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SHORT COMMUNICATION

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Prolonged presence of replication-competent SARS-CoV-2 in mildly symptomatic individuals: A report of two cases

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Abstract

It has been estimated that individuals with COVID-19 can shed replication-competent virus up to a maximum of 20 days after initiation of symptoms. The majority of studies that addressed this situation involved hospitalized individuals and those with severe disease. Studies to address the possible presence of SARS-CoV-2 during the different phases of COVID-19 disease in mildly infected individuals, and utilization of viral culture techniques to identify replication-competent viruses, have been limited. This report describes two patients with mild forms of the disease who shed replication-competent virus for 24 and 37 days, respectively, after symptom onset.

KEYWORDS
cell culture, coronavirus, SARS-CoV-2

Maria C. Mendes Correa and Fabio E. Leal contributed equally to this study.

1 | INTRODUCTION

Increasing evidence indicates that during the COVID-19 pandemic, SARS-CoV-2 RNA can initially be identified in infected individuals 1–3 days before symptom onset.[1–3] Viral load, as measured by reverse-transcription polymerase chain reaction (RT-PCR), reaches its highest level during the first week of symptom onset, followed by a gradual decline over time. The mean duration of flu-like symptoms in individuals with mild-to-moderate SARS-CoV-2 infections varies from 11.5 ± 5.7 days.[4] Previous studies estimated that replication-competent virus could be found in COVID-19 patients up until 20 days after onset of symptoms.[3–9] However more recently, prolonged shedding of the viable virus has been described in immunocompromised patients. In a recent review, Beran et al.[10] described a few studies among immunocompromised patients, who reported positive viral cultures with a median time of 26 days (interquartile range, 19–94.5).

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This report describes two SARS-CoV-2-infected women with mild disease in which the virus, shown to be replication-competent, persisted for longer periods of time than has been reported previously.[4,11]

2 | METHODS

The described cases were participants in The Corona São Caetano Program, a primary care initiative offering COVID-19 care to all residents of São Caetano do Sul, Brazil.[11] Briefly, residents of the municipality with symptoms consistent with COVID-19 were encouraged to contact the Corona São Caetano platform via the website (accessed at https://coronasaocaetano.org/) or by phone. Participants were invited to complete an initial screening questionnaire that included information on the type, onset, and duration of symptoms. Those meeting the suspected COVID-19 case definition were then called by a medical student to undergo a risk assessment. Individuals meeting pre-defined triage criteria for the mild disease were offered a home visit in which a self-collected nasopharyngeal swab was obtained for analysis.

Individuals positive for SARS-CoV-2 by RT-PCR were followed up to 14 days (a maximum of seven phone calls) after completion of their initial questionnaire that detailed sociodemographic data, clinical comorbidities, and body mass index (BMI). It also included information on the onset, type, duration of symptoms, and recent contacts. Participants were contacted every 48 h by a medical student who completed another risk assessment and recorded any ongoing or new symptoms. The purpose of the follow-up was to assess the evolution of clinical variables. In cases where patients were judged to be deteriorating or developing severe disease, they were assigned to secondary care services and advised to contact the platform for a new consultation if new symptoms developed. Among 1583 confirmed COVID-19 patients with mild forms of disease included in this platform, from April 13 and May 13, 2020, the mean duration of COVID-19 symptoms was 15 days.[11]

2.1 | Virus identification: RNA extraction, PCR amplification, and viral culture

All specimens were handled according to laboratory biosafety guidelines. Nasopharyngeal samples were subjected to total nucleic acid extraction with the QIAamp Viral RNA Kit (Qiagen), according to the manufacturer's instructions. Samples were then subjected to RT-PCR (RealStar® SARS-CoV-2 RT-PCR Kit 1.0; Altona Diagnostics) followed by DNA amplification (Roche LightCycler® 96 System).

Viral culture for SARS-CoV-2, conducted in a biosafety level-3 facility, utilized Vero CCL81 cells (ATCC® CCL-81™) in Dulbecco minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics/antimycotics.

