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Viral Load and Antibody Responses in an Asymptomatic/Minimally Symptomatic SARS-Cov2 Positive Cohort in Sri Lanka

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1. Abstract

Asymptomatic patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are silent carriers of the disease. We aimed to characterize their dynamics of disease occurrence, viral shedding and antibody responses using a cohort of asymptomatic/ minimally symptomatic patients from Sri Lanka during the first wave of COVID-19. The cohort includes the first 26 of a cluster of 936 SARS-CoV-2 positive Navy personnel that were reported. Sequential nasopharyngeal swab and blood samples were collected at various time points up to nine months. RT-PCR was done to measure SARS Cov2 viral loads, while blood samples were subjected to ELISA assays to detect SARS-CoV-2 IgM, IgG antibodies and neutralization assays. Eight (31%) patients were symptomatic at the time of diagnosis while 13 (50%) were pre-symptomatic and 5 (19%) remained asymptomatic. Fever was the commonest (59.69%) symptom. The viral loads at the time of diagnosis showed no significant difference among the symptomatic, asymptomatic and pre-symptomatic patients (p<0.05). The duration of viral shedding seemed longer in the pre-symptomatic group, and by day 21, all patients were negative for SARS-CoV-2 RNA. Early IgM seroconversion was observed in the pre-symptomatic group. IgM peaked at day 14 for all the three groups and was detectable even at 21 day. The symptomatic patients showed a significantly higher level of IgM as compared to the pre-symptomatic patients (p<0.05). A positive response of IgG was at the peak at day 21, which was detectible up to 9 months. There were four patients who expressed no antibodies despite their moderate viral positivity. Majority of patients with high antibody responses and high viral loads, also had fever. Symptomatic, asymptomatic and pre-symptomatic patients exhibited different kinetics of clinical/viral and antibody responses to SARS-CoV-2, allowing us to group them into four distinct categories. Such categorization may help to separate and identify the patients with minimal symptoms who may require an extra dose of the vaccination for long term protection. The results show that after 9 months, immunity is waning in this cohort of largely healthy young males and neutralizing antibodies are present at significant levels in <15% of the patients infected with a high viral load. Therefore, SARS-CoV2 asymptomatic or mildly symptomatic infections may not provide long lasting immunity after a single infection, for a majority of the individuals making them vulnerable to the new variants of SARS-CoV2 that may arise in the future. An expanded study is needed to further validate these findings.

2. Background

The epidemic of COVID-19 due to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS Cov-2) has been a serious threat to public health worldwide and there are over 332 million confirmed

cases of infections and ~ 5 million deaths (As of 19th January 2022). In Sri Lanka, as by the same date, over 500,000 laboratory confirmed cases and over 15,000 deaths were reported [1].

Transmission of the virus by asymptomatic individuals has been reported since the early stages of the outbreak [2, 3]. They are described as those who are tested positive by RT-PCR, but lack symptoms [4]. Asymptomatic infections are an important aspect of SARS-CoV-2 infection, because they are considered significant contributors to COVID-19 spread [5]. The dynamics of asymptomatic infections in Sri Lanka, in a tropical climate, are still poorly understood and information concerning SARS-CoV-2 transmission and viral shedding duration between symptomatic and asymptomatic SARS-CoV-2 patients remain controversial.

The extent of asymptomatic infections also seems highly variable [6]. A subset of asymptomatic patients develops symptoms after the onset of diagnosis and is known as pre-symptomatic patients [4]. Several studies have looked into the characteristics of asymptomatic and pre-symptomatic infections, such as their viral loads and viral shedding, in comparison to symptomatic patients [7-13]. Although they confirm the existence of transmission by asymptomatic individuals, the findings are contradictory when comparing viral loads and virus shedding between symptomatic/ pre-symptomatic and asymptomatic infections. However, asymptomatic carriers are silent spreaders, and warrant attention in terms of disease prevention to contain the disease [14].

