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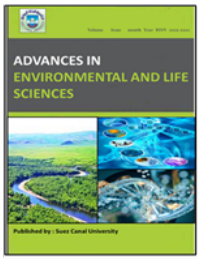

The Frequency of SARS-CoV-2 Anti-spike RBD IgG Antibodies in the Fully Vaccinated and Recovered COVID-19 Infected Individuals

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Abstract

Background: SARS-CoV-2 genome encodes different kinds of structural and nonstructural proteins. The spike structural protein is the prime target for vaccine manufacturing, drug development, and diagnostic assays.

Methods: In this cross-sectional study, we assessed the developed Receptor Binding Domain (RBD) protein in 520 serum samples. The samples were stratified according to vaccine status into two groups: Group 1 consisted of 274 pre-vaccinated samples collected from April to December 2020 (109 were asymptomatic contacted with COVID-19-patients; 100 patients had positive RT-PCR test for SARS-CoV-2 infection, and 65 were convalescent-COVID-19 cases), and group 2 consisted of 246 post-vaccinated participants with or without past-infection recruited from students, coworkers, and staff members from November 2021 to March 2022. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was performed to evaluate the seroprevalence of the SARS-CoV-2 immunoglobulin G (IgG) across both groups.

Results: The sensitivity and specificity of the RBD-developed protein were 88.4% (95% CI: 84% - 92%) and 100% (95% CI: 88%- 100%), respectively in the pre-vaccinated group. 213 out of 274 of the pre-vaccinated samples were positive. Meanwhile, the RBD-developed protein provided 100% RBD IgG seropositivity in the recovered COVID-19 cases. Moreover, the results were seropositive in all post-vaccinated candidates (n=246) and the results were reliable in 70 negative pre-pandemic sera specimens.

Conclusion: The developed S-RBD protein has the potential to be a successful screening method in COVID-19-vaccinated or infected individuals.

1. Introduction

Zoonotic viruses like the SARS-associated coronavirus (SARS-CoV) can infect humans, triggering mild to severe illness. SARS-CoV-2 is a non-segmented positive-strand RNA (+ssRNA) enveloped virus that is genetically classified into Betacoronaviruses [1]. SARS-CoV-2 spreads primarily through human airborne droplets, causing systematic and respiratory diseases [2], [3]. SARS-CoV-2 genome encodes different kinds of structural and nonstructural proteins. The SARS-CoV-2 structural proteins; include spike (S), nucleocapsid (N), envelope (E), and membrane (M); are mainly responsible for cell membrane attachment, viral fusion, immune evasion, antibodies neutralization, and viral pathogenesis [4]. Particularly, S protein is cleaved into two non-covalently associated subunits; S1 subunit which is composed of a receptor-binding domain (RBD) that enhances the angiotensin-converting enzyme 2 (ACE2) binding

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capability, and the S2 subunit, which mediates the viral fusion to enter the host cell [5]. Therefore, S protein is the prime target for vaccine manufacturing, drug development, and diagnostic assays. Most of the companies that developed the current vaccines used the S protein, whether using the full length of spike protein or the S1 RBD form [6] [7].

Likely, the S-RBD-based kits are powerful targets for COVID-19 detection. There are two main laboratory approaches for SARS-CoV-2 diagnosis: serological-based and molecular-based methods. The foremost serological techniques for the detection of antibodies against COVID-19 infection are enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunoassays (CLIA), and point-of-care tests (POCT). Meanwhile, the fundamental molecular-based tool is the reverse transcription–polymerase chain reaction (RT-PCR) [8]. RT-PCR is considered as the gold standard test for the diagnosis of COVID-19; however, it may lead to false negative results in some cases. Ai et al. revealed that molecular-based tools may result in ambiguous results for about 29% of patients when compared with their positive chest CT results [9]. Moreover, there are several complicated issues, such as sampling, sample transportation, necessity of skillful operators, which is hard to implement in some labs, and the optimal time of viral detection. Those issues may contribute to a misleading PCR test. Alternatively, the serological assays are easy-to-handle, well-established in low resource settings, and provide potential benefits in the outbreaks [10]. Most commercial serological kits are designed to identify the generated antibodies against S and N proteins. However, reactivity to those proteins distinguishes between past-infection and active immunization. Therefore, based on our developed proteins described in a previous study [11], we believed that our developed proteins could detect SARS-CoV-2 antibodies, which will provide a cost-effective serological test.

