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Relative Quantification Method as A Tool for Viral Load Assessment In COVID-19 Patients at A Tertiary Care Hospital

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ABSTRACT

Background: Since March 2020 till date, there has been a continuous increase in the epidemic curve in our country. The 2-ΔΔCt method is commonly used for viral load assessment in relative quantification methodology. The viral load detection has important implications in assessing the transmission risk associated with high viral load in any patient and protecting the disease's progression and thus holds an important role in early antiviral treatment for all critically ill patients with COVID-19. The aim of the study is to use the relative quantification method ($2^{-\Delta\Delta Ct}$ method) to calculate the viral load in non hospitalized population.

Methods: 285 were confirmed positive for SARS-CoV-2 infection using Bio-Rad real-time RT-PCR test. Relative quantification was calculated by the difference of the comparative threshold cycle (ΔΔCt) between RdRp gene and RNase P genes, from the clinical sample and the positive control tested simultaneously. The association between Ct values and various factors related to disease was calculated using appropriate statistical tests.

Results: The age ranging from 10-19 years showed the highest mean viral load ($2^{\Delta\Delta}$ Ct value= 80.41), which was not detectable after day 9. The mean viral load 2^-ΔΔCt in SARS-CoV-2 infected patients without any comorbidities was 30.79, while patients with comorbidities showed a moderate viral load 22.52. Fever and myalgia was the most common clinical presentation and were associated with mean viral load of 29.41.

Conclusion: Viral load information is required to help us understand the trends in various populations. In addition, reporting the viral load using the reference material and quantifying it using the quantification method will be more useful rather than using only Ct value for viral load analysis.

Keywords: *Relative Quantification; SARS CoV-2; Viral Load; Ct Value.*

1. INTRODUCTION

An acute respiratory infection is the most common health issue affecting all age groups and contributes to the world's disease burden. [1] Human coronaviruses (HCoVs) are also enveloped in single-stranded positive-sense RNA viruses among many other RNA viruses[2] . The COVID-19 pandemic is responsible for the wider spread of infection thus resulting in more morbidities and mortality as compared to previous coronavirus diseases [3] . The first cluster of patients presenting with respiratory illness was reported in Wuhan, China in 2019 December [4].

Since March 2020 till date, there has been a continuous increase in the epidemic curve in our country. Most of the COVID 19 patients complained of mild symptoms while only 14% individual presented with severe acute respiratory symptoms and required hospitalization [5] . SARS CoV-2 patients present with a varied range of clinical features ranging from asymptomatic to severe respiratory disease. Hence, it is important to evaluate the infectiousness and severity timely and provide the necessary treatment at the earliest[6] . The respiratory route (droplet) followed by the orofecal route is the most common mode of transmission for COVID-19. SARS CoV-2 also known as COVID-19 disease is known for its highly infectious nature, therefore it was important to develop rapid and effective diagnostic tools to have an early diagnosis of the infection and thus prevent its rapid spread [7] . The real time PCR is the gold standard diagnostic method available for SAR CoV-2 detection and became more popular in 2019. The most common sample preferred for RT-PCR testing is oro-nasopharyngeal swabs as many patients do not produce sputum easily[8] . There are different types of RT-PCR test interpretation (qualitative, quantitative, or semi-quantitative) used in SARS CoV-2 diagnosis. For quantifying the viral load, the Ct value cannot be used directly, due to its limitation [9] . Two methods are used for quantification namely, absolute quantification which directly analyses the gene dose and relative quantification method which compares the target gene changes with the reference sample. In relative quantification methodology, the 2-ΔΔ*C*t method is commonly used for viral load assessment [10,11] . As it is easy to perform, Bustos P et al have suggested its use in the study where the viral load was analyzed in mild SARS CoV-2 infected patients [12] . The viral load detection will also have important implications in assessing the transmission risk associated with high viral load in any patient which will protect against the progression of the disease and thus hold an important role in early antiviral treatment for all critically ill patients with COVID-19. There is a scarcity of studies which looked into the viral load analysis in mild to moderate disease. In India, our study is the first to use the relative quantification method ($2^{-\Delta\Delta Ct}$ method) to calculate the viral load in non hospitalised population.