During the first COVID-19 outbreak in Sri Lanka, asymptomatic individuals from the Navy camp Welisara, who had direct and indirect contacts with a few symptomatic COVID-19 confirmed cases, tested positive for SARS COV-2 using Real Time-PCR (RT PCR). This study is based on the first group of 26 male, military individuals (not vaccinated against COVID-19 at the time) and found to be asymptomatic/minimally symptomatic, tested positive for SARS CoV-2, from a cluster of 936 COVID-19 positive Navy personnel., After the diagnosis, this asymptomatic and minimally symptomatic NAVY cohort was quarantined for 21 days at Colombo East Base Hospital, Mulleriyawa.

Currently in Sri Lanka, COVID-19 infected subjects are kept under quarantine for 10-14 days irrespective of their symptoms or viral loads [15]. This leads to a heavy burden on the functioning of the economy as well as unfavorable impacts on positive individuals. Clear understanding of the initial viral loads, viral clearance and antibody response on minimally symptomatic and asymptomatic young patients without major co-morbidities will be important for economic reasons in deciding the duration of quarantine.

We conducted a prospective study for 9 months from the initial diagnosis to understand the differences in clinical characteristics, viral loads, viral shedding and antibody responses among asymptomatic and minimally symptomatic SARS CoV-2 confirmed first group of navy personnel during the first wave of COVID-19 in Sri Lanka. It should be noted that this study was conducted before any of the individuals received any COVID-19 vaccination, therefore this data represents largely naïve individuals who are being exposed for the first time in Sri Lanka. In the future it will be important to identify/characterize the immune response of asymptomatic individuals infected by new viruses such SARS-CoV2, to contain the spread before it reaches epidemic/pandemic levels. To our knowledge this report is one of the first descriptive studies on an asymptomatic and minimally symptomatic SARS CoV-2 infected group for over 6 months from a tropical environment such as Sri Lanka.

3. Methods

3.1. Study population and study design

The first 26 of the 936 Navy personnel, who were laboratory confirmed with SARS CoV-2 RT-PCR during the first wave of the pandemic in Sri Lanka, and admitted to the Colombo East Base Hospital were included. This study was conducted as a descriptive longitudinal study. The participants were followed up for 9 months from the initial diagnosis.

3.2. Demographic & clinical data and sample collection

Demographic and clinical data were obtained from patients using an interviewer-administered questionnaire by a telephone conversation after obtaining informed written consent as well as from clinical records by a trained medical practitioner. Nasopharyngeal swabs and 3ml of blood were collected from the study participants on days 07, 14, 21 to determine the viral positivity and SARS CoV-2 IgM and IgG antibodies and specimens were transported to the testing laboratory according to the standard operating procedures. An additional blood sample was obtained at 9 months from the initial diagnosis from consented subjects., to determine the prevalence of SARS- COV-2 antibodies in this cohort. The serum separation was done by centrifugation at 3,000 rpm for 10 min, and serum samples were stored at -80oC until tested.

3.3. Molecular assays

Viral RNA was extracted from nasopharyngeal samples with QiampR viral RNA kit (QIAGEN, Hilden, Germany). Real Time-PCR (RT-PCR) testing for the RNA extracts was carried out on the same day of collection using a commercially available viral nucleic acid extraction kit (Spinster, Malaysia) and PCR assay kit (Altona, Germany) as per the manufacturer's instructions. The specimens were considered positive if the cycle threshold (Ct) values were ≤39.

3.4. Serological assays

SARS CoV-2 specific IgM and IgG levels in the patients' sera were determined using a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kit (Dia Pro, Italy) according to the manufacturer's protocols. The positive and negative responses were determined based on the cut-off absorbance value calculated based on the absorbance of the negative controls.