The purpose of this study was to determine the frequency of anti-spike RBD IgG antibodies in fully vaccinated and recovered COVID-19 infection individuals, as well as the efficacy of the first Egyptian ELISA kit developed for SARS-CoV-2 detection.

2. Experimental

3. Materials and Methods

This cross-sectional study was approved by the ethical committee of Faculty of Medicine, Suez Canal University with a reference number #4771. A total of 520 eligible Egyptian participants were enrolled and signed informed written consents. A questionnaire included the type of COVID-19 vaccine that was received, the date of vaccination, and a history of previous COVID-19 infection. Then, venous samples were obtained, and the participants were stratified into two groups (table 1). The pre-vaccinated group, 274 samples had been collected from an accredited private medical laboratory, Menoufia, Egypt from April 2020 to December 2020.

Of the 274 individuals, 109 were asymptomatic subjects contacted with COVID-19-patients; 100 patients had positive RT-PCR tests for SARS-CoV-2 infection, and 65 were convalescent-COVID-19 cases. PCR-confirmed and convalescent-COVID-19 cases went through mild to moderate symptoms and none were hospitalized. The suspected (n=109) and infected sera samples (n=100) were collected 5-14 days after the onset of clinical symptoms. Meanwhile, the median time of the sample collection for the recovered-COVID-19 subgroup was 40 days (IQR: 30-55 days).

The second group included 246 post-vaccinated with or without prior infection participants recruited from Badr Academy, Badr city, Cairo and Faculty of Medicine, Suez Canal University, Ismailia, Egypt from November 2021 to March 2022.

The samples were collected following firm inclusion criteria: age between 18 to 65 years, at least 14-days after the second dose of immunization or from the onset of symptoms, and subjects who had been fully vaccinated for less than 6 months' duration.

On the other hand, the exclusion criteria were: (A) individuals who had been fully vaccinated for more than 6 months or subjects with a single dose of a vaccine; (B) symptomatic participants with common cold or flu symptoms at the time of the study; and (C) immunocompromised patients.

A control group was assigned from pre-pandemic samples, 70 serum samples that had been collected from healthy individuals from November 2018 to June 2019.

Table 1: Questionnaire for participants

Questions
What type of vaccine did you receive?
Date of 1st dose of COVID vaccination.
Date of 2nd dose of COVID vaccination
Date of (3rd dose) booster dose (if present).
Were you previously infected with COVID-19?
Date of previous COVID-19 infection.
What was the result of COVID-19 PCR test (If present)?
Did you have COVID-19 symptoms during the last 3 months?
Were you contacted COVID19-patients during the last 3 months?
Date of last common cold flu symptoms.

3.1. Statistical analysis

All analysis was performed using Microsoft Excel data analysis tool (Office 365).

3.2. Serological assay

We used the developed recombinant SARS-CoV-2 receptor binding domain (RBD) of spike protein to assess the seroprevalence of SARS-CoV-2 immunoglobulin G (IgG) across different groups and compared it to another commercially available ELISA kit (Tecan, Hamburg Germany GMBH).

3.3. Evaluation of IgG response by Indirect ELISA

Indirect ELISA was performed to measure the seropositivity of the S-RBD IgG antibodies in both groups. The presence of antibodies in the samples was analyzed by the SARS-CoV-2 IgG ELISA kit, Tecan, Hamburg, Germany GMBH (Cat. #: 30177447), following the manufacturer's instructions. The TECAN IgG ELISA kit has 100% sensitivity, and 99.5% specificity.