2. METHODS

This cross-sectional prospective study was carried out at BSL-2 Virology lab, as part of a routine diagnostic test of COVID-19. All COVID-19-positive patients (Admitted/Home isolated) who agreed to give consent for participation were included in the study irrespective of symptoms. Patients who turned out to be negative by RT-PCR for COVID-19 and /or were positive for COVID-19 with antigen test only; were excluded from the study.

Study Plan:

The nasopharyngeal/oropharyngeal swabs collected for RT-PCR were transported in viral transport media to the BSL-2 virology laboratory with proper cold chain maintenance. All the nasopharyngeal swab samples received in the BSL-2 Virology Laboratory were tested for SARS-CoV-2 infection using Bio-rad real-time RT-PCR.

Research Strategy:

Viral RNA extraction was done within 2 hours of receiving the sample in the laboratory by either automated or manual procedure according to the manufacturer's instructions. The extracted RNA was subjected to amplification in the thermocycler using the amplification kit available. The RT-PCR was conducted with primers and probes targeting the RdRp and N genes and positive reference/control. The amplification curves were analyzed on the system and results were considered positive when Cycle threshold value (Ct value) of both target genes was ≤ 35 and was considered negative when the Ct value of both the target genes was more than 35. The particular assay was considered valid only when the cycle threshold of the positive control was ≤ 35. All individuals confirmed positive for SARSCoV-2 infection were further tested according to the ICMR guidelines. Relative quantification was calculated by difference of the comparative threshold cycle (ΔΔCt) between RdRp gene and RNase P genes, from the clinical sample and the positive control tested simultaneously, using following formulas: 2ΔΔ*C*t (Δ*C*tsample-Δ*C*tcontrol), ΔCt sample = (Ct value of sample ORF1ab − Ct value of sample RNase P), and ΔCt control = (Ct value of positive control ORF1ab − Ct value of positive control RNase P. Association

between Ct values and various factors related to disease was calculated using appropriate statistical tests.

Ethical Considerations: The ethical approval was given by the Institutional Ethics Committee of SMS&R and Sharda Hospital, Sharda University with ref no. SU/SMS&R/76-A/2022/62.

Statistical analysis

The statistical analysis was conducted using SPSS 28, employing various methods to explore key aspects of the data. Differences in mean viral load across different age groups and over time were examined to identify significant variations in viral load dynamics. The effect of time (Day 0, Day 3, Day 6, Day 09, D12 , D14) on viral load was assessed using Repeated Measures ANOVA, determining if there were significant changes in viral load over the observation period. Statistical significance was determined with a threshold of $p < .05$.

3. RESULTS

Out of 4762 tested patients for COVID-19 infection, 285 were confirmed for SARS CoV-2 infection by RT-PCR. The viral load was analyzed in all the confirmed SARS CoV-2 positive patients using relative quantification under various parameters.

I. Age-wise distribution of SARS CoV-2 viral load (N=285):

The viral load detected by relative quantification was analyzed in various age group ranges of all SARS-CoV-2 positive patients **[Figure 1, Table 1].** The positive patient with age ranging from 10-19 years showed the highest mean viral load ($2^{\Delta\Delta}$ Ct value= 80.41). while in age group of elderly patients more than 80 years old showed a very low mean viral load($2^{\Delta\Delta}$ Ct value= 4.206). Similarly, age groups of 20-29 years old and 70-79 years old also showed a mean viral load low ($2^{\Delta\Delta}$ Ct value =11.62 and $2^{\Delta\Delta}$ Ct value =13.01) respectively. An increase in mean viral load was observed in the age group ranging from 40 to 70 years old (40-49: $2^{\Delta\Delta}$ Ct value= 41.68; 50-59: $2^{\Delta\Delta}$ Ct value=66.27). On analysis of gender-wise distribution, no significant difference in viral load was observed among males and females.