3.5. Neutralization Assays

Serially diluted patient's heat inactivated plasma (serial 2-fold dilutions from 1:20) were mixed with the same volume of virus (hCoV-19/Japan/TY-WK-521/2020, GISAID accession no. EPI_ISL_408667) and incubated at 37℃ for 1 hour. The virus antibody mixture was inoculated in replicates of two for each dilution using 12-well plates. The plates were incubated at 37 ℃ in 5% CO2 for 60 minutes. After the incubation, 2 mL (12-plates) of overlay media [5 g of Methylcellulose (Wako Pure Chemical Industries Ltd., Osaka, Japan), 12 g of Avicel RC 591 (FMC Biopolymer, United States), and 9.4 g of EMEM powder were dissolved in 1 L of DDW] was added to each well. After three days of post infection, cells were fixed with 4% of paraformaldehyde and stained with 0.25% crystal violet (Wako Pure Chemical Industries, Japan). The number of plaques were counted by naked eye. The neutralization titer, PRNT50 was defined as the highest plasma dilution which reduced the number of plaques by 50% compared to the control wells in which antibody was absent.

3.6. Statistical Analysis

Statistical analyses were performed using Mann Whitney test.

4. Results

The mean age of the study population is 31 years (range 25-42) and all are males. At the time of diagnosis all were either asymptomatic (n=18) or expressed mild symptoms (n=8) (Table 1). Eighteen of them were known to have a history of contact with individuals who had symptoms suggestive of COVID-19 within the military base prior to their diagnosis. Patients who have symptoms at the time of initial RT PCR diagnosis for SARS CoV-2 are defined as symptomatic. Those who are asymptomatic at the initial diagnosis but later developed symptoms are regarded as pre-symptomatic. Those who remained symptoms free throughout were considered as asymptomatic [4]. Accordingly, out of the 26 participants, 8/26 were symptomatic, 13/26 were pre-symptomatic and 5/26 remained asymptomatic, during the study period.

Out of the 8 symptomatic patients, symptoms persisted more than 7 days only in 2 patients. One of these two patients continued to show symptoms over the 21 days, while the other was relieved of symptoms by the day 14. The mean symptom duration was 9.62 days. Out of the pre-symptomatic subjects, 5 and 1 had symptoms more than 7 and 14 days respectively, and all were symptoms free at day 21. The onset of symptoms in the pre-symptomatic patients was within 7 days of the diagnosis, and the symptoms persisted for a mean duration of 10.23 days. Commonest symptoms observed was fever (57.69%) followed by sore throat (23.08%). Majority (21/26) participants did not have any co-morbidities and were not on any medication. Five participants had a past history of dengue. Of the 21 symptomatic and pre-symptomatic subjects, 14, 5 and 1 became symptom free at D7, D14 and D21 respectively.

On day 7 and 14, respectively, 19 and 24 patients became symptoms free, while 7 and 2 patients were symptomatic. However, 16/19 of these asymptomatic patients on day 7, were still positive for SARS CoV-2 by RT-PCR. It was further noted that 5 of these asymptomatic but RT-PCR positive patients, showed CT values between 20 and 28, indicating of their high positivity in infection. By day 14, only 3/24 asymptomatic patients were positive for SARS CoV-2 by RT-PCR and all showed CT values above 28, indicating of their reduced positivity in infection. Of the 7 patients who were symptomatic at the day 7, 5 were tested positive in RT-PCR. On day 14, only 2 patients were with symptoms, and they were tested negative by RT-PCR. The results do not indicate any correlation between the presence of symptoms and the positivity by RT-PCR testing, throughout the period of infection.

Further, sequencing and phylogenetic assignment of named global outbreak lineages (PANGOLIN) analysis showed that nine of the ten SARS CoV-2 strains from this study, which were subjected to whole genomic sequencing belong to the B.1.3 lineage, while the other strain belongs to the parent lineage B.1 (un-published data).

