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The extent of infectious SARS-CoV-2 shedding in an Argentinean cohort

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ABSTRACT

Background To analyze the infectious extent of severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) in different settings where prevention strategies are critical to limit infection spread, we evaluated SARS-COV-2 viability to guide public health policies regarding isolation criteria and infection control.

Methods We attempted viral isolation in 82 nasopharyngeal swabs from 72 patients with confirmed SARS-COV-2 infection. Study population was divided into four groups: (i) Patients during the first week of symptoms; (ii) Patients with prolonged positive PCR; (iii) Healthcare workers from a hospital participating of an outbreak investigation, with SARS-COV-2 infection confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and (iv) Recipients of convalescent immune plasma (CIP). Vero Cl76 cell-line (ATCC CRL-587) was used in assays for virus isolation. Plasma samples of CIP recipients were also tested with plaque-reduction neutralization test.

Results We obtained infectious SARS-COV-2 isolates from 15/84 nasopharyngeal swabs. The virus could not be isolated from upper respiratory tract samples collected 10-day after onset of symptoms (AOS) in patients with mild–moderate disease.

Conclusion The knowledge of the extent of SARS-CoV-2 infectivity AOS is relevant for effective prevention measures. This allows to discuss criteria for end isolation despite persistence of positive PCR and improve timing for hospital discharge with consequent availability of critical beds.

Keywords COVID-19, infectivity, isolation, public health, SARS-CoV-2

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) spreads rapidly causing coronavirus disease (COVID-19) epidemic outbreaks with significant number of deaths worldwide, in addition to the potential collapse of healthcare systems. Efforts to mitigate virus spread require prompt detection of cases and close contacts, followed by a quick implementation of control strategies to prevent secondary transmission from infected individuals. Therefore, identification of SARS-COV-2 cases constitutes the first step to reduce viral transmission. Infected individuals are identified with reverse transcriptase polymerase chain reaction (RT-PCR)

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test, which is a molecular assay that detects and amplifies genomic sequences (ribonucleic acid, RNA) of the virus, largely used for diagnosis, screening and surveillance of SARS-COV-2. Although a positive PCR allows identification of infected patients, it does not discriminate between infectious and non-infectious virus.[1] To take proper effective actions to stop/control infection propagation, it is important to determine if each infected person can indeed spread the virus and determine if transmission-control procedures (isolation/quarantine) are required.

Inoculation of clinical samples into susceptible cell-culture lines is the virological method to demonstrate virus viability, transmission and infectivity. SARS-COV-2 studies regarding viral viability focus on two aspects: extent of SARS-COV-2 infectivity after onset of symptoms (AOS) and patient's infectious potential based on viral-load measurement by cycle threshold (Ct) values. Different studies have demonstrated absence of infectious virus in samples collected 8-day AOS.[2–5] Moreover, the likelihood of culturing this virus declined to 6% in samples obtained 10-day AOS.[6] On the other hand, the association between SARS-COV-2 RNA detection and infectivity remains controversial, since the data differ in terms of correlation between Ct values and culturable viruses.[1,3–6]

Our study evaluated SARS-COV-2 viability in samples from the upper respiratory tract (URT) of four groups of patients. Since the significance of detecting RNA of SARS-COV-2 by RT-PCR in terms of viral viability remains controversial, we analyzed the extent of viral infectivity in different settings where prevention and/or control strategies are critical to limit the spread of infection and for take occupational/health-related measures. We intend to provide scientific evidence to understand the SARS-COV-2 dynamics to guide public health policies regarding isolation criteria and evaluation of current therapies.

Material and methods

Patients

We attempted viral isolation in 82 nasopharyngeal swabs (NPS) obtained from 72 SARS-COV-2 infected patients from a single hospital. Study population was classified into four groups:

Group 1. Patients during the first week of symptoms; samples collected in April 2020, at the beginning of viral spread in Argentina.

Group 2. Patients with prolonged RNA shedding in respiratory samples. Samples were collected from the first cases identified in this region in June/July 2020 and studied to provide scientific evidence for counselling Public Health Institutions of Cordoba province. At that time, criteria for ending isolation in patients with persistent RNA SARS-COV-2 detection were unclear and the advice was to remain isolated until negative RNA detection.