Nasopharyngeal samples were inoculated into a Vero cell culture in plastic bottles (Jet biofilm, 12.5 cm^2 area, 25 ml capacity) and incubated in a 37°C incubator in an atmosphere of 5% CO_2. Cultures were maintained for at least 2 weeks and observed daily for evidence of cytopathic effects (CPEs). At least two subcultures were performed on each sample. The detection of CPEs was investigated using an inverted microscope (Nikkon) and the presence of virus in supernatants from cultures showing CPEs was determined by specific RT-PCR, as described above. RT-PCR analysis was performed using RNA extracted from culture supernatants obtained two passages after the initial inoculation in cell culture.

2.2 | Ultrastructural examination

A standard quantity of cells from the culture flasks inoculated with samples from patients 1 and 2, after at least two passages, were transferred to a 1.5 ml centrifuge tube containing 1.2 ml 3% glutaraldehyde in phosphate-buffered saline (PBS) at pH 7.4. A subsequent fixation occurred in a mixture of 1 vol. 3% osmium tetroxide in PBS plus 1 vol. aqueous 3% potassium ferrocyanide. Dehydration was performed by emersion in a series of increasing ethanol concentrations and 100% acetone. Embedding was in LX Epon. Ultrathin sections were obtained with an Ultracut microtome. Observations were carried out in a 20-20 Jeol Electron microscope.

2.3 | Ethics

The study was approved by the local ethics committee (CAPPesq, protocol No. 13915; dated June 03, 2020).

3 | RESULTS

From April 13th until June 4th, 3652 suspected COVID-19 cases were tested by RT-PCR, of whom 940 (25.7%) were positive in the Corona São Caetano Program. Among four patients who were tested twice by RT-PCR due to persistence of symptoms, two remained RT-PCR-positive in both evaluations. Due to the persistence of symptoms and prolonged positive RT-PCR result, it was decided to investigate the replicative capacity of their SARS-CoV-2 viral infection.

3.1 | Clinical cases

Case 1 is a woman, 51 years old, whose first contact was April 13, 2020. She denied any previous comorbidity. Her BMI at initial contact was 31.9 kg/m^2. She reported first experiencing a dry cough, headache, asthenia, arthralgia, and myalgia 20 days previously (March 24). She denied ever having a fever. On April 15 (22 days after the onset of symptoms), a nasopharyngeal swab tested positive for SARS-CoV-2 RNA. Subsequently, she developed nausea, vomiting, anosmia, and ageusia. Significant symptoms persisted and a second nasopharyngeal swab test for SARS-CoV-2 RNA performed on May 1st (37 days after the onset of symptoms) was also positive. Most symptoms gradually resolved, but on May 15, she still complained of mild headache and asthenia.

Case 2 is another woman, 48 years old, who on May 11th began experiencing fever, headache, sore throat, cough, asthenia, rhinorrhea, arthralgia, myalgia, and nausea. She contacted the São Caetano platform and on May 16th a nasopharyngeal swab test for SARS-CoV-2 RNA was positive (5 days after the onset of symptoms). She also denied any previous comorbidity and her BMI was 20.4 kg/m^2. Her symptoms persisted and a second nasopharyngeal swab test for SARS-CoV-2 RNA performed on June 4th (24 days after the onset of symptoms) was positive. She remained symptomatic, with asthenia and headache until June 17th.

As symptoms were relatively mild in both women they were advised to remain at home. They did not undergo additional testing or receive any treatment and there was gradual improvement in their clinical conditions. As already mentioned, due to the persistence of symptoms and a prolonged positive RT-PCR result, it was decided to investigate the replicative capacity of their SARS-CoV-2 infection. Swab samples obtained at Day 37 (Case 1) and Day 24 (Case 2) were inoculated into Vero CCL81 cells and diagnostic tests were performed on the cell culture supernatant and intracellular fractions, as described above.

CPEs were observed in the Vero cell cultures incubated with samples from both patients after three passages (Figure 1B) and the presence of replicating SARS-CoV-2 in culture supernatants was confirmed by real-time RT-PCR.

