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ORIGINAL ARTICLE

SARS-CoV-2 antibody responses in COVID-19 confirmed cases in a university hospital

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Abstract

Introduction: COVID-19 diagnosis relies on the detection of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) genome or antigen. Antibodies have limited use for diagnosis but contribute to serological studies and epidemiologic analysis. We aimed to elucidate anti-N and anti-S antibody responses among patients with PCR-positive and indeterminate results, and/or those with rapid molecular test-positive results. *Materials and Methods:* 267 serum samples from 199 patients were collected from April 2020 until November 2021. The timing of serum sampling was determined from the diagnostic testing date. Anti-N and anti-S were tested using the ECLIA platform. The patients' COVID-19 clinical categories were retrieved from an online system, and data were analysed using SPSS, version 28. *Results:* 82.4% of patients' PCR were positive, while 17.6% were indeterminate. Overall sero-reactivity rate for anti-N and anti-S is 54.3% and 65.3%, respectively. The sero-reactivity rate and level of anti-S antibodies peaked at day 9 to 14 post-PCR but declined after day 15. Anti-N has significantly higher sero-reactivity rate ($p<0.001$) in the PCR-positive group. Anti-S sero-reactivity rate was significantly higher in mild COVID-19 infections ($p=0.027$). *Conclusion:* In a cohort with the majority belonging to the pre-vaccination period during the COVID-19 pandemic, sero-reactivity of the SARS-CoV-2 antibodies were highest from week two following laboratory diagnosis. Currently, the clinical utility of SARS-CoV-2 antibody testing is limited due to the current endemicity of the virus as well as the vaccinated population. Further research could explore how viral evolution and immunisation impact antibody responses and the detection of the various antibodies.

Keywords: COVID-19 nucleic acid testing, COVID-19 serological testing, nucleocapsid protein, SARS-CoV-2, spike glycoproteins

INTRODUCTION

Coronavirus disease (COVID-19) is an infection caused by the novel Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) virus. It was first reported in late December 2019 in Wuhan city and rapidly spread throughout the globe, causing a pandemic within a few months. After around three years, the number of confirmed cases markedly increased to more than 600 million, with 6.8 million deaths recorded.[1]

The preferred initial diagnostic test for COVID-19 is by detection of SARS-CoV-2 RNA in respiratory samples using nucleic acid amplification tests (NAATs) such as reverse-transcription polymerase chain reaction (RT-PCR) and rapid molecular tests.[2,3] NAATs are characterised by rapid detection, high sensitivity and specificity, and are regarded as the "gold standard" for virus detection.[4] PCR allows SARS-CoV-2 RNA detection 1 to 3 days prior to the onset of symptoms and can persist for up to 2 weeks in severe cases.[5]

However, PCR can give false negative results in patients in the late stage of infection when the viral load is very low and is influenced by pre-analytical factors such as sampling variability.[6] PCR can produce indeterminate results, which indicates that only one of the two or more gene targets was identified. Indeterminate PCR

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results can be considered presumptive positive results, given the high specificity of NAAT assays[7], but most of the time, repetition of the test or re-testing of new specimens is required.

Serological assays measure antibodies that remain detectable in the serum even after the acute phase of illness. Although it may not be well-suited to detect acute infections, it may be applied in some circumstances. The Infectious Diseases Society of America (IDSA) suggests antibody testing can be used to demonstrate evidence for prior SARS-CoV-2 infection three to five weeks after symptom onset, using an assay detecting IgG, IgG/IgM, or total antibodies to nucleocapsid protein. Serologic testing may assist with the diagnosis of multisystem inflammatory syndrome in children and may be a useful metric to identify immunocompromised patients who are candidates for immune therapy if they test negative for anti-S antibody.[8]

In addition, the detection of anti-SARS-CoV-2 antibodies allows the identification of individuals who mounted strong antibody responses and who could serve as donors for the generation of convalescent plasma therapy and enable the accurate determination rate of infection in an affected area, which is an essential variable to determine the case fatality rate.[9,10]

SARS-CoV-2 infection elicits the development of IgM and IgG antibodies, which are the most useful for assessing antibody response. Unlike the typical pattern of elevated IgM in the acute phase followed by IgG, these antibodies appear nearly simultaneously in serum in SARS-CoV-2 infection.[9] The median seroconversion timing has been observed around two weeks[11,12] with a seroconversion rate range from 90%-100%.[12,13] The antibodies produced are reactive to the SARS-CoV-2 virus's primary structural glycoproteins, which are the spike glycoprotein (S), envelope (E), membrane (M), and nucleocapsid (N). The S is essential as it contains a receptor-binding domain (RBD) in the S1 subunit, which is responsible for virus entry into the host cell via binding with the angiotensin-converting enzyme 2 (ACE2) receptors.[14] This specific antibody against the RBD has been identified to have a strong neutralizing capacity[13,15] and is a target for developing vaccines and plasma convalescent therapy.[16] The N protein encapsulates viral RNA and is essential for viral transcription, replication, packaging of the genome into virions, and blocking the host cell's cell cycle process. This protein is highly immunogenic and extensively produced during infection[17,18], making it a suitable candidate for a marker of recent COVID-19 infection.