Group 3. Healthcare workers (HCW) from a hospital participating in an outbreak investigation, which had a nosocomial outbreak at the end of July 2020. HCW presented SARS-COV-2 infection confirmed by RT-PCR. As a guideline for discharge timing, NPS from all HCW with history of mild–moderate disease who were free of symptoms on Day 10 but had persistent positive PCR were further evaluated by virus cultures. At that time, researchers were not reporting finding of infectious isolates from samples taken 8–10 days AOS, worldwide.

Group 4. Recipients of convalescent immune plasma (CIP).

We assessed virus infectivity in patients that received transfusion of specific neutralizing antibodies (NAbs); we analyzed blood samples obtained before transfusion, 72-h after transfusion (AT) and after clinical improvement (144-h AT). Two doses of 200 ml of COVID-19 convalescent plasma with high NAbs titres ($\geq$1/80) were administrated in early stages of the disease. We also evaluated four NPS from transfused patients with clinical improvement AT but prolonged shedding of RNA in respiratory samples.

Viral isolation

Vero Cl76 cell-line (ATCC CRL-587) was used for virus isolation assays. Cultured cell monolayers were maintained in their respective medium in T25 flask or Leyton tubes. Clinical samples were prepared by centrifugation at 7.500 rpm for 5 min, passed through a 0.22-μM filter. After removing the medium from cell flasks or tubes, filtered samples were inoculated into Vero cells and incubated at 37°C and 5% CO_2 for 1 h. Later, cell cultures were re-fed with 1× Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% foetal bovine serum (FBS) plus penicillin–streptomycin and cultured for 5 days. The development of cytopathic effect (CPE) was examined daily. SARS-COV-2 CPE specificity was confirmed by RT-PCR from RNA extracted from culture supernatants. Negative cell-culture supernatants were further passaged onto fresh Vero monolayers up to three times until discarding any evidence of infection. In parallel, non-inoculated Vero cell cultures were used as negative controls.

Neutralizing antibodies

We tested plasma samples for their neutralization capacity against SARS-CoV-2 (hCoV-19/Argentina/PAIS-G0001/

2020, data base: GISAID, accession number ID: EPI_ISL_499083) by plaque-reduction neutralization test. Virus neutralization test was carried out in 24-well plates. Vero Cl76 cells (ATCC CRL-587) were sowed 48-h before the infection. Plasma samples were heat-inactivated by incubation at 56°C for 20 min and centrifuged at 10 000 rpm 30-min before use. Treated-samples were 2-fold dilution and then an equal volume of virus stock containing 100 plaque forming units (PFU) was added to each corresponding well until reaching final dilutions ranging from 1/10 to 1/320. Cells were incubated with 0.5% agarose with DMEM supplemented with 2% FBS during 4 days at 37°C in a 5% CO_2 incubator. Once the incubation time was completed, 24-well plates were fixed and inactivated using a 10% formaldehyde/PBS solution and stained with crystal violet 1%. NAbs titres corresponded to the maximum dilution of plasma that neutralized 80% of the PFU, compared with PFU from the viral controls included in the test.

Results

Group 1

NPS from 10 patients collected between 3 and 5 days AOS were inoculated in cell cultures and viruses were successfully isolated in all of them. Five of 10 samples showed CPE within 48 h, 3/10 within 72 h and 2/10 after one blind subculture. Media of RT-PCR Ct value was 25 (range = 21–28). Average age of this group was 75 years old (range = 65–90); 80% were females.

Group 2

We analyzed NPS from 25 patients in whom prolonged RNA SARS-COV-2 detection (18–90-day AOS) was the only criteria to maintain isolation. Average age was 57.84 (range = 14–92), 11 (44%) were males. Ten patients with repetitive positive RT-PCR between Days 30 and 90 AOS had history of mild/moderate disease. Other seven patients, older than 80 years, had persistent positive PCR at Days 30, 35, 51, 54, 65, 84 and 91 AOS, respectively and in consequence, they could not be discharged from hospital because no nursing home would receive them. Eight patients with positive PCR between Days 18 and 29 AOS were also included in this group since they have comparatively mild disease with symptoms that lasted < 48 h. Patients with risk factors for severe COVID-19 (16/25 patients) remained hospitalized for observation and control and 9/25 patients were isolated at home. All patients were clinically ready for hospital discharge; however, due to prolonged RNA shedding through respiratory samples, they had no criteria for ending social isolation.