In addition, by electron microscopy, aggregates of elongated and spheroid particles ranging in size from around 60 nm to 140 nm with peripheral spike-like projections consistent with the morphology described for SARS-CoV-2[11] were observed (Figure 2). The major and minor axes of the virus profiles were 100 and 58 nm, respectively. Measurements of the orthogonal long and short axes of several virus particles, located close to two cells in the same preparation had the following mean dimensions with the corresponding standard error of the mean, respectively, 90 ± 4.5 nm (n = 22) and 62 ± 5.1 nm (n = 22). Viral particles were seen mainly at the cell periphery and eventually inside cytoplasmic vacuoles (Figure 2).

4 | DISCUSSION

Two women positive for SARS-CoV-2 presented with flu-like symptoms that persisted for a longer time than is typical.[4,12] This led to the collection of a second nasopharyngeal swab at 24 and 37 days, respectively, after symptom onset that resulted in the identification of the replication-competent virus in both women. To the best of our knowledge, there are no previous reports of the replication-competent virus being isolated 3 weeks after symptom onset among mildly symptomatic immunocompetent adults who do not require hospitalization.

Prolonged shedding of viable virus (>20 days) has been reported in some adults, either with severe COVID-19 or among immunocompromised patients.[10] However, both of the cases reported here did not report any previous comorbidity and presented with a mild form of the disease with no clinical complications or need for hospitalization.

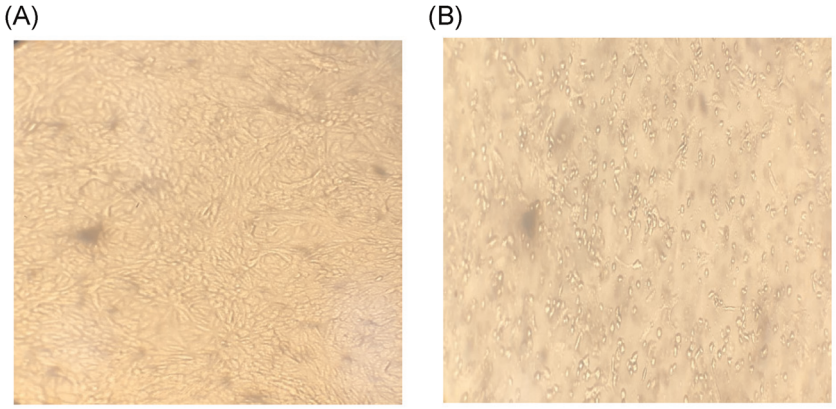

FIGURE 1 Induction of cytopathic effects on Vero cells after inoculations with nasopharyngeal swabs from women with SARS-CoV-2. Cultured Vero cells were untreated (A) or inoculated with material from nasopharyngeal swabs of patients positive for SARS-CoV-2 (B)