Both anti-N and anti-S antibodies were generally detectable at about the same time[13,19], and they appeared as early as one week after the disease onset.[20] In this study, we aimed to elucidate the antibody response in patients treated in our centre who were tested for COVID-19 and compare the specific antibody response of anti-N and anti-S assay for patients with PCR-positive and indeterminate results and/or rapid molecular test-positive. We also looked into the level of anti-S RBD antibodies in our study patients. The association of demographic data and severity of COVID-19 illness with the antibody results were also analysed.

MATERIALS AND METHODS

Study design and population

This is a cross-sectional study over a period of two years among patients who were tested for SARS-CoV-2 in our centre. The patients were admitted to our centre due to various reasons such as acute respiratory symptoms, COVID-19 illness and pre-operative purposes. This study includes patients who were 18 years old and above. They were identified from the laboratory information system, whose PCR result was reported as positive (two genes detected) or indeterminate (one gene detected out of two genes tested). Patients with positive rapid molecular test (IDnow™) were also included. Ethical approval was obtained from the Research and Ethics Committee of Universiti Kebangsaan Malaysia (UKM), approval number JEP-2021-295.This study is supported by UKM Fundamental Fund, project code FF-2021-170.

SARS-CoV-2 RT-PCR test

Nasopharyngeal samples and oropharyngeal samples were tested with RT-PCR. All of the specimens were handled under a biosafety cabinet (BSC) according to laboratory biosafety guidelines for SARS-CoV-2. Viral RNA was extracted directly from clinical samples using a viral RNA kit as directed by the manufacturer. RT-PCR was performed from the extracted RNA, for semi-quantitative detection of SARS-related Coronaviruses (E-gene) and specific SARS-CoV-2 (*RdRP*-gene) detection.

SARS-CoV-2 Rapid molecular test
Nasopharyngeal samples were tested using IDnow™, which employs isothermal nucleic acid amplification method for the qualitative detection of *RdRP*-gene. The test was carried out per the manufacturer's instructions for use. For the purpose of this study analysis, the result of this test is grouped together with RT-PCR as "PCR".

Sample collection for antibody testing
Serum samples were collected from identified patients at different points of time during admission until discharge or death. We classified the sample timing into two time points based on days from RT-PCR or rapid molecular test results, which is Point 1 (0 to 7 days) and Point 2 (8 days or more). The date range of serum sample collection is from April 2020 to November 2021.

Two to three millilitres of blood were collected in plain or gel-separator tubes and transported to the microbiology laboratory in a triple-layer packaging box. Blood was centrifuged at 3500 rpm for 10 minutes and aliquoted into two microcentrifuge tubes for storage at -80°C until the day of testing.

Sample analysis for antibody testing
The testing was performed in batches in the Virology Serology Unit, Department of Diagnostic Laboratory Services, HCTM. Patients whose serum were inadequate for antibody testing were excluded from the study.

In-vitro determination of SARS-CoV-2 antibodies was performed using Elecsys® Anti-SARS-CoV-2 specific antibody (Roche Diagnostics) that uses electrochemiluminescence immunoassay (ECLIA) via Roche cobas® e601 or e401 platform. Prior to analyses on patients› sera, calibration was performed, and quality controls were passed as per the manufacturer's instructions.

Anti SARS-CoV-2 test (Qualitative determination of anti-N)
Qualitative determination of (total antibody IgG and IgM) anti-nucleocapsid (N) was performed based on quantitative signal cut-off index (COI) value but with binary interpretation (reactive or non-reactive). The COI for reactive antibody response was 1.0 and above.

Anti SARS-CoV-2 S test (Quantitative determination of anti-S RBD antibody)
Quantitative measurement of anti-spike (S) protein RBD was obtained from a calibration curve specifically generated by the instrument via 2-point calibration and a master curve provided by the manufacturer. The COI for reactive antibody response was 0.8 U/ml, above which the sample is considered positive.

Data collection and analysis
The socio-demographic data and clinical information were obtained from the online C-HEtS (Caring Hospital Enterprise System) record. The clinical stage of COVID-19 cases was classified as per the Ministry of Health guidelines. The clinical stage and clinical description are as follows:

1: Asymptomatic
2: Symptomatic, no pneumonia
3: Symptomatic, with pneumonia
4: Symptomatic, with pneumonia requiring supplemental oxygen
5: Critically ill with multiorgan involvement

Data entry and data analysis were done using IBM® SPSS® Version 28.0.0. Numerical variables were summarised using means and standard deviations of normal distributions, while the median and interquartile range was used to display data that was not normally distributed. Categorical variables were summarised using frequency and percentage and analysed using chi-square testing. The association among the socio-demographic data, clinical characteristic, PCR results and antibody results were analysed using a chi-square test adjusted with likelihood ratio.

Shapiro-Wilk normality testing reveals that the data on anti-S antibody levels is not normally distributed ($p<0.001$). Thus, the analysis of anti-S antibody level and its relation to severity and PCR results was performed using non-parametric tests, such as Wilcoxon Signed Rank test, Kruskal-Wallis and Mann-Whitney U test where applicable.

RESULTS

Characteristics of the enrolled COVID-19 cases
A total of 267 serum samples were collected from 199 patients. Serums with sufficient volume for antibody testing for both assays were

included in the data analysis.