Approximately 30% (8/26) and 27% (7/26) of COVID19 patients showed detectable levels of IgM and IgG respectively on day 7 (Figure 2a & 2b). Majority of those who had IgM on day 7 belonged to the pre-symptomatic group (6/13) while it was only one patient each from the other two groups. By day 14, the percentage of patients presented with antibodies increased to 65% (17/26) for both IgM and IgG (Symptomatic group; 62.5% for both antibodies, Asymptomatic group; 40% and 60% for IgM and IgG, and Pre-symptomatic group; 70% and 77% for IgM and IgG respectively). By day 21, 80% (20/25) of patients had developed IgG, and 72% (18/25) were presented with positive IgM responses. (Symptomatic group; 75% and 62.5%, Asymptomatic group; 50% and 50%, and Pre-symptomatic group; 77% and 100% for IgM and IgG respectively). There were 2 symptomatic patients and 2 asymptomatic patients who did not show detectable levels of any antibodies, throughout the study period. Comparatively, low antibody response levels were observed in the asymptomatic patients as detected on day 7, 14 and 21. The level of antibody responses were significantly higher in symptomatic patients as compared to pre-symptomatic patients for IgM on day 14 (1.41±0.83 vs 0.44±0.12, p<0.05) and day 21 (1.25±0.77 vs 0.50±0.21, p<0.05), and for IgG on day 21(1.76±0.76 vs 1.00±0.57, p<0.05). However, only 11 of the 26 patients presented detectable levels of IgG, by 9 months after the initial diagnosis. Further, only 13(52%) and 4(17%) patients showed neutralizing antibodies, at 21 days and 9 months respectively.
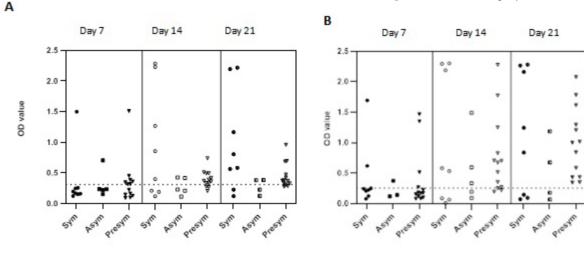
There was no significant difference in the CT values, in RT-PCR detection at the initial diagnosis for SARS CoV2, among symptomatic, asymptomatic and pre-symptomatic patients (N gene; 21.99±6.87 vs 29.62±7.52 vs 23.59±5.14, ORF gene; 22.93±6.91 vs 30.93±8.11 vs 24.00±5.18, p<0.05). However, the CT values of ma-

jority of patients (6/8) in the symptomatic group were clustered at very low values (within 16-21), implying high viral loads, and those of the asymptomatic group (3/5) were clustered at a high value, meaning a low viral loads (within 33-38) (Figure 2c). The CT values for pre-symptomatic group appeared distributed across a wider range (14-38) with the majority (10/13) having CT value between 20-28. Twenty-one out of 26 patients (81%) remained RT-PCR positive for SARS CoV2 after 7 days of initial diagnosis with significant levels of viral loads. All patients in the symptomatic and asymptomatic groups were negative for infection by RT-PCR on day 14. Three pre-symptomatic patients remained positive beyond day 14.

Based on the CT values in RT-PCR assay and the antibody responses, the 26 patients in the study cohort could be broadly categorized into 4 different categories as shown in Table 2. The patients in the first category (n=4), had mostly low viral loads (CT values from 27-38) but did not develop any antibody responses during the first 21 days or at the 9 month. The patients in the second group (n=2) showed high viral positivity, were presented with IgG, but no IgM was measured at detectable levels during the study period. None of the patients in the first and the second categories had fever. The third and the fourth categories of patients showed high viral loads, and also had developed both IgM and IgG at detectable levels. We further noted that the third category mostly included the patients with very high viral loads, and the level of IgM detected in patients of this category were also generally high. Most of the patients in the third category were symptomatic, also including the 6/8 symptomatic patients, who were presented with very low CT values at the initial RT-PCR diagnosis (within 16-21). Except for the one asymptomatic patient in this category, all the others had fever (90%, 9/10). The patients in the fourth category were largely pre-symptomatic and only four of them had fever (40%). Though IgM was detected in the patients of this category, the levels were low.
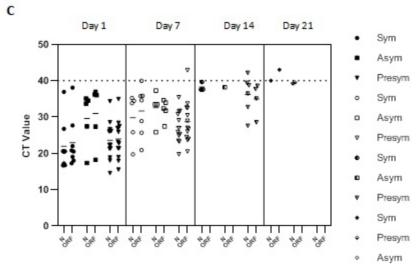