To evaluate the RBD-IgG antibodies in the sera using the developed protein, briefly, 96-well Immunolon-I microtitration plates (Nunc, IL, USA) were coated with RBD protein diluted in sodium carbonate - bicarbonate buffer, pH 9.6 (1 protein: 50 coating buffer) (100μl/well) overnight at 4°C. After washing with phosphate-buffered saline (PBS: pH 7.4), the wells were blocked with 4% skimmed milk in PBS (1x), pH 7.4, for 1 h at 37 °C (200μl/well). Then, the PBS-washing step, 100 μL/well of each serum sample diluted 1:100 in PBS (pH 7.4) containing 1% skimmed milk was added in duplicates to the wells and incubated for 1 h at 37 °C. Then, the goat anti-human IgG conjugated with alkaline phosphatase (ALP) (Cat.#:31310, Invitrogen) was added at a dilution of 1:1000 in 1% skimmed milk and incubated for 30 min at room temperature. To develop the color, the p-Nitrophenyl Phosphate (PNPP) substrate solution (Cat.#:34047) was added after three washing cycles. After 15 min of incubation at room temperature, 100 μl of sodium hydroxide (3M) as the stop solution was added. The absorbance values were measured at 405 nm using TECAN infinite F50 micro-plate reader. The results were qualitative and expressed as a positive/reactive or a negative/nonreactive.

4. Results:

Five-hundred and twenty Egyptian candidates were categorized into two groups 1) non-vaccinated with a history of the SARS-CoV-2 infection group (n=274, 115 females, and 159 males), 2) post-vaccinated with/without the past-infection group (n=246, 113 females, and 133 males). The median age of the pre-vaccinated participants was 34 years (27-44ys) (Table 2).

Between April and December 2020, 175 infected (5-14 days after the onset of symptoms) and convalescent COVID-19 participants were recruited (mean time after onset of symptoms was 40±9 days).

The median age of the post-vaccinated was 26 years (21-36 yr). Two-hundred forty-six had been vaccinated from September 2021 to February 2022. They were immunized with the available vaccines in Egypt (Table 3). The median time after the second vaccination dose was 85 days.

According to the history of SARS-CoV-2 infection

Table 2: Socio-demographic data of the pre-vaccinated andpost-vaccinated subjects included in the study (n=520)

	Pre-vaccinated n=274	**Post- vaccinated n=246**
Age; years: Median (IQR)	34 (27-44)	26 (21-36)
Gender; n (%)		
Female	115 (42%)	113 (46.7%)
Male	159 (58%)	133 (54%)
Average duration after 2nd dose of the vaccination	-	85 days
Occupation of post-vaccinated		
University Student	-	104
Faculty staff member and coworkers	-	142
History of COVID-19 infection in the last 3 months in pre- and post-vaccinated individuals; n (%)		
No COVID-19 symptoms	-	49 (20%)
Flu-like symptoms	-	110 (45%)
Positive COVID PCR test and recovered- COVID-19	165(60.2 %)	87 (35%)
Asymptomatic and contacted with COVID19- patients	109 (39.8%)	-

Table 3: The vaccinated participants' numbers withdifferent vaccines included in the study (n=246)

Vaccine type	**Males**	**Females**	**Total**
Sinovac-CoronaVac	27	33	60
AstraZeneca	29	23	52
Sinopharm	18	32	50
Pfizer–BioNTech	25	20	45
Sputnik V	30	2	32
Janssen	4	3	7
Total	133	113	246

in this group, 87 (35%) cases had been infected between September and October 2021 and had positive SARS-CoV-2 RT-PCR. While 110 (45%) participants reported that they had no RT-PCR test report, although they had mild to severe flu-like symptoms and fever during the three months before the sample collection. The remaining 49 (20%) participants stated that they had no suspected COVID-19 symptoms during the last three months. Seventy samples were obtained as a control group (39 females and 31 males).

4.1. Verification of the recombinant protein against the commercial kit

The synthetized RBD protein was developed in the Recombinant proteins laboratory (RPL), Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt [11].

Our primary goal was to validate the RBD protein and assess the seroprevalence of IgG against SARS-CoV-2 in a variety of clinical cases. We compared our results by testing the same samples with the TECAN SARS-CoV-2 IgG ELISA kit (n=520). The cutoff value was measured and calculated based on the manufacturers' instructions. In parallel, the performance of the developed protein was assessed by testing the RBD-IgG antibodies in the same serum samples from pre-pandemic (n=70), suspected (n=109), infected (n=100), COVID-19-recovered pre-vaccinated cases (n=65), and vaccinated individuals (n=246). Coating with 1:50 of

antigen produced a reliable signal in the different kinds of sera that titrated with serum dilution (1:100). The pooled negative control was run on each plate to determine the cut-off. The cut-off was calculated as the mean of O.D. of duplicated negative samples (mean value ≤0.05) and then by adding 0.1 to the average value, the cut off was ≥0.15. Hence, the results were expressed as reactive (positive) or nonreactive (negative), and the result was considered positive when the absorbance reading was ≥0.15.