Table 1 displays the patients' demographic data and the severity of COVID-19 illness. The ages of the patients ranged from 18 to 91 years, with a median interquartile range (IQR) of 41 (26-61) years. The majority of them were male (68.9%) and under 30 years old (32.2%, n=64). The Malay ethnic group recorded the highest number of cases (56.8%, n=113), followed by Chinese (20.6%, n=41) and foreigners (19.1%, n=38) who originated from Bangladesh, Indonesia, Myanmar and India.

The majority of the study patients had SARS-CoV-2 PCR results that were positive (82.4%, n=164) while the rest were indeterminate (17.6%, n=35). Based on the Integrated Laboratory Management System (ILMS) record, all patients with indeterminate PCR had no prior or subsequent positive PCR or rapid molecular results. More than half of the patients were either asymptomatic or had mild upper respiratory tract symptoms (59.3%, n=118), and 21 patients (10.6%) for whom the COVID-19 category was not stated in the discharge summary. If we exclude the 'unknown COVID-19 category' patients and classify the clinical severity; there were 83.7% (n=149) patients who were non-severe (Category 1, 2 and 3) and 16.3% (n=29) were severe (Category 4 and 5). All patients with indeterminate PCR results (n=20) had non-severe COVID-19 illness, and the association is statistically significant (p=0.036) (Table 2).

Table 3 shows the data association between patient age and the COVID-19 category. The mean age of patients in categories 1 and 2 was 33.9 and 38.45 years, respectively, whereas it soared to 70.25 years in category 5. The mean age increased significantly in the more severe COVID-19 illness category (p<0.001).

Table 1: Demographic data, PCR results and COVID-19 category

		Numbers (n)	**%**
GENDER	Female	60	30.2
(n=199)	Male	139	69.8
RACE	Chinese	41	20.6
(n=199)	Foreigner	38	19.1
	Indian	6	3
	Malay	113	56.8
	Other	1	0.5
AGE GROUP	18-29 years	64	32.2
(n=199)	30-39 years	33	16.6
	40-49 years	26	13.1
	50-59 years	22	11.1
	> 60 years	54	27.1
PCR RESULTS	Positive	164	82.4
(n=199)	Indeterminate	35	17.6
COVID CATEGORY	1	74	37
(n=199)	2	44	22.1
	3	31	15.6
	4	25	12.6
	5	4	2
	N/A	21	10.6
SEVERITY	Non-Severe (Category 1-3)	149	83.7
(n=178) *	Severe (Category 4-5)	29	16.3

*Patients with unknown COVID-19 category were excluded

Table 2: Association between PCR results and severity of COVID-19 illness (n=178)

SEVERITY	PCR RESULT		Total n=178	X^2	p value
	Indeterminate n (%)	Positive n (%)			
Non- severe	**n (%)**	129 (81.6%)	149 (83.7%)		
Severe	0 (0.0%)	29 (18.4%)	29 (16.3%)	4.84	0.04
Total	20 (100.0%)	158 (100.0%)	178 (100.0%)		

The Summary of Antibody Results

The serum samples were classified into two time points based on the 'days from PCR result', and sero-reactivity rates were calculated based on these time points. Point 1 is '0 to 7 days' (n=187), and Point 2 is '8 days or more' (n=80). The median (IQR) days for Point 1 and Point 2 are one day (0-3 days) and ten days (9-12 days), respectively. The longest period from PCR result to serum sampling is 45 days in a patient with positive PCR result; however, this patient did not have a sample at Point 1, and the result at Point 2 is non-reactive for both assays.

Table 4 summarises the antibody results for both assays. The overall sero-reactivity rate (regardless of the timing of the sample) for anti-N and anti-S antibodies was 54.3% (n=108) and 65.35% (n=130), respectively. Combining both assays, the overall sero-reactivity rate slightly increases to 67.3% (n=134). Cohen's κ was used to see if the antibody testing of the patients yielded comparable sero-reactivity rates between the two assays. There was moderate agreement between the two assays, $\kappa = 0.67$ (95% CI, 0.568 to 0.772), $p < 0.001$.

Looking into the different timing of serum samples, the sero-reactivity rate during Point 1 was 46.52% (n=87) and 59.89% (n=112) for anti-N and anti-S, respectively. The rate increased at Point 2 to 63.75% (n=51) for the anti-N assay and 77.5% (n=62) for the anti-S assay. In Figure 1, the sero-reactivity rate is plotted against four different time points. We observed that the highest sero-reactivity is at day 8 to day 14 post-PCR, and it decreased after 14 days.

The highest proportion of patients with reactive antibody results are those with COVID-19 Category 1, which is 39.8% (n=43) for anti-N assay and 44.6% (n=58) for anti-S assay (Table 4). However, when we compare the sero-reactivity rate between the patients with severe and non-severe illness (excluding those with unknown COVID-19 category), we found that mild infection is associated with reactive anti-S (p=0.027), but not anti-N, which suggests that anti-S assay is more sensitive and can detect more participants with reactive antibody even in a patient with mild infection.