Figure 1: Molecular and serological data following 21 days after the initial diagnosis of the 26 SARS CoV2 infected patients.

A: IgM responses (OD measurement of ELISA assays) on day 7, day 14 and day21

B: IgG responses (OD measurement of ELISA assays) on day 7, day 14 and day21

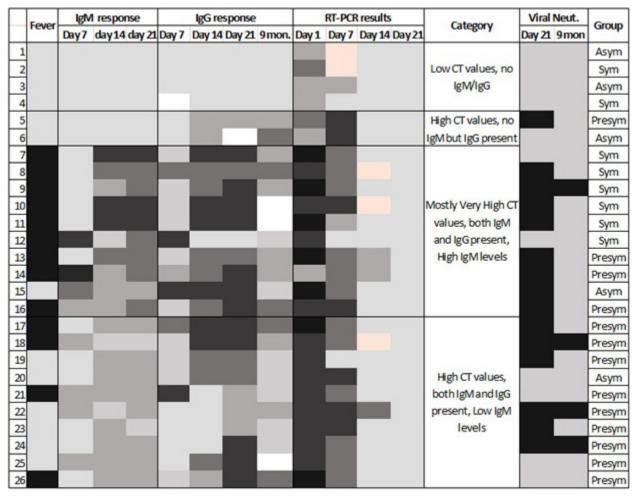C: Viral loads as indicated by the CT values on day 1, day 7, day 14 and day 21

	Fever	IgM response			IgG response				RT-PCR results				Category	Viral Neut.		Group
		Day 7	day 14	day 21	Day 7	Day 14	Day 21	9 mon.	Day 1	Day 7	Day 14	Day 21		Day 21	9 mon	
1													Low CT values, no IgM/IgG			Asym
2																Sym
3																Asym
4																Sym
5													High CT values, no IgM but IgG present			Presym
6																Asym
7													Mostly Very High CT values, both IgM and IgG present, High IgM levels			Sym
8																Sym
9																Sym
10																Sym
11																Sym
12																Sym
13																Presym
14																Presym
15																Asym
16																Presym
17													High CT values, both IgM and IgG present, Low IgM levels			Presym
18																Presym
19																Presym
20																Asym
21																Presym
22																Presym
23																Presym
24																Presym
25																Presym
26																Presym

Figure 2: Categories of patients based on serology and viral positivity

	RT-PCR	IgM	IgG	Fever	Viral Neut
no	>40		<cutoff	No	<1:40
m	35-40	cutoff-(cutoff+0.2)			
H	28-35	(cutoff+0.2)-1.0			
VH	20-28	<1.0			
	<20			Yes	>1:40
		No data			
Inconclusive					

The colour code for the table 2: For RT-PCR results, the colour code is based in the CT values. For IgG/IgM the colour code is based on the absorbance measurements of ELISA assays. For fever, the colour code indicated the presence or absence of fever. For viral neutralization, the serum dilution for PRNT50 compared to the control, less than 40 indicates no viral neutralization, whereas that above 40 indicates viral neutralization.