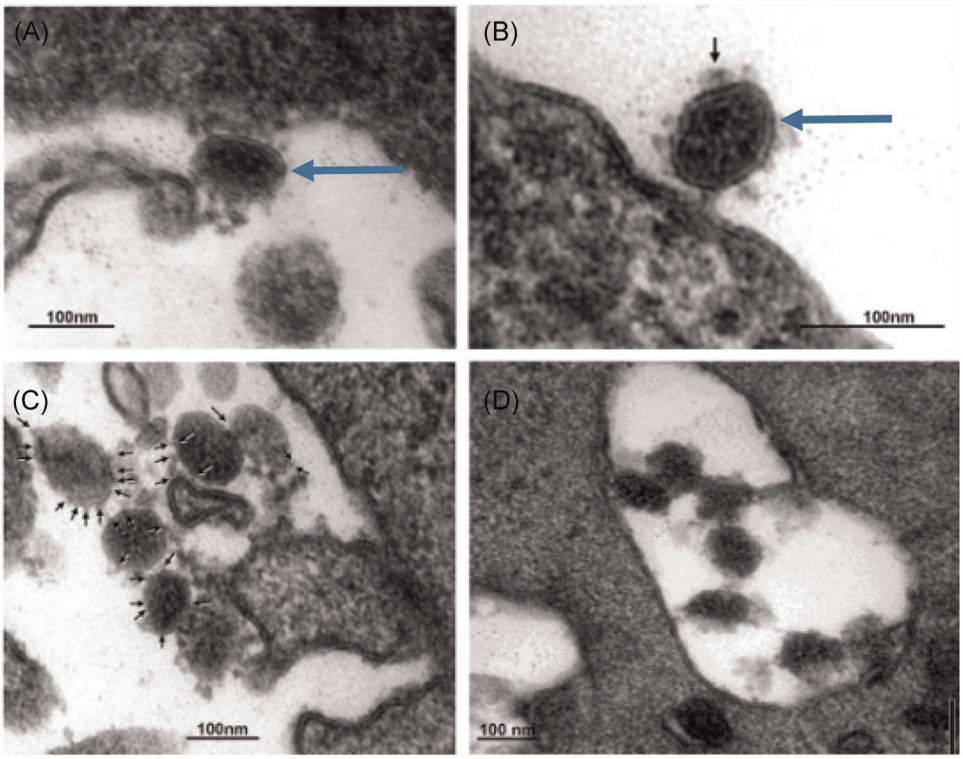

FIGURE 2 Electron microscopy of Vero cells inoculated with nasopharyngeal samples from women infected with SARS-CoV-2. (A–D) Representative thin-section electronmicrographs of the detection of SARS-CoV-2. Long blue arrows indicate elongated and spheroid viral particles, respectively, attached to the cell border membrane in (A) and (B). (B) A small arrow points to a virus spike. (C) Small arrows indicate petite and longer virus spikes. (D) Several viral particles inside a cytoplasmic vacuole

It may be relevant that Case 1 presented with a BMI that placed her in the obese range. Clinical and epidemiologic studies have indicated that obesity increases the risk of severe complications and death from SARS-CoV-2 infection.[13,14] Similarly, it has been shown that obesity may influence influenza virus transmission. Among symptomatic and asymptomatic adults, obesity increased the duration of influenza A shedding by 104%.[15] Indeed, it has recently been proposed that adipose tissue in individuals with obesity may act as a reservoir for more extensive viral spread, with increased shedding, immune activation, and cytokine amplification.[16] More investigations are needed to explore the possible association of obesity with prolonged SARS-CoV-2 persistence and contagion.

According to WHO-updated recommendations on the criteria for discharging SARS-CoV-2-positive individuals from isolation, patients must be clinically recovered (symptom-free).[17] Our data reinforce that even mildly symptomatic individuals are potentially contagious.

Recently, there have been descriptions of individuals who initially tested positive for SARS-CoV-2 RNA, became virus-negative but subsequently again became PCR-positive.[18,19] This may be due to either reinfection following exposure to another infected person or by reactivation of the latent virus.[20] Re-infection, latent virus reactivation, and prolonged viral shedding may represent unique presentations of this infection in different patients or, alternate phases of the same infection. Immunological and clinical characteristics of individual patients, as well as genomic characteristics of the involved viral strains, may help determine the natural history of COVID-19 and the different phases of the disease, as described above.

A limitation of our study was that we were unable to analyze genomic characteristics of the involved viral strains in these two cases, so the possible involvement of viral variants in persistence and viral shedding could not be evaluated. In addition, we did not establish the length of time that replication-competent virus was present in both individuals. In both cases, there was a short time interval between the onset of symptoms and confirmation of the viral presence and the time interval between collection of the first and second swab sample was ≥15 days.

Further clarification of the frequency of presumed prolonged infectivity, as illustrated by the cases described in this communication, will be defined by prospective follow-up studies involving a greater number of individuals. Nevertheless, this report highlights that individuals with prolonged but mild symptoms can remain positive for the replication-competent virus, highlighting the need for such individuals to exercise appropriate precautions to avoid potential transmission of SARS-CoV-2 in their community.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

All authors made a significant contribution at different stages of the work reported and contributed to data interpretation. Maria C. Mendes Correa and Fabio E. Leal wrote the first draft of the paper and all authors participated in writing subsequent drafts. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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