We also compare the sero-reactivity rate between positive and indeterminate PCR results (Table 4). The highest sero-reactivity rate is observed in patients with positive PCR results; however, this is only statistically significant for the anti-N assay ($p<0.001$). We observed that anti-S assay detects more patients with reactive antibodies, which implies higher sensitivity of the assay.

Summary of patients with indeterminate PCR results

There were 35 patients with indeterminate PCR results. Twenty of the patients had mild COVID-19 infection (Category 1 and 2) while another 15 patients, the COVID-19 category

Table 3: Association between age and COVID-19 category (n=178)

COVID CATEGORY	Mean age (Std. Deviation)	n (%)	F	p value
1	33.96 (16.198)	74 (37.2%)	21.27	<0.001
2	38.45 (16.864)	44 (22.1%)		
3	56.26 (18.072)	31 (15.6%)		
4	60.48 (14.748)	25 (12.6%)		
5	70.25 (10.210)	4 (2.0%)		
Total	43.49 (19.842)	178 (100%)		

Table 4: Summary of antibody result (anti-N and anti-S)

Antibody assays		**Anti-N n (%)**		**Anti-S n (%)**		
Antibody results		**Reactive**	**Non-reactive**	**Reactive**	**Non-reactive**	**Total (n)**
Overall sero-reactivity (n=199)		108 (54.3)	91 (45.7)	130 (65.3)	69 (34.7)	199
Sero-reactivity (days from PCR) (n=267)	Point 1: 0-7 days	87 (46.5)	100 (53.5)	112 (59.9)	75 (40.1)	187
	Point 2: ≥ 8 days	51 (63.8)	29 (36.3)	62 (77.5)	13 (22.5)	80
COVID CATEGORY (n=199)	1	43 (21.6)	31 (15.6)	58 (29.1)	16 (8.0)	74
	2	31 (15.6)	13 (6.5)	34 (17.1)	10 (5.0)	44
	3	16 (8.0)	15 (7.5)	16 (8.0)	15 (7.5)	31
	4	14 (7.0)	11 (5.5)	15 (7.5)	10 (5.0)	25
	5	0 (0.0)	4 (2.0)	0 (0.0)	4 (2.0)	4
	N/A	4 (2.0)	17 (8.5)	7 (3.5)	14 (7.0)	21
Severity (n=178)	Non-severe	90 (60.4)	59 (39.6)	108 (72.5)	41 (27.5)	149
	Severe	14 (48.3)	15 (51.7)	15 (51.7)	14 (48.3)	29
X^2 (p-value)		**1.47 (0.225)**		**4.90 (0.027)**		
PCR results (n=199)	Positive	101 (61.6)	63 (38.4)	110 (67.1)	54 (32.9)	164
	Indeterminate	7 (20.0)	28 (80.0)	20 (57.1)	15 (42.9)	35
X^2 (p-value)		**20.09 (<0.001)**		**1.256 (0.262)**		

were not recorded. All of them had serum samples sent at Point 1 (0-7 days), and only four had a second serum sent after 8 days. Although the overall sero-reactivity rate in PCR-positive is higher (Table 4), the anti-S assay detected a substantial number of patients (57.1%, n=20) with reactive antibodies in the indeterminate PCR group compared to anti-N (20.0%, n=7).

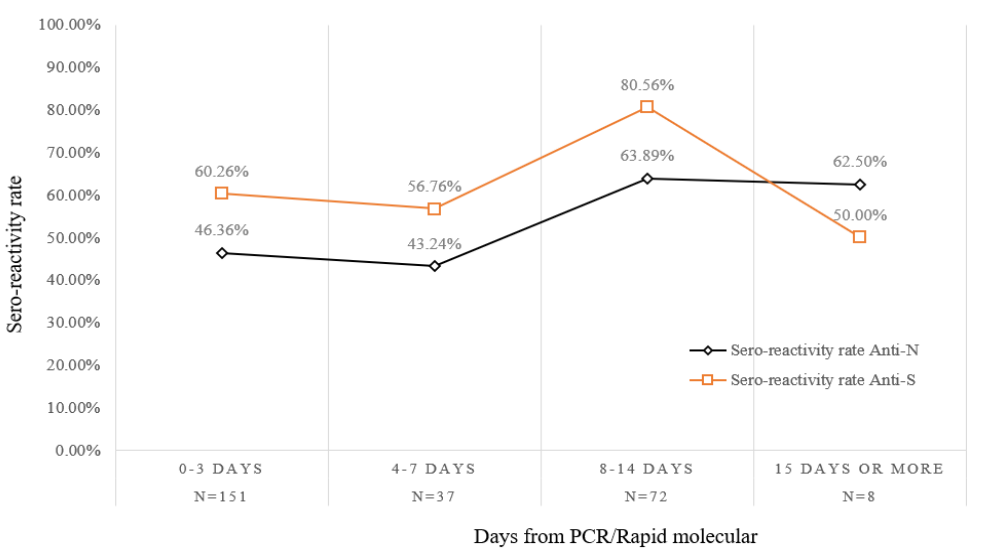

Figure 1. Sero-reactivity rates according to days from PCR or Rapid molecular results (n=267).

