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Lower nasopharyngeal viral load during the latest phase of COVID-19 pandemic in a Northern Italy University Hospital

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Abstract

Objectives: A milder clinical course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been anecdotally reported over the latest phase of COVID-19 pandemic in Italy. Several factors may contribute to this observation, including the effect of lockdown, social distancing, lower humidity, lower air pollution, and potential changes in the intrinsic pathogenicity of the virus. In this regard, the clinical severity of COVID-19 could be attenuated by mutations in SARS-CoV-2 genome that decrease its virulence, as well as by lower virus inocula.

Methods: In this pilot study, we compared the reverse transcription polymerase chain reaction (RT-PCR) amplification profile of 100 nasopharyngeal swabs consecutively collected in April, during the peak of SARS-CoV-2

epidemic, to that of 100 swabs collected using the same procedure in May.

Results: The mean Ct value of positive samples collected in May was significantly higher than that of samples collected in the previous period (*ORF 1a/b* gene: 31.85 ± 0.32 vs. 28.37 ± 0.5 , $p < 0.001$; *E* gene: 33.76 ± 0.38 vs. 29.79 ± 0.63 , $p < 0.001$), suggesting a lower viral load at the time of sampling. No significant differences were observed between male and females in the two periods, whilst higher viral loads were found in (i) patients over 60-years old, and (ii) patients that experienced severe COVID-19 during the early stages of the pandemic.

Conclusions: This pilot study prompts further investigation on the correlation between SARS-CoV-2 load and different clinical manifestation of COVID-19 during different phases of the pandemic. Laboratories should consider reporting quantitative viral load data in the molecular diagnosis of SARS-CoV-2 infection.

Keywords: COVID-19; Ct value; Italy; SARS-CoV-2; viral load.

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Introduction

Starting at the end of February 2020, the current coronavirus disease 2019 (COVID-19) pandemic has heavily affected Italy, with most cases clustered in Lombardy. Given the possibility of novel pandemic waves, the identification of potential host-related and virological markers that predict specific disease outcomes would be crucial from both a medical and an epidemiological perspective [1–3]. As of May 29th 2020, the clinical picture of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has considerably changed in Italy and in Lombardy, with significant reduction in the number of new cases, paralleled by a decrease in the number of severe cases needing ventilatory support in Intensive Care Unit (ICU) [4]. Social distancing and lockdown procedures adopted by the Italian Government have likely contributed to reducing inter-human SARS-CoV-2

transmission, therefore explaining the observed decrease in the number of cases [5]. However, the different factors causing the observed more benign course of the infection remain to be elucidated, including the possible role of viral factors.

Patient age, sex (especially in the first phase of pandemic) and comorbidities have been convincingly identified as important predictors of severity in COVID-19 [6–10]. Unfortunately, no virological marker has been identified as associated with the clinical stratification of patients with COVID-19. In the absence of clear molecular evidences of viral attenuation, other viral parameters deserve deeper investigation, such as the level of detectable viral load. This aspect has only been partially addressed during the peak of SARS-CoV-2 pandemic, with controversial results and only a few studies correlating the viral load in different biological samples to a worse clinical outcome [11–16]. In this regard, it is interesting to note that, in a model of SARS-CoV-2-infected macaques, higher viral loads were detected in nasal swabs of aged macaques compared to younger individuals [17].

In this pilot study we sought to compare the reverse transcription polymerase chain reaction (RT-PCR) amplification profile of 100 nasopharyngeal swabs consecutively collected in April during the peak of the epidemic in Lombardy, to that of 100 swabs collected during a later period (e. g., in May), characterized by a lower relative amount of clinically severe cases. In particular, we used cycle threshold (Ct) as a commonly used correlate of viral load in the sample (i. e. the lower the observed Ct value, the higher the amount of virus within the tested sample).

Materials and methods

Samples

Swabs were collected using FLOQSwabs® (COPAN) in UTM® Universal Transport Medium (COPAN). One hundred consecutive nasopharyngeal swabs were randomly collected from COVID-19 patients during two different phases of Lombardy COVID-19 pandemic, each. Period 1 spans from April 7th to 10th 2020 and corresponds to a steep increase of the pandemic curve and a high relative amount of severe cases; Period 2 spans from May 12th to 19th 2020 and corresponds to a steep decrease of cases and lower relative amount of ICU admissions. For each sample, sex, age and need of hospitalization were recorded. The study was reviewed and approved by San Raffaele Hospital IRB in the COVID-19 Biobanking project “COVID-BioB” N° CE: 34/int/2020 19/ March/2020 ClinicalTrials.gov Identifier: NCT04318366.