The results were reliable and comprehensive to the developed protein, as all samples were seropositive in post-vaccinated group, and nonreactive in the pre-pandemic group. Remarkably, 213 out of 274 of pre-vaccinated samples were positive. Particularly, the pre- and post-vaccinated samples recorded positive IgG with the average values of optical density (O.D.) equaled to 0.98, 1.14 respectively. The 70 pre-pandemic samples were nonreactive (average O.D. = 0.05), we found an average absorbance value of 0.05 for the RBD-developed protein compared to 0.08 for the commercial kit, as shown in Figure 1.

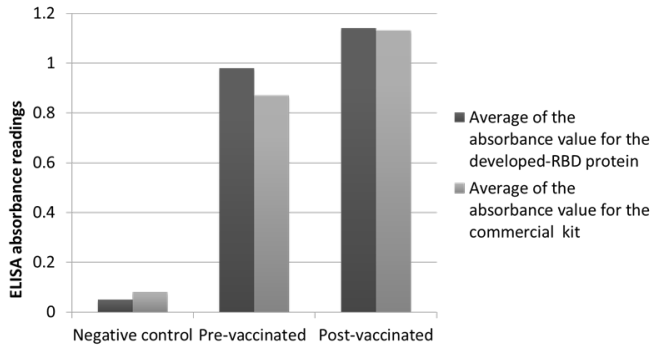

Figure 1: Comparison between the average absorbance values for the commercial kit and developed-RBD protein

Figure 1 illustrates that the average of the absorbance for the developed protein in the pre-vaccinated group was slightly higher than the commercial kit O.D. values (O.D.=0.98, 0.87 respectively). Meanwhile, in the post-vaccinated group, the developed protein and TECAN IgG ELISA kit had approximately the O.D. values (O.D.=1.14, 1.13 respectively). When the developed S-RBD protein was evaluated across the three groups, the O.D. readings values for the post-vaccinated samples were the highest (Figure 2).

4.2. Sensitivity and specificity

Overall, in the pre-vaccinated group, the RBD-developed protein showed a sensitivity of 88.4% (95% CI: 84% - 92%) and 100% (95% CI: 88%-100%) specificity. The Negative Predictive Value (NPV) and the Positive Predictive Value (PPV) were 47.5% (95% CI: 39.6% - 55.7%) and 100% respectively as shown in the table 5. For the COVID suspected cases in this group, 80 out of 109 cases reported a positive PCR test. Only 65 specimens were positive RBD-IgG when using the developed protein compared to 80 samples were seropositive by TECAN SARS-CoV-2 IgG kit (table4). In contrast, all of the 29 negative PCR asymptomatic individuals were seronegative by our developed RBD protein. Whereas 25 were nonreactive and 4 individuals were reactive when TECAN SARS-CoV-2 IgG kit was performed. Therefore, the sensitivity, specificity, the PPV and the NPV in this group were 84.2 % (95% CI: 75.30% - 90.88%), 100% (95% CI: 88%-100%), 100%, and 65.9% (95% CI: 54.86% - 75.47%) respectively.

Regarding the infected pre-vaccinated group, 17 patients out of 100 had nonreactive antibodies test; however, they had an infection and reported positive PCR test. While TECAN SARS-CoV-2 IgG kit tested only four nonreactive samples. For the 65 convalescent-COVID19 participants, the RBD-developed protein provided 100% RBD IgG seropositivity by the developed protein and TECAN kit (table 4 and 5).

We also observed that the Sinopharm applicants' subgroup had the lowest average of the IgG absorbance in contrast to their counterpart (Sinovac-CoronaVac subgroup) (average O.D.=0.5, 1.01 respectively). Interestingly, we noticed that the average of IgG absorbance of Sputnik V participants' sera was the highest value compared with mRNA-based (i.e., Pfizer–BioNTech) and viral vector-based candidates (i.e., AstraZeneca and Janssen) (average O.D.= 1.82, 1.34 respectively) (Figure 3).