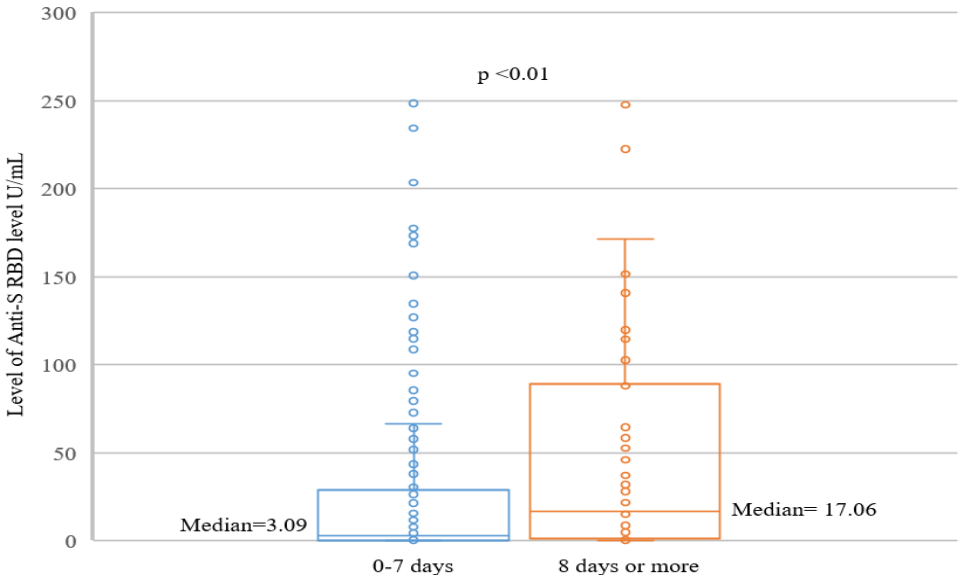

Figure 2. Comparison of median antibody level at Point 1 (0-7 days) and Point 2 (8 days or more) (n=199).

Analysis of anti-S RBD antibody (quantitative assay)

The COI value in the anti-S assay quantitates the level of total antibody (IgG and IgM) against the spike protein receptor binding domain. The minimum limit of detection of the assay is 0.4 U/mL, and the maximum is 250 U/mL, with the cut-off for a reactive result is 0.8 U/mL. We did not perform further dilution for samples with an antibody level above 250 U/mL.

The median (IQR) antibody level for the samples at Point 1 and Point 2 is 3.09 (0.4-29.34) U/mL and 17.06 (1.22- 88.84) U/mL, respectively (Figure 2). Related-samples Wilcoxon Signed Rank test demonstrates the difference of antibody level in Point 1 and 2 is statistically significant ($Z=-5.47$, $p<0.001$), indicating that anti-S antibody levels increase approximately after 1 week. However, five out of 68 patients had a second sample sent at Point 2 with decreased antibody levels and four patients had non-reactive antibodies even after 14 days post-PCR. We observe the highest median level of antibody developed is after day 9 to day 17 post-PCR result (Figure 3), and pairwise comparison of the median between '0-3 days' and the peak '9-14 days' is statistically significant ($p<0.001$). The level reduced to 2.23 U/mL in the samples taken after 14 days; however, statistical analysis shows a non-significant result ($p=0.067$), likely due to the low number of samples.

Independent-sample Mann-Whitney U test was performed to evaluate if there is a difference in the anti-S antibody level distribution between the severity of COVID-19 illness and PCR result (indeterminate vs positive). The tests show no significant difference in anti-S levels between COVID-19 severity and PCR result at Point 1 and 2 (Table 5). Similarly, the comparison of median antibody level for these variables is also not statistically significant (Figure 4 and 5).

As expected, the magnitude of the antibody level increases over time, though a small number of patients may have an early decrease in antibody level and become non-reactive. In addition, the magnitude of antibody level is not associated with either PCR result or severity of COVID-19 illness. All patients in Category 5 (n=4) had samples at Point 1 only, and the results were non-reactive (0.4 U/mL).

DISCUSSION

This study looked at the antibody response (anti-nucleocapsid and anti-spike protein) of patients with SARS-CoV-2 whose PCR or rapid molecular tests reported positive and indeterminate results. A total of 199 patients were enrolled, with serum samples sent at various timing from admission until discharge or death. The patients were recruited during the first year and a half of the COVID-19 pandemic, with the serum sample collection date ranging from April 2020 to November 2021. The

