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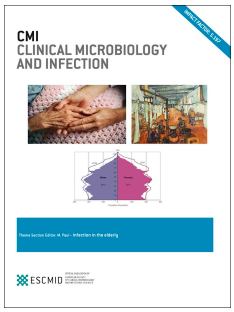

Prolonged SARS-CoV-2 cell culture replication in respiratory samples from patients with severe COVID-19

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ABSTRACT

Objectives: This study compares the infectivity of SARS-CoV-2 in respiratory samples from patients with mild COVID-19 with those from hospitalised patients with severe bilateral pneumonia. In severe COVID-19, we also analysed the presence of neutralising activity in paired sera.

Methods: We performed cell cultures on 193 real-time reverse transcription polymerase chain reaction respiratory samples, positive for SARS-CoV-2, obtained from 189 patients at various times, from clinical diagnosis to follow-up. Eleven samples were obtained from asymptomatic individuals, 91 samples from 91 outpatients with mild forms of COVID-19, and 91 samples from 87 inpatients with severe pneumonia. In these patients, neutralising activity was analysed in 30 paired sera collected after symptom onset >10 days.

Results: We detected a cytopathic effect (CPE) in 91 (91/193, 47%) samples. Viral viability was maintained for up to 10 days in the patients with mild COVID-19. In the patients with severe COVID-19, the virus remained viable for up to 32 days after the onset of symptoms. Patients with severe COVID-19 presented infectious virus at a significantly higher rate in the samples with moderate to low viral load (cycle threshold value $\geq$26): 32/75 (43%) versus 14/63 (22%) for mild cases ($P < 0.01$). We observed a positive CPE despite the presence of clear neutralising activity (NT50 >1:1024 in 10% (3/30) of samples.

Conclusions: Patients with severe COVID-19 might shed viable virus during prolonged periods of up to 4 weeks after symptom onset, even when presenting high cycle threshold values in their respiratory samples and despite having developed high neutralising antibody titres.

INTRODUCTION

SARS-CoV-2, a novel human coronavirus that emerged in Wuhan (China) in late 2019,[1,2] has been responsible for the largest pandemic in a century.

The use of real-time reverse transcription polymerase (rRT-PCR)[3] as a diagnostic and follow-up tool for SARS-CoV-2 infection has led to hypotheses regarding infectivity duration, the possibility of reactivation, and even reinfection.[4] Although rRT-PCR is the gold standard diagnostic method, it is less useful as a follow-up technique, because samples from patients who have overcome either mild or severe SARS-CoV-2 infection still have detectable viral RNA for variable periods of time.[5-7] In the absence of diagnostic methods with reliable quantification, the cycle threshold (Ct) value obtained in amplification has been employed as a semiquantitative measure and has been proposed as a parameter for elaborating approaches to removing patients from isolation.[8] Establishing a reliable cut-off Ct value is difficult, given the large number of available rRT-PCR-based diagnostic tests; the need to use more than 1 molecular test in most clinical laboratories to meet growing demand, and the use of different types of samples during patient follow-up. Hence, the importance of establishing the duration of virus viability in various clinical situations. The assessment of SARS-CoV-2 viability will help establish criteria for isolating patients.

The role of anti-SARS-CoV-2 neutralising antibodies in controlling viral excretion has recently been evaluated,[9,10] finding differences in the titres achieved and antibody persistence, depending on illness severity. It has also been suggested that the presence of neutralising antibodies is correlated with the lack of viral viability in respiratory samples.[7]

This study compared viral detection by rRT-PCR and the infectivity of SARS-CoV-2 in respiratory samples from patients with mild COVID-19 with those from hospitalised patients with severe bilateral pneumonia. In those patients with severe COVID-19, we also analysed the presence of anti-SARS-CoV-2 immunoglobulin G (IgG) and the neutralising activity in paired sera with respiratory samples, as well as the correlation between its presence and viral viability.

METHODS

Design, setting, and ethics

This retrospective study focused on respiratory samples obtained during a 2-month period that met the following requirements: (1) clinical record is available; (2) collection on viral transport medium that ensures virus viability; (3) sufficient residual volume after routine diagnostic assays; (4) samples processed with the same rRT-PCR assay; (5) when a reduction in Ct values was detected during follow-up. The study was approved by our institutional review board (Reference CEIm: 20/232).