Table 1: Clinical data of study participants

	Frequencies (n=26)
Mean age	31
Gender -Male	100%
Symptoms	
Fever	15 (57.69%)
Cough	5 (19.72%)
Shortness of breath (SOB)	0 (0%)
Sore throat	6 (23.08%)
Diarrhea	1 (3.85%)
Anosmia	3 (11.5%)
Other (Headache, fatigue, runny nose)	5(19.72%)

Comorbidities	
No Comorbidity	21 (80.77%)
Diabetes mellitus	1 (3.87%)
Hypertension	2(7.69%)
Chronic Kidney disease	1(3.87%)
Chronic liver cell disease	0 (0%)
Malignancies	0 (0%)
Other	2 (7.69%)
Medication use	
No medications	24 (92.31%)
ACE inhibitors/ARB	2 (7.69%)
Steroids	0 (0%)
Other immunosuppressive drugs	0 (%)
Past history of infections	
Pulmonary TB	0/26 (0%)
Dengue	5/27 (19.23%)
Substance use	
Alcohol	23/27 (88.46%)
Nicotine	16/27 (61.54%)

5. Discussion

We report the dynamics of asymptomatic or minimally symptomatic cases infected with SARS CoV 2 using a cohort of 26 patients from Sri Lanka. These patients have not received any COVID-19 vaccines during the study period. This cohort was largely symptom free at the time of diagnosis (18/26). Thirteen of the 18 participants without symptoms at the initial diagnosis have become pre-symptomatic within the first 7 days after the initial diagnosis. All the symptomatic or pre-symptomatic patients expressed only mild symptoms. Ninety percent of the strains of these cohort belong to the B.1.3 lineage and with this study, these patients become the first to be identified as being infected with this strain in Sri Lanka (un-published data).

The data from literature is unclear on the dynamics of viral loads, as indicated by the Ct value in the RT-PCR detection, and viral shedding among the symptomatic, asymptomatic and pre-symptomatic patients. In a few studies it was found that SARS-CoV-2 viral load was significantly lower in pre-symptomatic COVID-19 patients than in symptomatic ones [10, 16, 17]. We too observed that the majority of the patients from the symptomatic groups had high viral loads as indicated by low CT values while the majority of the asymptomatic patients had low viral loads as indicated by very high CT values. Based on viral load, antibody responses and antibody neutralization assays, we categorized the cohort into 4 groups and we did not find a significant difference in the viral loads among the symptomatic, asymptomatic and pre-symptomatic patients groups, which could be due to the low sample number in our study. In addition, none of the patients had severe symptoms.

Though we did not exactly know the date of exposure for these patients, based on the date of the initial diagnosis, the pre-symptomatic group had a longer duration of viral shedding as compared to the symptomatic and asymptomatic groups (< 21 days vs <14 days). A study done in 2021 has shown lower level of viral loads and shorter viral shedding time in asymptomatic carriers when compared to symptomatic patients and viral load was significantly lower in presymptomatic COVID-19 patients than in symptomatic ones [17]. Additionally, the viral shedding duration was significantly longer in pre-symptomatic COVID-19patients than in asymptomatic carriers in that group as reported by others [17].

According to a study carried out by Cheng et al 2021 on viral shedding in duration of SARS-Cov-2 infected mildly symptomatic patients isolated in a community facility it was shown that close interaction between positive patients increases the duration of viral shedding than in the patients who are isolated individually [18]. Similarly, our study cohort was quarantined together which could have resulted in the longer duration of viral shedding, suggesting the importance of proper management of mildly symptomatic patients in quarantine centers in a more efficient manner to reduce the period of virus shedding.

Notably, detection of viral RNA does not necessarily mean that infectious virus is present in respiratory specimens, and caution is required when applying virus shedding duration that was calculated based on RT–PCR to assess infection potential. However, the ability to sequence full length SARS-CoV2 virus from these samples suggest that intact virus is present in this group. As evident from this study, it is an important consideration that these asymptomatics/presymptomatics can be silent carriers for the disease given their high viral loads and the longer period of viral shedding.