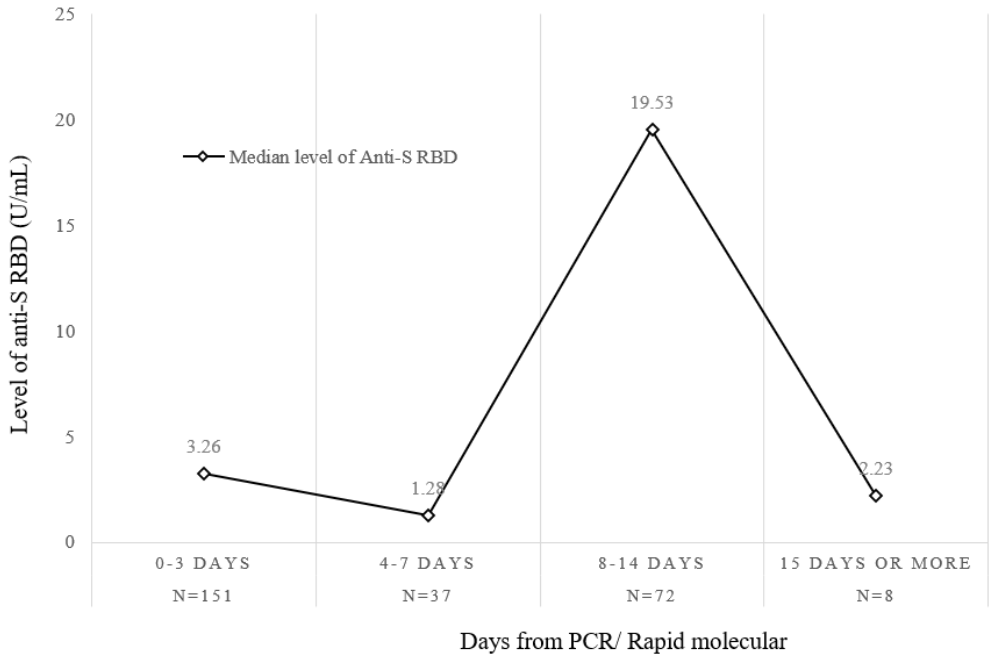

Figure 3. Median anti-S receptor-binding domain (RBD) level according to days from PCR or rapid molecular result (n=267)

Malaysian national COVID-19 vaccination programme was implemented on 24th February 2021 for healthcare workers and in May 2021 for the general population. We have 43 patients whose samples were collected after February 2021, and unfortunately, these patients' vaccination status was unavailable.

More than half (52.3%, n=104) of samples were obtained from April to May 2020 during the second wave of COVID-19 in Malaysia, which was associated with the lineage B.6 SARS-CoV-2. The following third wave, from September 2020 to March 2021, is predominantly caused by lineage B.1.524 and B.1.466.[21] Alpha (B.1.1.7) and beta (B.1.351) variants were subsequently detected around December 2020. Infection by the beta variant increased at the early fourth wave but then was replaced by the delta (B.1.617.2) variant from June 2021 to January 2022.[22] However, we could not conclude the effect of the different circulating variants on antibody results in our study.

Almost 70% of our study patients were male, which could be due to the fact that a significant number of them were from clusters involved in

Table 5: Comparison of anti-S receptor-binding domain (RBD) quantitation level between severity and PCR result; Mann-Whitney U test analysis (n=178)

Sample timing	Variable		Mean rank of antibody level (n)	Mann-Whitney U	p-value
Point 1: 0-7 days	Severity	Non-severe	87.12 (141)	1534.00	0.11
		Severe	70.81 (27)		
Point 2: 8 days or more		Non-severe	38.49 (72)	143.50	0.99
		Severe	38.63 (4)		
Point 1: 0-7 days	PCR result	Positive	94.87 (152)	2528.00	0.64
		Indeterminate	90.23 (35)		
Point 2: 8 days or more		Positive	40.64 (76)	141.50	0.81
		Indeterminate	37.88 (4)		

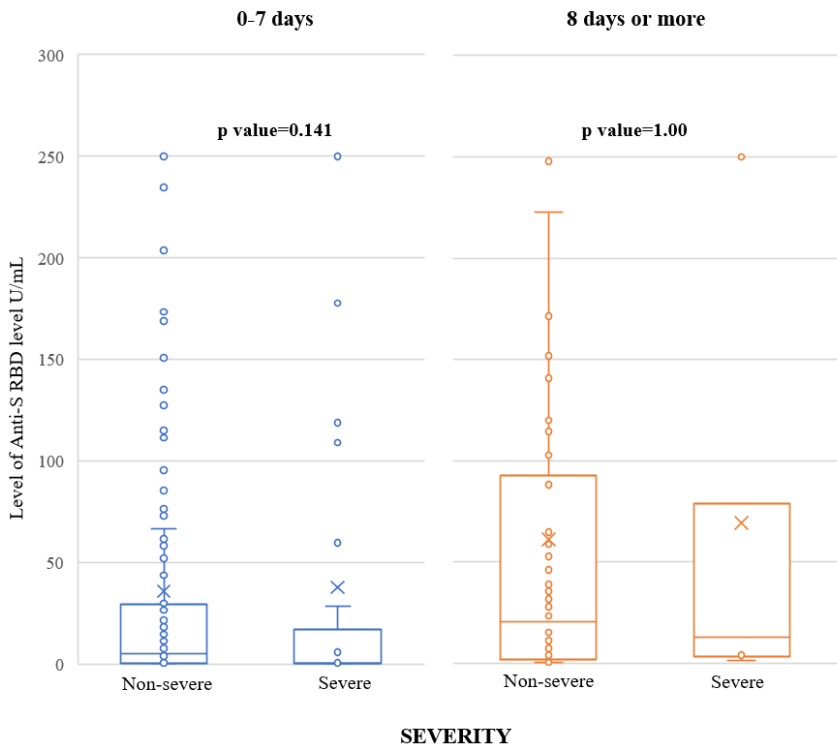

Figure 4. Comparison of antibody level according to the severity of COVID-19 illness (n=267).