Samples and patients

A total of 193 respiratory samples (186 nasopharyngeal exudates and 7 bronchial aspirates) were processed by rRT-PCR and cell culture. All the samples were from adult patients. Ninety-one samples were obtained from 91 patients with COVID-19-compatible symptoms who did not require hospital admission and who were mostly health care workers (HCWs (n = 76) attending the Occupational Health and Safety Service for a first consultation or follow-up after a first positive rRT-PCR sample. Eleven samples were collected from a different group of 11 asymptomatic individuals in whom the virus was detected during pre-surgical or delivery screening for hospital

admission or during contact studies. Ninety-one samples were obtained from 87 hospitalised patients with severe COVID-19 pneumonia. The diagnosis of severe COVID-19 was established by respiratory, laboratory, and radiographic findings. Samples were obtained at various time points covering the time from clinical diagnosis to follow-up during hospital care. Bronchial aspirates were collected during the follow-up of patients admitted to the intensive care units (ICUs).

Microbiological methods

Nasopharyngeal samples were collected with flocked swabs in universal transport medium (UTM) (Copan Diagnostics, Brescia, Italy). A previously published rRT-PCR protocol for detecting the E gene[3] was adapted for processing on the Panther Fusion Hologic (San Diego, CA, USA) automated molecular diagnostic platform, using its open access functionality.[11] The Ct value obtained in this assay was employed as a measure of relative quantification throughout the study.

For the cell culture, an aliquot (250 µL) of the residual sample was decontaminated using gentamicin and amphotericin B, inoculated into 24-well plates on Vero E6 cells (ATCC CCL-81), and cultured in Medium 199 supplemented with L-glutamine and 10% foetal bovine serum. The plates were incubated in a 5% carbon dioxide atmosphere for 5 days. The development of a cytopathic effect (CPE) was examined daily. SARS-CoV-2 CPE specificity was confirmed by immunofluorescence (shell-vial technique) by using a commercial anti-SARS-CoV-2 N protein (Rockland Immunochemicals, Inc., Limerick, PA, USA) as the primary antibody and a goat anti-rabbit IgG labelled with Alexafluor 488 (Abcam, Cambridge, UK) as the secondary antibody. Upon CPE observation and at the end of the cell culture incubation period, culture supernatants were collected from each well and an rRT-PCR was performed, which was confirmed positive if it was at

least 3 Ct lower than the original sample. All cell culture-related procedures were performed at a biosafety level 3 facility.

Specific anti-SARS-CoV-2 antibody detection

For 27 patients with severe COVID-19, we study a serum sample collected at least 10 days after symptom onset, paired with the analysed respiratory sample. In total, we analysed the presence of IgG and neutralising antibodies[6] in 30 serum samples through an IgG anti-SARS-CoV-2 chemiluminescent immunoassay (Abbott Laboratories) and neutralisation assays. We employed the SARS-CoV-2-pseudotyped recombinant vesicular stomatitis virus-expressing luciferase system to test the neutralising activity. Virus-containing transfection supernatants were normalised for infectivity to a 0.5–1 multiplicity of infection and incubated with the serum sample dilutions at 37°C for 1 h in 96-well plates. After the incubation, 2 x 10^4 Vero E6 cells were seeded onto the virus-plasma mixture and incubated at 37°C for 24 h. Cells were then lysed and assayed for luciferase expression. We calculated the 50% neutralisation titre (NT_{50}) using a nonlinear regression model fit with settings for log (inhibitor) versus normalised response curves.

Data analysis

We recorded and analysed the demographic data, COVID-19 severity, symptom onset to test time (STT), whether the patient was undergoing immunosuppressive therapy at the time of infection, Ct values, and CPE detection. NT_{50} neutralising activity was correlated with viral viability in the paired respiratory samples.

Quantitative variables are described using median and interquartile range (IQR) and were compared using the Mann–Whitney U test. Categorical variables are expressed

as relative frequencies and were compared using Fisher's exact test. P-values <0.05 were considered statistically significant. The statistical analysis was performed using GraphPad Prism v8 software.

RESULTS

Patient and sample descriptions

The mean age of the asymptomatic patients was 52.9 years (range 22–76), and 45% (5/11) were women**.** The mean age of the patients with mild COVID-19 was 40.7 years (range, 20–81), and 75% (68/91) were women. This mean age and sex distribution are due to the fact that most of the individuals included in this group are HCWs. Inpatients with severe COVID-19 had a mean age of 65.2 years (IQR 17–94), and 34% were women (30/87).

The patients with mild COVID-19 consulted for their symptoms earlier (mean 3.2 days [range 1–10], median 3 days [IQR 2–3]), than those with severe COVID-19 (mean 7.5 days [range 3–27], median 6 days [IQR, 4–10]; $P < 0.001$).

Seven (7/87, 8%) patients with severe COVID-19 were admitted to the ICUs and underwent mechanical ventilation.

In total, 7 (7/87, 8%) patients with bilateral pneumonia died, presenting a higher median age than the patients with bilateral pneumonia who recovered (80.0 vs. 64.5 years, $P < 0.01$).