Virus culture assays were negative for all patients and Ct values of the samples in RT-PCR were > 30.

Group 3

Twenty three HCW free of symptoms at least 72-h before Day 10 AOS, who tested positive for RNA SARS-COV-2 on Day 10 AOS were recruited. Average age: 36.7 (range = 26–58); 5 (21.74%) were males. Media Ct value of RT-PCR was 31 (range = 24–38, median: 33 and mode: 32). Virus cultures were negative for all samples.

Group 4

Ten patients were recruited during the first week of symptoms; all had comparable clinical course and were eligible for COVID-19 CIP at Day 6 AOS. Average age was 54.8 years old (range = 32–70) and 7 (70%) were males. Before transfusion, NPS and blood samples were collected for inoculation into confluent cell monolayer and NAbs detection, respectively. Subsequent samples were obtained AT and/or clinical improvement: 6 at 72 h and 4 at 144 h. Media Ct value before transfusion was 28 (range = 18–32, median = 28), 32 at 72 h (range = 28–36, median: 32) and 28 at 144 h (range = 24–34, median: 28). Before transfusion, 6/10 (60%) patients were negative for Nabs and viral isolation was achieved in five of the six patients. The highest Ct value in a culturable sample was 32. Among the other four patients with detectable NAbs before transfusion, the titres were 1/20, 1/20, 1/40 and 1/40, respectively. Viruses could not be isolated from NPS in any of these four patients. NAbs at 72 h were detectable in 9/10 patients, titre media: 1/80 (range = 1/10–1/320) and in 10/10 patients at h 144, titre media: 1/320 (range = 1/10–1/320). Viable viruses could not be isolated from NPS in any sample collected 72 and 144-h AT.

Within this group, we also evaluated 4 NPS from transfused patients with clinical improvement AT but without criteria for hospital discharge due to persistent RNA shedding in respiratory samples, even 16 days after symptoms improvement. All had detectable NAbs at time of sampling, three had titres of 1/320 and one 1/80. Moreover, no viable viruses were isolated from these NPS samples.

Discussion

We obtained infectious SARS-COV-2 isolates from 15/84 NPS. The viruses could not be isolated from URT samples collected after Day 10 AOS in patients with mild/moderate disease. Our data are in agreement with studies that have iden-

tified viable virus until 8–10-day AOS.[2–6] Since early October 2020, strategies to discontinue isolation in Córdoba are in line with recommendations from the World Health Organization (WHO).[7] Data emerging from our study support current guidelines that recommend isolation of confirmed Covid-19 patients for at least 10-day AOS or 10-day after the first positive test if asymptomatic. After that period, patients can be released if they have been 72-h free of symptoms.

Although the extent of virus detection and load differ between patients, viral RNA generally becomes undetectable about 2-week AOS.[7] However, persistent SARS-COV-2 replication has been demonstrated in severe COVID-19 cases for a period of 32-day AOS.[8] On the other hand, viral ARN detection by RT-PCR can remain positive for weeks beyond the infectious period in asymptomatic patients or those recovered from mild/moderate disease. Beyond 8–10-day AOS or 10-day after the first positive test in asymptomatic patients, the presence of viral RNA may not represent viable virus.[2–5] Taking this into consideration, decisions on infection control need to be supported by infectious evidence; otherwise, unnecessary measures (isolation/quarantine) could be inaccurately applied. We assessed viral viability in NPS from 25 patients with prolonged RNA SARS-COV-2 detection. This cohort awaited a negative PCR for a prolonged period of time in order to leave isolation. No culturable viruses were obtained from any of these samples 10-day AOS. These data sustain current guidelines and criteria based on the WHO recommendations and also support that awareness of SARS-CoV-2 viral shedding is of primary importance for effective prevention and control measures.[1] In a situation characterized by constraints in the healthcare system, mainly represented by limited availability of hospital beds, there are great concerns about patients occupying critical beds due to COVID-19 as well as pressures for early hospital discharge following clinical improvement and end of the infectious period.