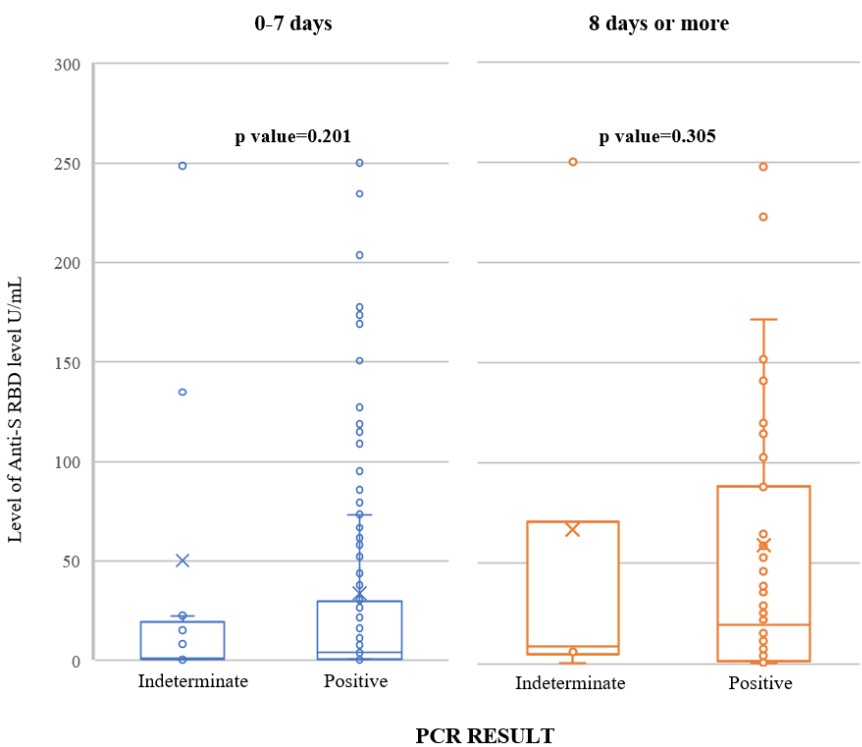

Figure 5. Comparison of antibody level (anti-S RBD) according to PCR results (Independent sample median test) (n=267)

a religious movement (Tabligh group) who were all male. The majority of the samples in our study were obtained at Point 1 (93.97%, n=187), and only 80 patients had samples sent at Point 2. The low number of patients with samples at Point 2 was due to the changing discharge policy implemented throughout the study period. At the beginning of the pandemic, the mandatory quarantine period in the hospital was 14 days, with negative PCR results as a requirement prior to discharge. However, later on, patients who were fit for discharge between day 3 to day 5 were allowed for home quarantine without negative PCR results as a discharge requirement.

The overall seroconversion rate for the anti-S assay and anti-N is 65.3% and 54.3% respectively. We observed a higher seroconversion rate in the PCR-positive cohort which is 61.6% for anti-N ($p<0.001$) and 67.1% for anti-S ($p=0.262$). Although the percentage is lower, our data is concordant with the clinical sensitivity claimed by the manufacturer, which is 88.6% for anti-S compared and 60.2% for anti-N assay; for serum samples tested within the first week of post-PCR confirmation. The sensitivity increases to 100% for Anti-S assay and 99.5% for anti-N assay after 14 days post-PCR confirmation.[23] Findings from a previous study on serum samples more than two weeks post-symptoms onset, nucleocapsid protein antibody assay demonstrates 100% sensitivity compared to spike protein antibody (91%).[24] Anti-N assay is also slightly more advantageous, allowing for two days earlier antibody detection compared to anti-S assay.[25]

Findings from our study showed that more than 50% of patients seroconverted to reactive antibody. This sero-reactivity rate is very much lower compared to previously published studies which range from 90 to 100%.[12,13,26] Those studies, however, had more than two time points of sample collection for most patients with a more extended period of observation, which was up to 39 days post-symptom onset. The lower seroconversion rate in our study could be attributed to a lack of samples at a later stage of illness, as we had only 80 patients with samples at Point 2. Moreover, we include patients with indeterminate PCR results who might be false-positive PCR results. Even so, our sero-reactivity rates were comparable to another study that looked into antibody response at the early stage of illness (within one week), which was 64.1%.[27]

Previous case reports described three COVID-19 cases in immunocompetent adults who did not develop antibodies even 40 days after symptom onset. Two of them had a mild infection, while the other had severe pneumonia.[28,29] This suggests that at least a small proportion of patients may not develop detectable antibodies against the virus.

In addition to specific antibody production, the immune response in COVID-19 also involves the cellular mechanism by CD4 and CD8 T lymphocytes targeted against the viral proteins. Current findings show that T cell responses were identified in almost 100% of COVID-19 convalescent patients[30], and further studies demonstrate that individuals exposed to SARS-CoV-2 may develop virus-specific T-cell responses even in the absence of detectable antibodies.[31,32] Thus, the concept of "cellular sensitisation without seroconversion" has emerged in COVID-19 infection.[33] This phenomenon is also seen in the closely related coronaviridae family, where a recent study demonstrated that MERS-specific cellular responses occur without antibody seroconversion in dromedary's slaughterhouse workers.[34]

Previous studies have demonstrated that severe COVID-19 infections were associated with a more robust antibody response[35,36] and individuals with negative COVID-19 serology were associated with asymptomatic and mild infection.[37] Contrary to expectations, the median antibody level in our study was higher in patients with categories 1, 2, and 3. A study on a cohort of young adults with non-severe COVID-19 infection demonstrated that neutralizing antibody titres did not correlate with symptoms, and asymptomatic patients are able to induce similar immunity as symptomatic patients.[38] However, the cohort did not include severe COVID-19 patients for comparison of the antibody response.