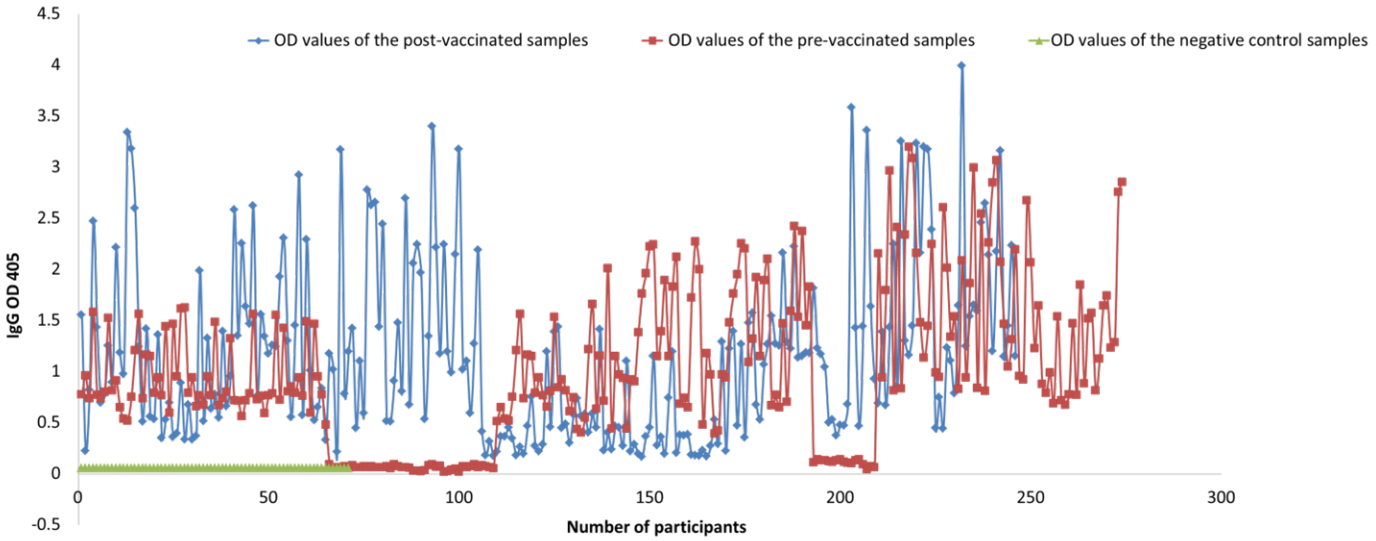

Figure 2: The scattered plot illustrates the reactivity of the negative control samples (n=70),Pre-vaccinated samples (n=274), and Post-vaccinated samples (n=246) in the developed-RBD SARS-CoV-2 protein.

Table 4: Comparison between the results of our developed RBDIgG and Tecan SARS-CoV-2 IgG ELISA in pre-vaccinated participants (n=274)

	RBD IgG		**Tecan**		**PCR results**	
	Positive	Negative	Positive	Negative	Positive	Negative
Asymptomatic n=109	65	44	84	25	80	29
Infected n=100	83	17	96	4	100	0
Recovered n=65	65	0	65	0	65	0
Total	213	61	245	29	245	29