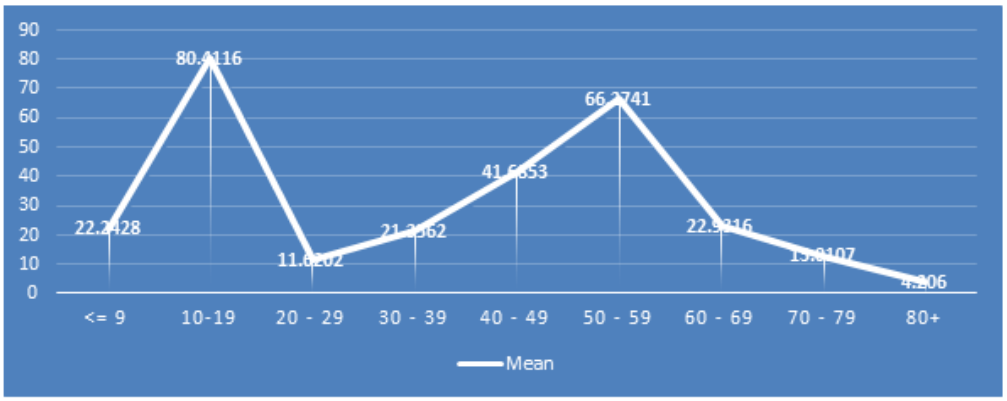

Figure 1: Agewise distribution of SARS CoV-2 viral load in 285 infected individual.

Table 1 Age wise distribution in SARS CoV-2 viral load (N=285)

AGE	Mean
<= 9	22.2428
10-19	80.4116
20 - 29	11.6202
30 - 39	21.3562
40 - 49	41.6853
50 - 59	66.2741
60 - 69	22.9316
70 - 79	13.0107
80+	4.206

II. Day-wise viral load distribution in all SARS CoV-2 infected patients.

The viral load difference in various age group ranges could be real or it could be affected by the day of testing after onset of symptoms **[Table 2 and Figure 2].**

To evaluate this theory, viral load was analyzed on every 3 rd day from the first day of the sample received in the laboratory i.e Day 0 (D0) , Day 3 (D3), Day 6 (D6), Day 9 (D09), Day 12 (D12) and Day 14 (D14) in all age group ranges. As shown in **Table 2,** the mean viral load is seen highest in patients of 10-19 years age range on D0 ($2^{\Delta\Delta Ct}$ =80.41). Later on , on 3rd day of the first day of testing which is D3, the mean viral load dropped down ($2^{\Delta\Delta Ct}$ =59.26). After day 9 , the viral load was not detectable in this age group of patients. Similarly, On D0, the mean viral load was 22.24 in less than 9 years of age group patients, which gradually decreased on D3, D6 and was not detectable after D09. In patients more than 60 years of age group to 80 years old, the mean viral load was detectable till Day 14 and then became negative.