The extent of T-cell response in relation to COVID-19 severity has not been fully defined, but clinical reports have shown that lymphopenia occurs in COVID-19 patients.[39] The mean T cell count in COVID-19 patients was lower than the normal range and severe patients tend to have lower lymphocyte counts compared to those who are non-severe.[40] In addition, COVID-19 patients have a disruption in T cell differentiation balance. Regulatory T

cells (Tregs) are significantly lower in severe cases compared to moderate cases.[41] There is an increased percentage of CCR6+Th17 cells and a decreased percentage of Treg cells, indicating activation of systemic proinflammatory T cell response, which may aggravate pathological immune damage and contribute to disease severity.[42]

All four patients in Category 5 in this study had negative antibody results, and they were elderly patients (over 60 years old) with multiple co-morbid conditions, namely diabetes, hypertension, and cardiovascular disease. Evidence showed that the higher level of antibodies in severe COVID-19 cases was not affected by increasing age.[43,44] Our contradictory result may be due to the fact that only Point 1 samples were available for antibody testing for the most patients in the severe disease category (only four patients had samples at Point 2). Moreover, a study found that the generation of specific antibodies occurs one week later in patients with severe COVID-19 compared to patients with mild or moderate disease.[45]

In our study, five patients had decreased antibody levels (anti-S) and two of them sero-reverted to non-reactive. Antibody response is expected to wean off by time, as demonstrated in a prospective study, a significant decrease in anti-S IgG levels is seen after three months post-PCR.[46] However, the majority of patients had antibodies that remained detectable up to 12 months after a positive PCR, and the decreased antibody level is associated with overweight and obese patients (BMI $\geq 25 kg/m^2$).[47]

Another study comparing the long-term evolution of anti-N and anti-S IgG demonstrates a significantly higher peak antibody level/cut-off ratio for anti-S compared to anti-N and lower persistence of anti-N compared to anti-S after one year of SARS-CoV-2 infection.[48] A large prospective, longitudinal study (n=4553) demonstrates robust increases of anti-N occur 100 days post-infection, and the linear mixed model predicts that anti-N antibodies may remain for at least 500 days in most patients.[49]

Vaccination for the general population in Malaysia commenced around May to June 2021, and there were three patients who had samples taken after that period of time with reactive anti-S and non-reactive anti-N. One patient had positive PCR while the other two had indeterminate results. The two patients with indeterminate PCR results showed negative PCR results on subsequent testing, suggesting that the reactive anti-S antibody was most likely due to vaccination.

The COVID-19 vaccines, such as Cominarty (Pfizer BioNtech) and COVAX (AstraZeneca) which use the spike protein as the main component, produce specific antibodies targeted to the protein. Thus, at that early stage of the pandemic, it is considered that specific antibody assays could be used to differentiate between vaccination and infection. This is based on a study that demonstrated the ability to distinguish individuals previously infected with SARS-CoV-2 from those who were vaccinated, using a serologic testing algorithm involving anti-S RBD and anti-N assay.[50]

However, development of vaccines using inactivated virus like CoronaVac (Sinovac) leads to production of both anti-spike and anti-nucleocapsid antibodies, making differentiation between past infection and vaccination more difficult. As of October 2022, 61.2%, 7.9%, and 29.8% of the Malaysian population have received the Pfizer, AstraZeneca, and Sinovac vaccines, respectively.[51] Therefore, interpretation of COVID-19-specific antibodies in a widely vaccinated population should be done cautiously and include a detailed vaccination history. Furthermore, high seroprevalence of anti-SARS-CoV-2 antibodies in most populations reduces the value of antibody testing for epidemiologic purposes.[8]

Extensive mutations in the spike protein gene lead to emerging variants of SARS-CoV-2. These variants demonstrate significantly increased resistance to neutralization by both convalescent plasma (9.4-fold) and vaccinee sera (10.3-12.4-fold).[52] Viral evolution might also affect antibody detection, potentially reducing assays' accuracy.

CONCLUSION

This study captured a cohort with the majority belonging to the pre-vaccination period during the pandemic. The seroreactivity of the SARS-CoV-2 antibodies was seen highest from week two following laboratory diagnosis, and the level of anti-S declined afterwards. The Anti-S assay exhibits greater sensitivity compared to the anti-N assay and detected more reactive results even in patients with mild infections or indeterminate PCR results. Accurate result

interpretations and understanding of the antibody responses require more extensive information about vaccination history. Currently, the clinical utility of SARS-CoV-2 antibody testing is limited due to the current endemicity of the virus as well as the vaccinated population. Further research could further investigate and explore how the dynamics of viral evolution impact the antibody responses and detection of antibodies.

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