Eighteen (18/87, 21%) patients with severe COVID-19 were undergoing immunosuppressive therapy when they acquired the infection (12 had malignancies, 3 were solid transplant recipients, and 3 had autoimmune diseases).

For the entire patient group, 109 samples were obtained at clinical diagnosis, and 73 were collected during patient follow-up. The median Ct value was 29.2 (IQR 26.0–32.3) for the inpatients' first samples (n = 63) and 25.2 (IQR 21.5–29.1) for the outpatients (n = 46) (P = 0.007). The 7 patients who died presented higher viral loads in the diagnostic sample than the other patients with pneumonia (median Ct values 21.0 vs. 29.5, P = 0.009). In contrast, first samples from the immunocompromised patients did not presented significantly lower Ct values (27.0 vs. 29.5, P = 0.2).

Cell culture

A CPE was detected in the cell culture in 91 (91/193, 47%) samples and was detectable in most cases in 72 h (Figure 1). Initial samples presented viral replication at a higher proportion than the follow-up samples: 69% (75/109) vs 15% (11/73) (P < 0.001). The mean collection time for the initial samples was 5 days (range 1–20, median 3, IQR 2–7), whereas for the follow-up samples it was 18.8 days (range 10–32, median 20, IQR 10–25).

The percentage of samples that presented viral replication for each of the patient groups is shown in Table 1, along with other sample data and patient demographics.

Correlation between virus viability and time from symptom onset

For the outpatients, a CPE was detected in 71% (17/24) of the samples obtained in the first week after symptom onset. In this group of patients with mild COVID-19, the maximum STT of a CPE-positive sample during follow-up was 10 days.

In the hospitalised patients with severe COVID-19, the virus was viable in 59% (16/27), 56% (9/16), and 64% (7/11) of the samples obtained in the first, second, and third week, respectively, and in 25% (2/8) of the samples obtained beyond the third week

STT. The maximum STT of a CPE-positive sample in the severe COVID-19 group was 32 days.

Figure 2 shows the distribution of samples analysed by the collection week after symptom onset, the percentage of samples with CPE in cell cultures in each week for both patient groups, and their statistical significance.

Correlation between virus viability and viral load (Figure 3)

In both the mild and severe COVID-19 groups, the samples that showed viral replication had significantly ($P < 0.001$) lower Ct values than the samples without viable virus (23.3 [IQR 20.5–28.0] vs. 36.4 [IQR 31.8–39.1], respectively, for mild COVID-19 and 27.7 [IQR 23.2–30.0] vs. 33.0 [IQR 30.4–38.0], respectively, for severe COVID-19).

The samples with higher viral loads (Ct ≤25) in both patient groups showed viable virus at a rate >90%. However, even the samples with low viral loads (Ct ≥35) could harbour viable virus, although at a much lower proportion (5% for mild COVID-19 and 15% for severe illness). Differences in viral viability between the outpatients and hospitalised patients were dramatic in the samples with moderate or low viral loads (Ct ≥26).

Patients with severe COVID-19 presented infective virus at a significantly higher rate (47%, 24/51) than outpatients (18%, 7/38) ($P < 0.01$).

In this regard, it is noteworthy that 2 of 7 bronchial aspirates presented CPE despite the fact that the median Ct value for this type of sample was 35.0 (IQR 32.6–38.9).

Correlation between viral replication and presence of anti-SARS-CoV-2 antibodies

Of the 30 sera collected with STT >10 days, 12 were paired with a CPE-positive respiratory sample, and 18 were paired with a CPE-negative respiratory sample.

In 7 samples, the presence of IgG and neutralising activity was not detected, 5 of which paired with CPE-positive respiratory samples. In the remaining samples, both assays were positive.

There was a significant difference between the NT_{50} geometric mean titre between the samples with and without CPE (107.2 vs. 699.69, $P = 0.04$). Most of the sera paired with CPE-negative respiratory samples (16/18, 89%) had an NT_{50} >1:80, whereas only 5/12 (42%) sera paired with CPE-positive respiratory samples had an NT_{50} >1:80, $P = 0.032$). This difference was not due to a greater proportion of samples from immunocompromised patients in the group of sera being paired with respiratory samples presenting CPE (25%, 3/12 vs. 11%, 2/18; $P = 0.32$).

Production of high neutralising antibody titres >1:1024 was present in almost half (14/30, 46.7%) of the samples. Despite this neutralising activity, viral replication was detected in 21% (3/14) of the paired respiratory samples.