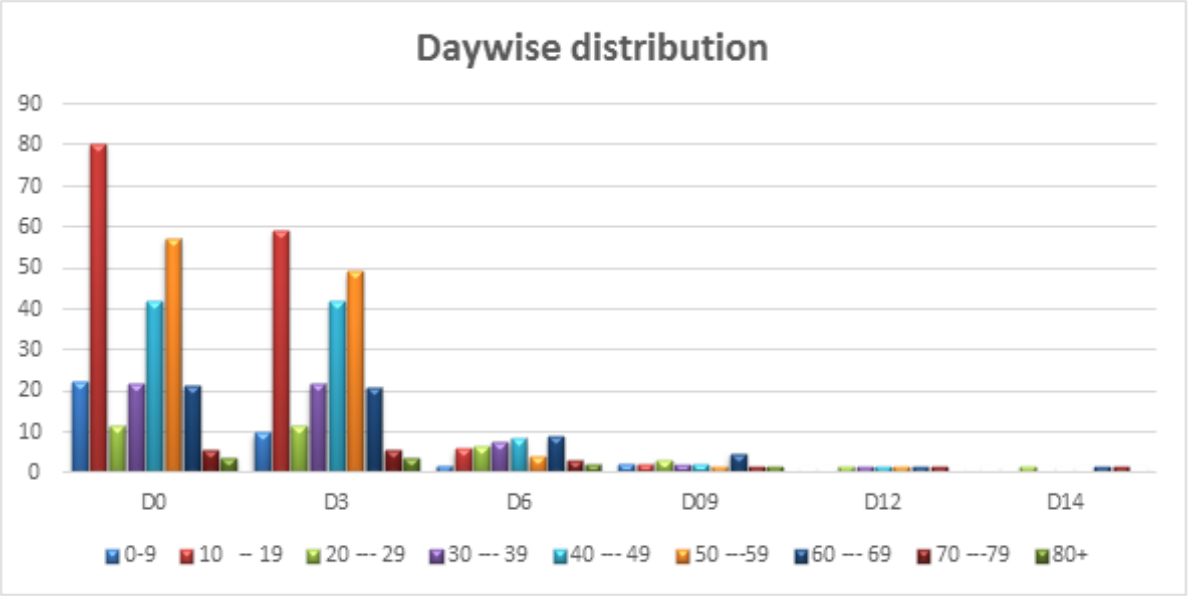

Fig 2: Mean viral load distribution in all SARS CoV-2 patients

Table2: Mean viral load distribution in all SARS CoV-2 infected patients on every 3rd day:

Age Range	D0	D3	D6	D09	D12	D14
<= 9	22.2428	9.761	1.873	2.361	-	-
10-19	80.4116	59.2644	5.9848	2.2136	-	-
20 - 29	11.6243	11.52	6.4571	3.2632	1.43	0.03
30 - 39	21.6918	21.6518	7.684	2.0629	0.06	0
40 - 49	41.6853	41.7737	8.4068	2.2722	0.01	0
50 - 59	57.2423	49.2406	4.0548	1.4174	0.02	0
60 - 69	21.0432	20.959	8.8625	4.7725	1.54	1.34
70 - 79	5.7508	5.7514	3.1014	1.1729	0.97	1.21
80+	3.8192	3.818	2.232	0.352	0	0.0

III. Analysis of viral load and clinical features:

Most of the patients included in this study presented with mild to moderate type of disease. As shown in **Table 3** and **fig 3.1** the mean viral load 2^-ΔΔCt in SARS-CoV-2 infected patients without any comorbidities was 30.79. As depicted in **Figure 3.3**, in all infected patients, fever and myalgia was the most common clinical presentation and was associated with 29.41. The patients presented with body aches and headaches were also associated with moderate viral load 29.18 **[Figure 3.2].**

While, the mean viral load was low in patients presented with cold, diarrhoea dyspnoea ranging from 0.52 -15.88 **[Figure 3.2].** When single and combined symptoms were analyzed in **Figure 3.3** and **Figure 3.4**, it was observed that sore throat,

myalgia, and cough were associated with low viral load ranging from 1.12 -14.21 or moderate viral load (34.64). In contrast, the presence of fever exclusively or combined with other symptoms was associated with high viral load ranging from 28.48-189.78 **[Figure 3.3 and 3.4].** The mean viral load in SARS-CoV-2 infected patients associated with comorbidities showed moderate viral load 22.52 **[Figure 3.5].** The mean viral load was observed high in patients associated with hypothyroidism with a value of 215.87. The patients with hypertension and asthma as comorbidity showed relatively moderate level of viral load ranging from 36.62 -48.62 **[Table 3 and Figure 3.5].**

Table 3: Analysis of viral load according to clinical features and comorbidities:

Variable	**(N,%)**	**Mean Viral load 2^-ΔΔCt**
Individuals without comorbidities	**254 (89.1)**	**30.79**
Clinical features		
Fever	246 (96.9)	29.41
Myalgia	246 (96.9)	29.41
Cough	202 (79.5)	24.28
Sore Throat	207(81.5)	22.05
Cold	136 (53.5)	15.88
Body ache and Headache	31(12.2)	29.18
Diarrhoea	2(0.8)	8.36
Loss of Taste	1(0.4)	54.95
Dyspnoea	1 (0.4)	0.52
Single Symptom		
Fever	12	28.48
Cough	10	8.78
Myalgia	5	2.91
Sore throat	3	1.12
Combined symptoms		
Fever+ Myalgia	14	189.78
Cough + Fever	10	14.21
Fever+ Myalgia+ Sore throat	25	94.54
Cough + Myalgia	8	34.64
Individuals with comorbidities	**31 (10.8)**	**22.52**
Comorbidities		
Diabetes Mellitus	12 (38.7)	3.33
Hypertension	8 (26)	48.62
Asthma	5 (16.1)	36.62
Hypothyroidism	2 (6.4)	215.87

Cardiovascular disease	2 (6.4)	1.27
Cancer	1 (3.2)	2.01
HIV	1 (3.2)	20.82

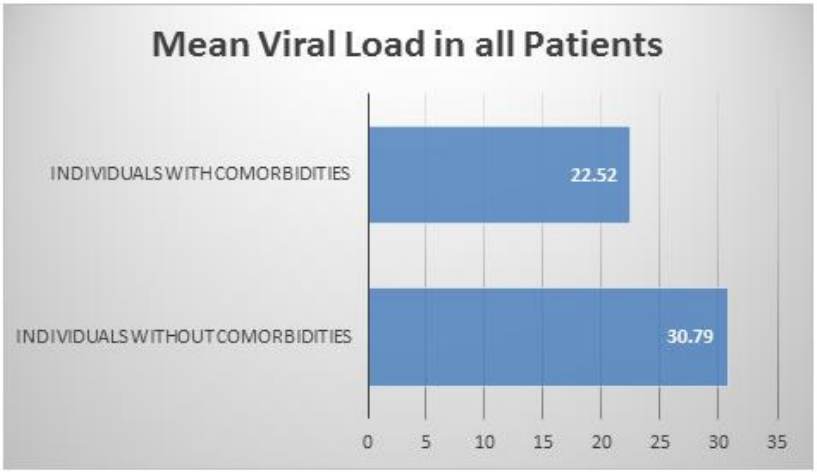

Fig 3.1 Mean Viral load in all SARS CoV-2 Patients

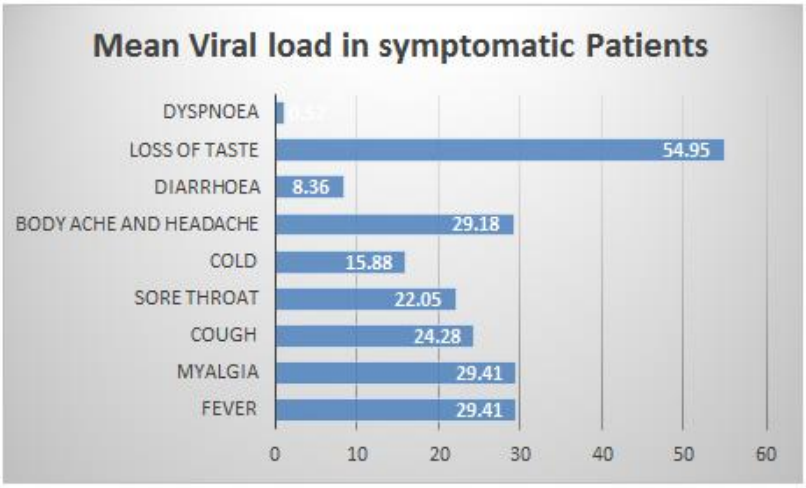

Fig 3.2 Mean Viral load in symptomatic SARS CoV-2 Patients

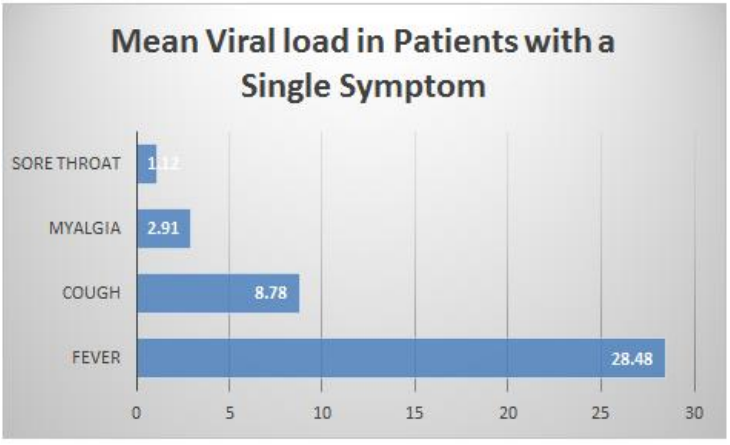

Fig 3.3 Mean Viral load in Patients with single symptom

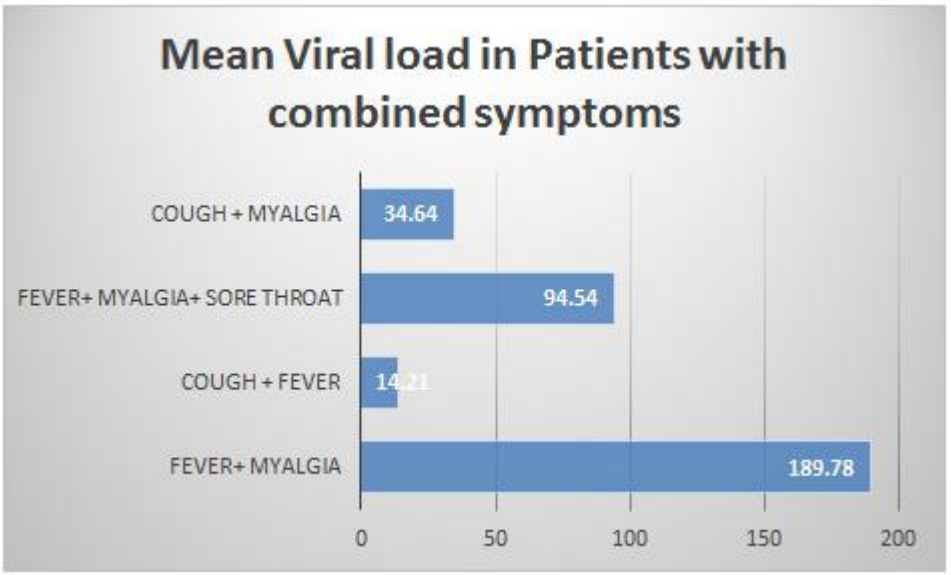

Fig 3.4 Mean Viral load in Patients with combined symptoms

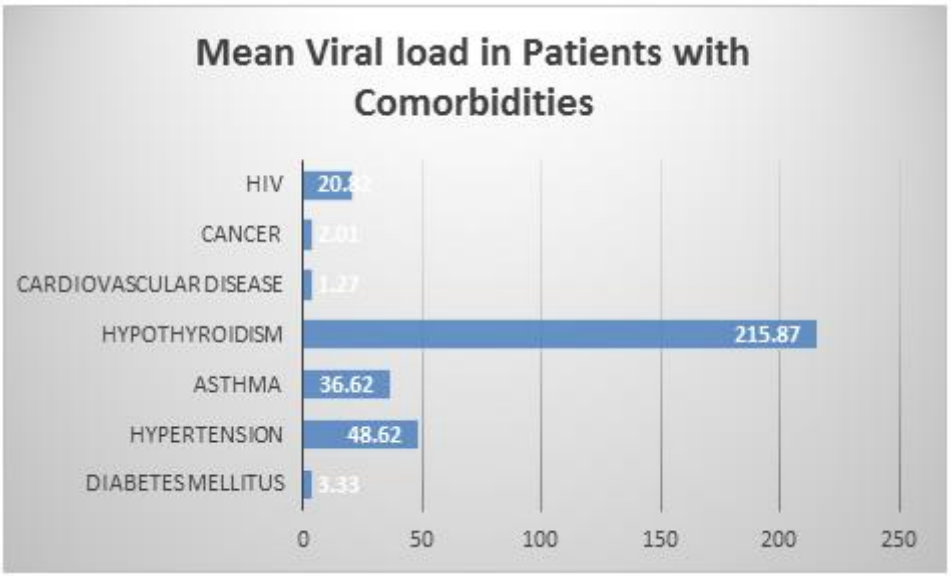

Fig 3.5 Mean Viral load in Patients with comorbidities

4. DISCUSSION

SARS CoV-2 also known as COVID-19 disease is known for its highly infectious nature, therefore it was important to develop rapid and effective diagnostic tools to have an early diagnosis of the infection and thus prevent its rapid spread [7]. Many studies have used Ct-value as a marker for monitoring the disease severity in viral respiratory disease, but its use has reported varying results [8]. Most of the studies done in the year 2020 included hospitalized or critically ill patients. There was a scarcity of studies that looked into the correlation of the progression of severity in mild or moderately infected individuals.