Transfusion of CIP from individuals recovered from COVID-19 is an experimental treatment currently analyzed. To date, we are conducting clinical studies on the efficacy of COVID-19 CIP and herein, we present some preliminary data from a subset of our cohort. In the group of CIP transfused patients, viruses could be isolated from patients in early stages of infection and negative detection of NAbs. This seems to be in line with a study by Van Kampen *et al.* (2021), which showed viral shedding in patients with low NAbs response.[9] Although data on NAbs titres in transfused recipients are still preliminary, seroconversion and high titres of NAbs seem to be an important variable to consider when assessed virus viability, transmission and infectivity. Ct values of pre-transfused patients were similar to values from NPS at 72 and 144 h, despite viable virus were not isolated after administration of CIP. These data suggest that AT, hospital discharge followed by home isolation could be implemented for patients with clinical improvement within a few hours AT, allowing a major availability of critical beds. However, larger studies would be necessary to support these findings.

A clear understanding of infectious viral shedding is critical to prevent transmission by infected individuals and pandemic control. Since viral detection by RT-PCR does not mean viable virus and given that viral culture is not always available, investigations to understand how RT-PCR detection relates to culturable virus are being carried out.[6] In our cohort, Ct value was not enough to discriminate patients shedding infectious virus. The findings obtained by different researchers, which correlate Ct values with viral load and culturable virus[3,5,6] stand against those who support that Ct values do not seem sufficient to discriminate samples harboring infective virus.[1,8] Bullard *et al.* demonstrated that infectivity is significantly reduced when Ct values are > 24 and La Scola *et al.* showed that patients with Ct above 33–34 are not contagious.[3,5] High Ct values may indicate low viral load, but these values also could be attributed to deficient sample collection, viral degradation or sampling in early or late stages of infection. Data presented by Romero-Gómez *et al.* about their SARS-COV-2 culturing experience show that the highest Ct value in samples with positive cultures were 36.08, 37.73 and 37.41 in samples taken 1-day AOS[1] and Singanayagam *et al.* estimated an 8.3% likelihood of recovering virus from samples with CT > 35.[6] An explanation for this controversy can be found in the report from La Scola *et al.*, who state that 'one limitation of their study is that it cannot be extrapolated to other hospitals because they used different systems for sample transportation, RNA extraction and PCR with different primers and probes'.[5] This limitation applies for all the data provided by different researchers and under different conditions. Reagent supplies also play an important role on reported data. In our case, RNA was extracted from NPS using different commercial kits on different platforms (manual, semi-automatic or automatic). The kits were purchased according to market availability since during the first months of the pandemic not all reagents were available in Argentina and/or the suppliers had limited storage. Also, as previously stated, the time of sample collection AOS seems to be an important variable to take into consideration.[1]

In conclusion, we did not isolate any viable SARS-COV-2 from URT samples collected after Day 10 AOS in patients with mild/moderate disease. Ct values were not enough to

discriminate patients shedding infectious virus. Our data support current guidelines for clinical decisions, which recommend isolation of confirmed COVID-19 cases for at least 10-day AOS or 10-day after the first positive test if asymptomatic; after that period patients can be released after been symptom-free for at least 72 h.

Authors' contributions

BS, AJJ, KBS, DLA, SL, BM, CC, DM, BMG and GSV conceived and design the study. BS, AJJ, KBS, DLA, SL, BM and GSV performed virus culture isolation and performed analysis and interpretation of the data. CC, DM coordinated sample collection. DM and BMG coordinated patient management and follow up. Staff from the Lab Central Córdoba working group performed RT-PCR and analyzed and interpreted the data. All the authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Ethics

This report has been prepared in accordance with specific local regulations (provision no. 32/2016, dated September 8, 2016, by the Council for the Ethical Evaluation of Health Research, Ministry of Health of the province of Córdoba, Argentina). The study has observed the ethical standards established in the Declaration of Helsinki of 1964 and its subsequent modifications.

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