DISCUSSION

A systematic review and meta-analysis of the duration of viral shedding and infectivity[12] have shown that, although the shedding of RNA in respiratory samples can be prolonged, the detection of viable viruses does not occur after more than 9 days of illness. Previous studies[5,7] have shown prolonged viral shedding in patients with severe COVID-19 and its relation to high viral loads. Although we observed a significant positive correlation between low Ct values and the presence of viable virus, this viral load estimate appears insufficient for discriminating samples harbouring infective virus. It is important to highlight that Ct values obtained for the same sample in different rRT-PCR assays can vary remarkably[13]; thus, the correlation between Ct value and viral viability should be determined for each assay.

Prolonged detection of viral replication has been demonstrated in immunosuppressed patients[14]; however, our results show that viral replication can also be detected in immunocompetent patients, even with moderate or low viral loads, for longer periods of time than those previously described.[7,12,15] It remains to be seen whether this finding is related to our higher cell culture positivity rate (51.6%) in patients with severe COVID-19 compared with that reported previously (9%),[7] due to technical factors such as cell line permissiveness to SARS-CoV-2.[16] Ideally, viral viability should be measured in human nasopharyngeal epithelium cell culture.

The use of different types of samples from the upper respiratory tract has been proposed for diagnosing SARS-CoV-2.[4] The demonstration that the nasal epithelium has the highest expression of the angiotensin-converting enzyme 2 virus cell receptor[17] indicates that nasopharyngeal exudate is the more suitable respiratory sample to investigate virus viability, which was the upper respiratory tract sample type analysed in our study.

We have found a positive correlation between serum neutralisation activity and SARS-CoV-2 nonviability in cell cultures. Nevertheless, we observed a positive CPE in patients with severe COVID-19, despite the presence of clear neutralising activity (NT_{50} >1:80). It remains to be seen whether this high level of neutralising antibodies plays some pathogenic role.[18,19] In our series, 2 patients who presented very high (>1:1024) NT_{50} titres required ICU admission and mechanical ventilation. Interestingly, this fact has been reported for patients with SARS-CoV-1 infection, in whom rapid production of high neutralising titres was associated with poor prognoses,[20,21] and recently for SARS-CoV-2 infection.[22,23] These apparently contradictory results can only be explained by performing longitudinal studies to assess the kinetics of viral replication and of

antibodies, as well as virus-specific T cell response, in patients with varying disease severity.

In summary, we detected a completely different pattern of SARS-CoV-2 viability in upper respiratory tract samples from mild cases, in which viral replication in the upper respiratory tract occurs for a short period (maximum STT, 10 days), compared with hospitalised patients with severe COVID-19, in whom viable virus can frequently be demonstrated during prolonged periods of up to 4 weeks, both in their upper and lower respiratory tract samples, even in the presence of high levels of neutralising activity. These results have important implications to discontinue isolation precautions, given we have demonstrated that immunocompetent patients with severe disease can shed viable virus for long periods of time. For mild COVID-19, quarantine should be extended to at least 10 days.

Transparency declaration

We declare that we have no conflicts of interest.

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Authors' contributions

M.D.F. and R.D. were involved in the design and supervision of this study; J.L., F.L., and M.D.F performed experiments; A.P.R. collected data; and M.D.F., J.L., A.P.R., and R.D. performed the data analysis. All the authors were involved in writing the paper and have approved the final version.

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Table 1. Main patient and sample data for all patient groups

	Asymptomatic	Mild COVID-19 HCW	Mild COVID-19 Non-HCW	Severe COVID-19 Immunocompromised	Severe COVID-19 Exitus	Severe COVID-19 Other Pneumonia
Number of patients (Total N=189)	11	76	15	18	7	62
Age Mean, (range)	52.9 (22-76)	40.2 (20-62)	43.1 (26-81)	59.1 (42-77)	79.28 (70-91)	65.7 (17-94)
Female sex Number (%)	5 (45)	59 (78)	5 (33)	6 (30)	3 (43)	24 (39)
Number of samples (Total N=193)	11	76	15	18	7	66
rRT-PCR Ct value Median, (IQR)	34.9 (21.3-39.5)	32.1 (26.0-37.6)	25.3 (24.0-35.8)	28.5 (22.6-35.9)	21.1 (19.9-26.4)	31.5 (28.2-34.9)
STT Mean, (range)	NA	9.5 (2.0-16.0)	7 (3.0-10.0)	8.5 (5.0-20.2)	5 (4.0-10.0)	9.5 (5.0-15.2)
CPE positive samples Number, (%)	5 (45)	31 (41)	8 (53)	11 (61)	6 (86)	30 (45)

HCW: health care worker; IQR: interquartile range; STT: symptom onset to test time; CPE: cytopathic effect; rRT-PCR: real-time reverse transcription polymerase chain reaction; Ct: cycle threshold