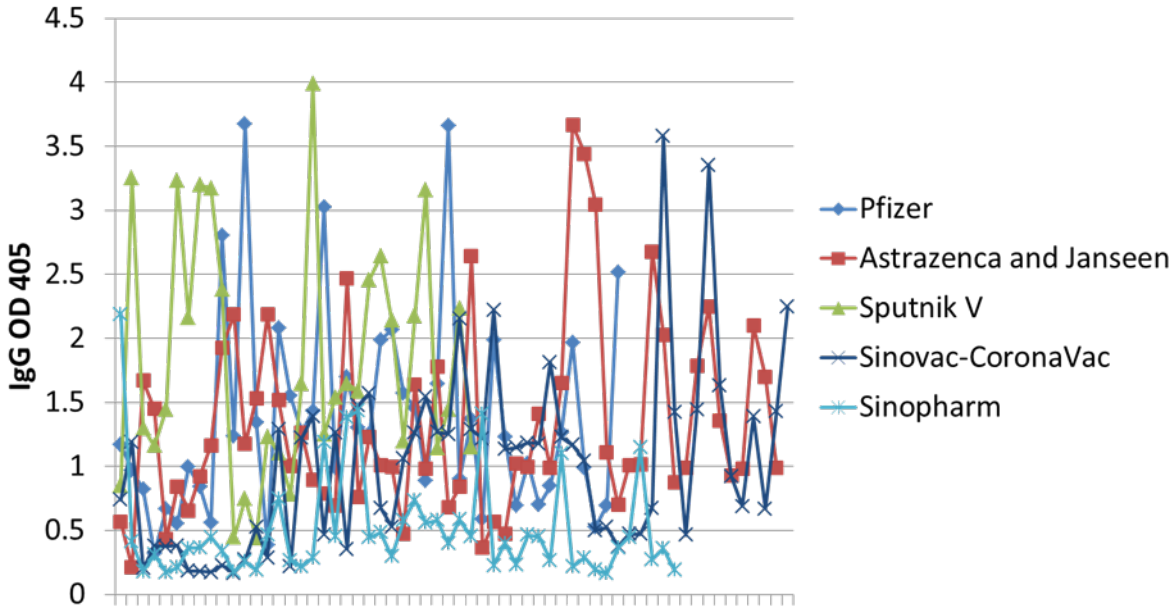

Figure 3: The absorbance of the RBD-IgG in the different types of vaccines

Table 5: Comparison between the sensitivity and specificity ofour developed RBD IgG and Tecan SARS-CoV-2 IgG ELISA in the pre-vaccinated subjects(n=274)

The pre-vaccinated subjects	Our RBD IgG
Sensitivity	88.4% (95% CI: 84% - 92%)
Specificity	Specificity 100% (95% CI: 88%- 100%)
Positive Predictive Value (PPV)	100%
Negative Predictive Value (NPV)	47.5% (95% CI: 39.6% -55.7%)
Asymptomatic cases	Our RBD IgG
Sensitivity	84.2% (95% CI: 75.30% - 90.88%)
Specificity	100% (95% CI: 88% - 100%)
Infected patients	Our RBD IgG
Sensitivity	83% (95% CI: 74.18% - 89.77%)
Specificity	—

5. Discussion

ELISA and chemiluminescence immunoassays (CLIA) are the most powerful and more sensitive serological assays that could be used to identify the immune response. Here, we applied the recombinant RBD of the SARS-CoV-2 spike protein that was developed using the Baculovirus Expression Vector System (BEVS) as previously described [11] on 520 pre- and post-vaccinated human sera and 70 pre-pandemic serum samples as a negative control using a qualitative indirect ELISA technique.

In this cross-sectional study of the vaccinated and pre-vaccinated non-hospitalized individuals who were asymptomatic or had mild-to-moderate symptoms of COVID-19, we found that our developed protein has the ability to detect the generated antibodies produced against SARS-CoV-2 infection and vaccination. Head-to-head comparison of the developed RBD protein against TECAN IgG ELISA kit proved that there were non-significant differences between results as the negative control group tested nonreactive and all post-vaccinated samples were seropositive. These findings are consistent with previous studies [12] [13]. Whilst the sensitivity of the developed RBD protein was 88.4% (95% CI: 84% - 92%) with 100% specificity (95% CI: 88%- 100%) in the pre-vaccinated group. Żak et al., exhibited a high sensitivity value that reached to 92.5% and 100% specificity [14]. In 2021, Yassine et al. evaluated the sensitivity of five different ELISA kits after different time points from symptoms onset (≤7->14 days) using serum samples. The sensitivity values varied from 57.1% (39.1%–73.5%) to 90.0% (77.0% –96.0%) [15]. Interestingly, another study that was conducted on dried blood spot samples reported 100% sensitivity (95% CI: 95.8-100%) and 96% specificity (95% CI: 88.7-99.1%) in the past-infected subjects [16].