Evaluation of SARS-CoV-2 RNA amount in clinical samples

Cycle threshold values (Ct) have been determined with Cobas® SARS-CoV-2 Test (Roche), which detects conserved regions for ORF-1a/b and E-gene regions on SARS-CoV-2 after RNA automatic extraction. Non-infectious plasmid DNA containing a specific SARS-CoV-2 sequence and a pan-Sarbecovirus sequence are used in the test as positive control. A non-Sarbecovirus related RNA construct is used as internal control. The test is designed to be used on the automated Cobas® 6800 Systems under Emergency Use Authorization (EUA). The test is available as a CE-IVD test for countries accepting the CE-mark.

Data analysis and statistics

Mean and standard errors from mean (SEM) of Ct measured in the two groups were used for comparing study cohorts. Statistical analyses were performed by using two tailed unpaired *t*-test with Welch's correction for comparisons between study cohorts. Prism 5 (GraphPad) software was used for both statistical analysis and graphical rendering of plots. $p < 0.05$ was set as significance threshold value.

Results

The stratification of patients according to the time of sample collection, sex, age and the need of hospitalization is shown in Figure 1A. For each tested sample, the Ct value has been reported for both molecular targets (ORF1 a/b and E) detected by the diagnostic kit used in the study. The mean Ct value of positive samples collected during the Period 2 was found to be significantly higher than the mean Ct value of samples from Period 1, suggesting a lower viral load (ORF 1a/b: 31.85 ± 0.32 vs. 28.37 ± 0.5 $p < 0.001$; E: 33.76 ± 0.38 vs. 29.79 ± 0.63 , $p < 0.001$) (Figure 1B). No differences could be observed in the mean Ct value of Cobas® 6800 System internal (34.10 ± 0.08 vs. 34.21 ± 0.11 , $p = 0.4$) and positive (ORF 1a/b: 33.52 ± 0.08 vs. 33.54 ± 0.06 , $p = 0.87$; E: 35.78 ± 0.09 vs. 35.79 ± 0.09 , $p = 0.94$) controls between the two periods, thus confirming the reliability of our observation.

The two cohorts of patients were then stratified for sex, in order to investigate further the observed difference. No significant differences were observed between sexes during Period 1 (ORF 1a/b: 28.96 ± 0.72 in men vs. 27.78 ± 0.79 in women, $p = 0.27$; E: 30.51 ± 0.86 in men vs. 29.07 ± 0.92 in women, $p = 0.26$). On the other hand, an increase in average Ct value was observed in Period 2 for both sexes, which was highly significant in women (ORF 1a/b: 30.63 ± 0.61 in men vs. 32.81 ± 0.27 in women, $p = 0.002$; E: 32.35 ± 0.72 in men vs. 34.86 ± 0.34 in women, $p = 0.002$).

Figure 1: Description of the analyzed cohorts and overall depiction of SARS-CoV-2 RNA load in nasopharyngeal swabs.

(A) Stratification of subjects belonging to the two analyzed pandemic periods based on sex, age and hospitalization. (B) Mean Ct values for ORF 1a/b and E targets, evaluated for the samples during the two time periods. (C) Mean Ct values for ORF 1a/b and E targets of under and over 60-year-old subjects during the two time periods. Means and error bars representing SEM indicated with red lines. ns: not significant; **** $p < 0.0001$.

Interestingly, a significant increase of mean Ct values was noted for both molecular targets in the two periods also in non-hospitalized subjects (ORF 1a/b: 28.42 ± 0.57 in Period 1 vs. 31.91 ± 0.35 in Period 2, $p < 0.001$; E: 29.87 ± 0.68 in Period 1 vs. 33.83 ± 0.42 in Period 2, $p < 0.001$). In the early pandemic phase, higher viral RNA amount was also observed for both molecular targets in hospitalized patients, approaching the threshold of statistical significance (ORF 1a/b: 28.06 ± 1.62 in Period 1 vs. 31.41 ± 0.78 in Period 2, $p = 0.08$; E: 29.32 ± 1.84 in Period 1 vs. 33.21 ± 0.96 in Period 2, $p = 0.08$).