We found that the antibody responses presented by pre-symptomatic patients were to be significantly lower than that of symptomatic patients. Though their antibody levels were moderate, it was interesting to note that pre-symptomatic patients showing an early IgM seroconversion as compared to the other two groups, which might have occurred even before the onset of symptoms. Further, the expression of IgM peaked at day 14 for all the three groups and was detectable even at 21 day. However, 19 and 24 patients were free of symptoms by day 7 and 14 respectively. The results suggest that positive IgM responses were measured mostly during the convalescent phase, 12.5% of the symptomatic group did not present IgM during their acute phase of illness. There was no noticeable difference in the occurrence of IgG antibodies among the three groups. 80% of patients were presented with IgG by day 21 from the initial date of diagnosis. Further, the IgG antibody percentage had declined to 35% of patients by the 9 months. Similar to our results, Long et al 2020 observed that IgG levels in a high proportion of individuals who recovered from SARS-CoV-2 infection start to decrease within 2–3 months after infection. Further, as measured at day 21, the antibodies were neutralizing in half of the cohort which decreased to one tenth of it. However, previous studies have shown that circulating antibodies against SARS-CoV1 or MERS-CoV last for at least 1 -2 years [18-21]. However, previous work on COVID-19 patients from Sri Lanka show that a small group of asymptomatic/mildly symptomatic individuals had a drastic decline in neutralizing antibodies after 90 days [23]. Our results agree with this even though after 9 months we find 3/17 high viral load symptomatics continuing to have neutralizing antibodies.

In this study, we observed four categories of SARS CoV2 infected patients based on their antibody levels and viral loads. The first category of patients is notable. The four patients in this category had no IgM/IgG despite the fact that two of them being symptomatic patients and one of them having a moderate viral load (CT value ~28) at the initial diagnosis. They may be representing individuals, whose host factors prevent them being infected, though they carry the virus. The third category of patients were also distinct; they were presented with very high viral loads, comparatively higher IgM levels, fever, and were mainly symptomatic. Symptoms like fever may be linked to higher antibody response as a result of having high viral loads. Generally, the occurrence of fever was low in the categories of patients with no or low antibody responses (first, second and fourth).

The T cell response has not been measured in this study due to the limited resources available for the study. Undoubtedly the T cell response plays a major role in providing long term protection and a number of studies have been carried out and in the context of these four categories it would be important to include T cell data [23].

This study suggests that SARS-CoV2 infection does not always provide long lasting immunity after a single infection for a majority and currently this is suggested with the increased number of re-infections that are being observed suggesting SARS-CoV2 acts more like the traditional seasonal coronaviruses [24, 25]. It can be stated that SARS-CoV2 asymptomatic or mildly symptomatic infections may not provide long lasting immunity, for a majority of the individuals making them vulnerable to the new variants of SARS-CoV2 that may arise in the future.

The data generated in this study by means of viral loads and antibody responses over the 21 days from the initial diagnosis would be useful in implementing control measures of the disease. However, further studies with a larger sample size over a longer period of time will be necessary to understand how viral positivity and the level of antibody responses will give an idea of long term protection.

6. Conclusions

Majority of asymptomatic patients, at the time of initial diagnosis, converted to pre-symptomatic status, but with minimal symptoms. These patients showed the longest viral shedding duration. Comparatively, symptomatic patients seemed to have higher viral loads than the asymptomatic patients, though the difference was not significant. Symptomatic, asymptomatic and presymptomatic patients exhibited different kinetics of IgG/IgM responses to SARS-CoV-2. The level of IgM responses was significantly higher in Symptomatic patients as compared to pre-symptomatic patients, at day 14 and 21. Symptomatic individuals with high viral loads also seem to have developed a good antibody response, which may further be related to having symptoms like fever. However, by 9 months immunity is waning in this cohort of largely healthy young males and neutralizing antibodies are present at significant levels in <15% of the patients infected with a high viral load. Therefore, SARS-CoV2 asymptomatic or mildly symptomatic infections may not provide long lasting immunity after a single infection, for a majority of the individuals making them vulnerable to the new variants of SARS-CoV2 that may arise in the future.

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