RBD-IgG antibodies are the critical element of immune protection against the viral infection. Zhao et al., demonstrated that 80% of the cases had seroconversion to IgG due to natural infection and developed by day 15 after disease onset [17]. In previous studies, the median time of the detection of IgG antibodies was 8-day or 11-day of the post-symptom onset with all of patients reaching IgG seropositivity by 19 days [18] [19]. The IgG persistence level would be maintained from 6 months to 1 year after the infection. Surprisingly, the disease severity would not contribute to the RBD-IgG persistence level [20]. In the current study, the sensitivity of the developed protein was lower in the asymptomatic who were PCR-confirmed during the early phase of illness (84.4%, 83% respectively) as the time of the sample collection from the symptom's onset was 5-14 days. This might elucidate why only 213 (77.7%) participants tested positive to IgG and why the sensitivity of the RBD-developed protein varied among the subgroups. Remarkably, seventeen samples out of the 100 RT-PCR-confirmed cases exhibited undetectable antibody level, so that the sensitivity slightly dropped to 83% which needs to re-test those samples two weeks later. While the median time of the sample collection for the recovered-COVID-19 subgroup was 40 days (30-55 days). Consequently, all the 65 specimens were seropositive when using our developed protein. TECAN showed the highest sensitivity in the infected, asymptomatic, and

recovered serum samples compared to our findings. Hence, these results point to the necessity for improving the sensitivity in the early phase of infection. Also combining IgM immunoglobulin test along with IgG may enhance the results.

The Ministry of Health in Egypt started the COVID-19 immunization March 4^{th}, 2021. More than 39.4 million are fully vaccinated and 3.4 million had a third dose. As by September 11^{th}, 2022, a total of 98,019,706 vaccine doses have been administered. COVID-19 mRNA and viral vectored-based vaccines such as Pfizer (BNT162b1, BNT162b2), Moderna, AstraZeneca and Johnson & Johnson vaccines are strongly immunogenic by activating the humoral and cell mediated immune responses that may provide the antibody longevity with rational safety profiles [21] [22].

Due to the hybrid and vaccine-induced immunity, the protection against specific disease or its severity has been emerged. Subsequently, the detection of the spike IgG level is the fundamental test for evaluation of the long-lasting immune response particularly in the vaccinated subjects. Prior studies addressed the longevity of the IgG immunoglobulin response after the active immunization. Several studies reported that the persistence of IgG antibodies was highest at the first 3 months and started to decline during 6-8 months after a full vaccination with mRNA-based and viral vector-based vaccines [12] [23] [24] [25] [26] [27]. In the current study, the median time after the full vaccination was 85 days, and all post-vaccinated samples were seropositive. However, 80% of the participants had a history of COVID-19 infection or its symptoms in the last 3 months. These results agreed with Lo Sasso, et al., who found that there is no significant difference in immune response between vaccinated subjects with or without SARS-CoV-2 natural infection [28].

The lowest average of the IgG in the Sinopharm candidates could be explained as the median time after the vaccination was 130 days in the Sinopharm subgroup whereas, the average period of sample collection for Sinovac-CoronaVac subgroup was 93 days. There may be another explanation, the limited cold-chain infrastructure or low colloidal stability of the components especially in the low resource settings may contribute to this issue. Meanwhile, the duration was 29 days after Sputnik V administration and 75 days for Pfizer–BioNTech, AstraZeneca and Janssen subgroups.

The strength point in our paper is the variation of the clinical cases. Due to the discrepancies in the immune status and age that may impact on the seroconversion [19], it would be beneficial to perform a further quantitative assay on a large sample size. In addition, evaluate the RBD-IgM antibody response in the pre-vaccinated samples. Besides, the determination of the anti-nucleocapsid (anti-N) antibody IgG in the post-vaccinated subjects may enhance the results to serve as an indicator of prior infection.

6. Conclusion

Overall, this study assessed the developed receptor binding domain (RBD) protein in the different clinical cases that were collected during different periods of time. The developed protein revealed high specificity in the pre-pandemic, pre-vaccinated and post vaccinated samples. Subsequently, this protein has the potential to become a successful screening approach for COVID-19 vaccinated individuals or infected cases. It will provide a cost-effective, specific, and valuable serological tool. We seek for radically achieving a sustainable cost reduction by reducing trade costs of COVID-19 detection tools. Therefore, the quantitative assay on a large sample size will be carried out in the further study.

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8. Declaration of Competing Interest:

The authors have no competing interests to declare that are relevant to the content of this article.

9. Authors' contributions:

All authors have contributed to the manuscript in significant way, have reviewed and agreed upon the manuscript content.

10. Ethical approval:

All data was collected in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee, Faculty of Medicine, Suez Canal University with reference number #4771.

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