mean + 3 × standard deviation. This developed ELISA's preliminary cut-off value was 0.24. Within the 50 negative samples used, the OD450 readings of 40 negative samples were less than the preliminary cut-off value (Fig. 7A), while ten samples were above this value, indicating false positive results. On the other hand, 73 samples of the positive samples ($n = 105$) predetermined with the same commercial kit SARS-CoV-2 IgG kit (Abbott, Ireland), were identified as positive (Fig. 7B) and 32 of these samples were identified as false negative. Similar results were obtained from three independent experiments. Next, the accuracy of this test was assessed using ROC analysis, and the cut-off value that provides the best sensitivity and specificity was determined. With an area under curve (AUC) of 0.751 ± 0.047 and a 95 percent confidence interval of 0.659 to 0.843, our in-house ELISA displayed remarkable accuracy. The cut-off value for 78.1% sensitivity and 72% specificity was 0.179 (Fig. 7C). The developed assay provides 69.5 % sensitivity, 88 % specificity, 75.5 % agreement, 92.3 % PPV and 56.5 % NPV which was estimated as described in statistical Analyses) (Fig. 7D).

4. Discussion

Coronavirus disease 2019 (COVID-19) is an emerging disease caused by the coronavirus 2 (SARS-CoV-2) that causes severe acute respiratory syndrome. Antibody testing is currently focused on the spike (S) and nucleocapsid (N) structural components of SARS-CoV-2. The S protein is formed of two subunits (S1 and S2),

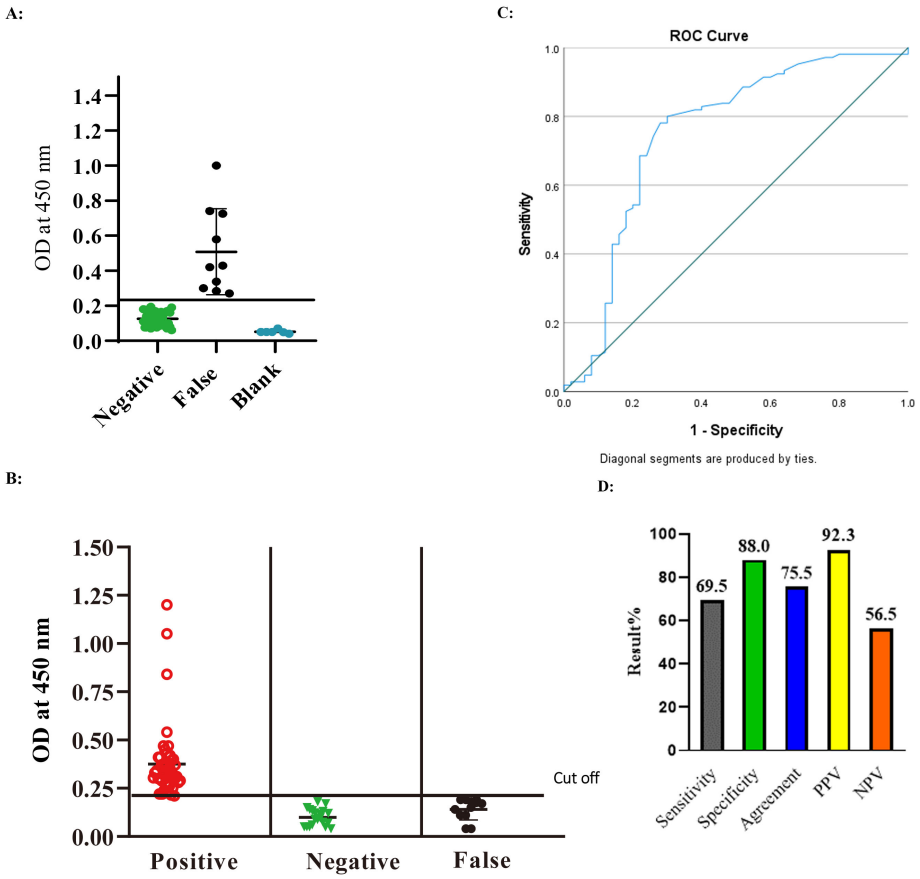

Fig. 7. A: The Cut off value of the ELISA assay. B: Testing of human sera for the presence of IgG antibody directed to SARS-CoV-2 RBD protein using the optimized ELISA conditions. C: Receiver operating characteristics (ROC) analysis of the optimized developed indirect ELISA. D: Sensitivity, Specificity, Agreement, PPV and NPV of the developed ELISA utilizing in-house expressed purified SARS-CoV-2 rRBD-6×His.