Stratifying the cohorts according to age, a significant difference in Ct values was observed both for patients ages < 60 years (ORF 1a/b: 30.15 ± 0.65 in Period 1 vs. 33.27 ± 0.24 in Period 2, $p < 0.001$; E: 27.03 ± 0.76 in Period 1 vs. 31.16 ± 0.45 in Period 2, $p < 0.0001$) and, more interestingly, also for high-risk older patients (ORF 1a/b: 31.86 ± 0.80 in Period 1 vs. 35.54 ± 0.27 in Period 2, $p < 0.0001$; E: 28.23 ± 0.88 in Period 1 vs. 32.88 ± 0.53 in Period 2, $p < 0.0001$) (Figure 1C).

A further stratification was performed in older patient, who were stratified in decades. Interestingly, for both ORF 1a/b and E molecular targets, lower Ct value means were observed in Period 1 compared to Period 2. However,

statistically significant differences were observed only for the cohorts at higher-risk, including subjects in the 80–89 years range (ORF 1a/b: 25.32 ± 1.37 in Period 1 vs. 31.38 ± 1.07 in Period 2, $p < 0.01$; E: 26.34 ± 1.63 in Period 1 vs. 33.20 ± 1.22 in Period 2, $p < 0.001$) and the over-90 years (ORF 1a/b: 24.14 ± 1.40 in Period 1 vs. 32.31 ± 0.72 in Period 2, $p < 0.001$; E: 24.67 ± 1.58 in Period 1 vs. 34.26 ± 1.02 in Period 2, $p < 0.001$) (Figure 2A, B). Interestingly, age-dependent differences were observed during Period 1 for both molecular targets, with lower Ct values (higher viral loads) in the oldest groups (Figure 2C, D). These differences in average Ct values were somehow less evident in Period 2, especially comparing the under-60 years cohort to the 80–90 years and the > 90 years cohorts (Figure 2C, D).

Discussion

The overall number of SARS-CoV-2-infected subjects in Italy has decreased considerably during the last few weeks of the COVID-19 pandemic [4]. The overall reduction of cases was paralleled by a reduction in the fraction of severe cases requiring hospitalization or ICU support [1]. The reasons underlying these changes are not completely clear

Figure 2: SARS-CoV-2 Ct values of nasopharyngeal swabs: inter-phase and intra-age analyses.

(A, B) Mean Ct values, for both ORF 1a/b and E targets, observed in different age decades in the two time periods. (C, D) Redistribution of mean Ct values of subjects stratified according to age by decades for the two time periods. The two molecular targets are reported. Means and error bars representing SEM are indicated with red lines. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and may include epidemiological, environmental and virological factors, such as the typical increase of temperature in May vs. April in Italy, a notable decrease of environmental pollution which may have contributed to reduce virus propagation, as well as the possible selection of viral variants characterized by attenuated virulence and pathogenicity [18, 19].

In this pilot study, we have measured SARS-CoV-2 load in nasopharyngeal samples collected during two different periods of the current outbreak, using Ct values of a commercially available diagnostic system as surrogate marker [20]. The most important message emerging from these results is that positive swabs collected during the more recent phase are characterized by lower viral load compared to those of the former period. Importantly, these changes are evident also in the oldest age cohorts, which were at higher risk of developing more aggressive form of illness and experiencing worse clinical outcomes during the first phase of the Italian outbreak [21]. Interestingly, the clinical picture has somehow changed during the last weeks in these cohorts as well [1, 4].

We are aware of some limitations in our study, including its single-center design, the limited number of enrolled subjects and the consequent difficulty of directly correlating what we have observed to the different clinical outcome of COVID-19 in the current latest phase of the epidemic. Moreover, we are aware that, although already

used in other clinical studies carried out on SARS-CoV-2, the Ct-based semi-quantitative approach may be influenced by several variables, such as the different number of cells in each sample, which was not directly assessed in our study [22, 23]. Nevertheless, we think that these findings deserve further and probably deeper investigation, even using more sensitive dedicated quantitative approaches [24].

A possible working hypothesis driven by our observation, to be confirmed in larger studies, is that lockdown measures and social distancing exerted a double effect on the population. On one hand, the measures certainly led to the decrease in the number of infected subjects. On the other, the new cases were infected by an average lower viral load as a consequence of social distancing, enhanced frequency of hand washing and widespread use of face-mask and other personal protective equipment (PPE), as possibly suggested by our observation and by other recent studies [25–27]. In the absence of confirmed molecular data on mutations in the viral genome leading to a less severe clinical manifestation in our geographical area, we think that the nasopharyngeal viral load should be carefully evaluated from a clinical and epidemiological perspective. Confirming this initial observation in larger multi-center cohorts of COVID-19 patients may provide valuable epidemiological information and help identifying patients at high risk of severe disease.

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