In our study, most of the cases involved were the non-hospitalized individuals, which could be due to the awareness of the disease and the government-led management guidelines available for mild to moderate cases. So, to evaluate the severity, viral load was used as the parameter for analysis, and the relative method of quantification was used to quantify the viral load, instead of only using the Ct value.

Though our study was conducted from 2022 – 2023, during which cases were seen to decrease and also the people were getting immune to the infection, which affected our total number of cases but still the positivity rate almost remained the

same, as observed. Out of the total samples received in the laboratory for SARS CoV-2 testing by RT-PCR, 285 cases were confirmed positive for the COVID-9 disease with a positivity rate of 5.9%. there were very few studies that have reported a positivity rate. One such study done by Sarkar B et al 2020 [13], reported 7% positivity rate in one month. The studies done before were mostly carried out during the initial phase of pandemic when there was unawareness of the infection pathogenesis and its severity, which led further to panic and thus led to testing in large numbers.

Previously, a study done by Sarkar B et al. 2020 [13], assessed the distribution of viral load in COVID-19-infected cases. In this study, viral load was categorized as high, moderate, and low by using only Ct value. In our study, viral load was analyzed using the relative method of quantification instead of only using the Ct value.

Analysis of viral load with various parameters

- **Age-wise distribution:**

In our study, in the>60 years of age group viral load was detected till day 14 , suggesting the presence of the virus for a longer period, which might suggest chances of contracting severe illness. Knudtzen FC et al. 2021 [8], have also reported similar findings of getting severe illness in > 60 years of age group. Bustos P. et al. 2021[12], have reported to have high viral load values up to 2nd week after onset of symptoms in mild cases. In SARS-CoV-2 infection, the neutralizing antibodies develop as the immune response. These neutralizing antibodies responsible for clearing out the virus starts developing within 1-2 weeks after symptom onset and peak after 4th week. In older people, as the immune system is weak and is not able to produce a sufficient number of antibodies, the virus remains for a longer period of time and thus could further lead to severe illness. In addition, elderly people also report having high levels of ACE-2 receptors in their alveoli, which is the receptor responsible for SARS-CoV-2 virus attachment.

On gender-dependent analysis, no significant difference was seen in our study. In the study done by Knudtzen FC et al. (2021) [8], the presence of the virus was found to be for a longer duration in men than in women. The author also reported the immune status difference in males and females which was related to hormone level differences. On the contrary, in our study, no immune analysis was carried out.

- **Day-wise viral load distribution:**

In our study, patients came for testing when symptoms were still mild or in initial stages, because of the general awareness of circulating pandemic disease. Many previous studies have conducted the viral load assessment but that was only qualitative using the Ct values as the marker. Yu X et al. 2020 [14], reported viral load assessment using Ct value from lower respiratory samples (sputum). In the above study, at the time of admission, viral load was low in mild to moderate cases while it was high in severe cases.