and the RBD in S1 is responsible for recognizing angiotensin converting enzyme 2, a human cell surface receptor [20]. Antibodies that target the RBD in S1 can neutralize SARS-CoV-2 by preventing it from infecting host cells, hence anti-RBD antibodies serve as both immunological and neutralizing antibodies (nAbs) [21]. Several successful vaccines based on the S protein are currently available and immunization campaigns have begun around the world [22–24]. Anti-RBD antibodies and nAbs serological tests are becoming increasingly important for assessing humoral immunity against SARS-CoV-2 not only after vaccination but also in the assessment of previous infections.

Antibodies to SARS-CoV-2 can now be tested using a range of methods, including point-of-care lateral flow-based devices, high-throughput immunoassay analyzers, and manual approaches like ELISA. The nucleocapsid protein (N), full-length spike protein (S), S1 subunit, and receptor binding domain (RBD) of the S protein are among the antigens studied and used to generate kits for serological testing [25]. These kits were approved by FDA, applied after using specimens of 60 to 120, and showed between 80 to 98% sensitivity. Because SARS-CoV-2 antibody assays use a variety of techniques and antigenic targets, they must be thoroughly assessed before being used in clinical settings [26].

Similar to numerous developing countries, Egypt relies on the import of serological kits, which is an expensive and time consuming strategy. In addition, a sustainable supply of imported kits cannot be guaranteed in case of flight restrictions, border closures or urgent need of the kits by the manufacturer's countries. Accordingly, developing a local SARS-CoV-2 serological assay is of particular importance for the current pandemic and may represent a prototype for the rapid diagnosis of future emerging pathogens.

Since the RBD of SARS-CoV-2 spike protein is one of the important domains of the virus used in vaccine trials, drug development, and diagnostic purposes, several mammalian expression systems were applied to

produce it [27,28]. Compared to prokaryotic expression systems, mammalian systems are costly, time consuming, sometimes have low production yield. However, mammalian systems produce efficiently folded and post-translationally modified proteins. To match our goal of developing a local SARS-CoV-2 serological assay, we based our assay on RBD cloned with an N-terminal 6× histidine tag in pET30b(+) and expressed it in *E. coli* BL21(DE3) Rosetta Gami. We reasoned that the prokaryotic expression would provide a relatively high yield of pure RBD in addition to being time and money saving and requiring less sophisticated equipment. In parallel, a study by Du and colleagues showed that rRBD of the spike protein of SARS virus produced in either *E. coli*, mammalian, or insect cells maintained intact conformation, which further motivated us to explore this possibility [29].

So far, we report for the first time the successful expression of SARS-CoV-2 rRBD in four different *E. coli* expression strains: *E. coli* BL21(DE3) pLysS, *E. coli* BL21(DE3) Arctic RIL, *E. coli* BL21(DE3) C43 and *E. coli* BL21(DE3) Rosetta Gami. Our results corroborate with Gao and colleagues work of successful expression of RBD with a C-terminal histidine tag in *E. coli* BL21 (DE3) Rosetta Gami from pET-28a(+) using 1 mM IPTG as an inducer [11]. The same study reported that the renatured RBD could efficiently bind to ACE2 as was also shown by He and colleagues for RBD expressed from *E. coli* (BL21) [10]. Our study adds an extra evidence of the feasibility of RBD expression in *E. coli* at different IPTG concentrations, induction durations, and temperatures. We successfully expressed the 30 kDa RBD protein using a low concentration of IPTG (0.25 mM) at 28°C for 4 hours (Supplementary figure 2a) or 20°C overnight (Fig. 2), purified and refolded the recombinant protein that can be used directly in several applications such as vaccine trials, functional binding with other proteins or drugs, and diagnostic approaches. Remarkably, under all our tested conditions and the previous reports, RBD was almost exclusively localized in inclusion bodies and required solubilization and renaturation before any downstream application.