In our study, the SARS CoV-2 virus was detectable after day 14 of onset of symptoms in more than 60 years of age group. Similarly, the study done by Huang Y. et al. 2020 [15] and Zou L. et al. 2020 [16], also reported the longer duration of shedding of virus beyond day 28 and day 14 days after the onset of symptoms respectively.

The duration of virus shedding helps in assessing the risk of transmission and thus protecting healthcare workers. By assessing the duration of shedding the effectiveness of management especially in severely ill patients can be monitored. The study done by Huang Y et al. 2020 [15], has shown that virus prolonged shedding seen in critically ill patients from the lower respiratory tract indicates that a longer period is required for clearance of viruses from these patients. The study conducted by Knudtzen FC et al. 2021 [8], also reported that viral RNA declines slowly in sputum as compared to throat swabs.

On the contrary, Wölfel R. etal. 2020 [17], reported viral load assessment using the standard curve method of quantification, which showed detectable viral load till day 5. The study reported successful virus isolation from the sample during the first week of symptoms. This shows successful isolation from early throat swabs with high viral load, suggesting potential virus replication in the tissues of the upper respiratory tract. To further confirm this, viral sub-genomic mRNA identification was done by the Wölfel R. etal. 2020 [17]. So, we can conclude from the study that active replication is seen in the first five days of symptom onset. Similarly in our study, peak viral load was detectable in the initial phase of symptoms thus supporting the above findings. Lescure F-X et al. 2020 [18], also reported to have high viral load in upper respiratory samples. These studies conclude that there is a high risk of transmission of infection to others in the initial days of symptoms [17,18].

In our study, viral load detection was done from nasopharyngeal samples in mild to moderate cases. The study showed to have high viral load in the first week of symptom onset and a decline in 2nd week. Similarly, Bustos P. et al. 2021 [12], conducted the detection of viral load in milder cases, which reported to observe a similar type of viral load peak. In our study, the virus was detectable even after day 14 which was in concordant with the findings observed by Bustos P. et al. 2021[12]. The study done by Lescure F-X et al. 2020 [18], has shown that viral load decreases over days in all patients except the critically ill and becomes negative over day 9 and day 14. Though our study was conducted in milder patients but in patients more than 60 years old showed detectable virus even after day 14, suggesting the longer presence of the virus in older age group cases.

Previous studies have reported that viral load in upper respiratory tract samples could be the marker of disease severity [12].

In our study also, viral load analysis was done on upper respiratory tract samples. As no other samples were included in our study, no comparison was carried out among different samples. On the contrary, in the study conducted by Knudtzen FC et al. 2021 [8] , viral load in respiratory samples was shown to be higher than compared to stool and serum samples, especially in patients with severe disease rather than patients with mild disease. The above study has also shown to report the comparison of viral load in milder and severe cases [8] . In our study no such comparison was done as our population included only mild to moderate cases. We can conclude here that knowing the viral load can help us in assessing the prognosis. To know timing of the peak and decline of virus in upper respiratory tract samples during the early stage of infection, will also help in the prevention of further transmission.

5. CONCLUSION

Using relative quantification, the 2-ΔΔCt method for viral load detection has its advantage, as it requires very few reagents, require no curve for interpretation and is less time-consuming. So, if the viral load of these patients could be communicated routinely to attending doctors or nursing staff, additional precautions and care could be taken to reduce the transmission among them. We can also justify the use of viral load detection in providing early antiviral treatment to COVID-19 patients, if effective, would reduce the risk of progression and thereby mortality.

Conflict of interest: The authors report no conflicts of interest.

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Authorship Contributions: All authors have made substantial contributions tothe conception and design of the study, acquisition of data, analysis, interpretation of data, drafting the article and revising it critically for important intellectual content. All authors approved the final submission of the article.

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