To the best of our knowledge, this study is among the unique studies establishing a diagnostic immunoassay using SARS-CoV-2 rRBD protein produced in *E. coli*. Our optimized ELISA test was used to detect IgG in human sera of infected COVID-19 patients, and showed sensitivity and specificity of 69.6% and 88%, respectively. Recently, Villafañe et al. [30], showed 100% serological concordance, using an in-house ELISA test based on recombinant RBD expressed in mammalian system, with commercial test based on the full-length spike protein (COVIDAR). However, the sensitivity of COVIDAR test, reported for IgG detection was 72% to 74% between 2 and 3 weeks from the onset of symptoms and seroconversion increased up to 90.4% after 3 weeks [31]. Moreover, Márquez-Ipiña and colleagues showed the reactivity of a shorter RBD fragment (N318-V510) expressed in *E. coli* BL21 strain C41 with 55 sera samples obtained from COVID-19 convalescent individuals [12]. However, the direct ELISA protocol reported was longer in time (including an 8 hours incubation for coating) and the assay time was only reduced by using a sandwich ELISA protocol [12].

Interestingly, we have tested the same sera samples used in this study, using the chemiluminescence kit from Abbott which uses the nucleocapsid protein (NP) as a coating antigen. These samples showed high IgG titer compared to their reactivity to our rRBD. Our results may correlate with the conclusion of Fafi-Kremer et al. [32] that a subset of patients may have an insufficient humoral immune response against a specific domain of the SARS-CoV-2 compared to their high immune reaction to another protein of the same virus.

To date, numerous mutations were identified in different antigens of the SARS-CoV-2 virus. Some of these mutations affected its interactions with the host cells and susceptibility to the disease. Barton et al. [33] concluded that mutations in the RBD enable the escape of immune responses such as K417N/T and the mutation E484K augments both ACE2 viral binding and also immune escape. This hypothesis may explain the discrepancy in the results within the samples we tested.

Although, both sensitivity and specificity are essential parameters for an assay, assessing the negative predictive values (NPV) and positive predictive values (PPV) of each assay are more valuable in determining how the assay can best be applied and interpreted. For any assay results, both PPV and NPV, respectively point to the probability that an assay can successfully determine whether individuals do or do not have a specific condition. Upon screening a percent of the population, both metrics are more significant than are sensitivity and specificity [34]. In our ELISA assay using the prokaryotic rRBD, PPV and NPV were 92.3% and 56.5%, respectively. The PPV value indicates reliable testing of the optimized in-house ELISA test.

We believe that our study had two limitations. First, sera were collected from 155 patients just before starting the vaccination protocols. Therefore, it was not possible to increase the number of samples especially that most vaccines available to date use the mRNA of S pro-

tein including the RBD. Second, more investigations are required to test the reactivity of our SARS-CoV-2 rRBD-6xHis with anti-SARS-CoV-2 IgM for diagnosis of early infection.

In conclusion, we successfully developed a rapid and sensitive in-house ELISA assay based on SARS-CoV-2 rRBD-6xHis expressed and purified from *E. coli*. Our in-house optimized assay detects anti SARS-CoV-2 IgG in human sera is an economical system that can be used in serological tests, functional and vaccine studies.

Author contribution

N.H. and E.A. carried out protein expression and purification. A.S., I.A., M.E., and M.S. conceived data assembly. A.E., A.T., and As.E. performed the immunological assays. A.T., A.E., N.H., Y.S. carried out manuscript writing. Y.S. was responsible of funding acquisition. Y.S., N.H., A.E., and A.T. designed the experiments. Y,S. demonstrated cohort management and supervision.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval

All the steps were approved by the Ethical Review Board of the National Research Center, Egypt (NRC#20192) according to Helsinki Declaration (1975).

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/HAB-220003.

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