Isolation of viable SARS-CoV-2 in feces suggesting possible fecal transmission of COVID-19

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Abstract

Background

SARS-CoV-2 excretion in stools is well documented by RT-PCR, but evidences that stools contain infectious particles are scarce.

Objectives

After observing a COVID-19 epidemic cluster associated with a ruptured sewage pipe, we search for such a viable SARS-CoV-2 particle in stool by inoculating 106 samples from 46 patients.

Results

We successfully obtained two isolates from a unique patient with kidney transplantation under immunosuppressive therapy who was admitted for severe diarrhea.

Conclusions

This report emphasizes that SARS-CoV-2 is also an enteric virus that is important to detect in the stool in cases of diarrhea, particularly after kidney transplantation. Immune-compromised patients are likely to have massive multiplication of the virus in the gastrointestinal tract and this report suggests possible fecal transmission of SARS-CoV-2.

Keywords: SARS-CoV-2; stools; cell culture; Covid-19

1. Background

In Wuhan, China, a new viral disease emerged in December 2019. The pathogen responsible for this disease was identified as a new strain of coronavirus, called Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2), and the associated disease was named "COronaVIrus 2019 Disease" (COVID-19) [1]. In nearly one year, SARS-CoV-2 infected millions of people in 212 countries and was responsible for more than 1.4 million deaths worldwide [2]. SARS-CoV-2 is highly contagious and considered as acquired through the respiratory tract after inhalation of particles or contact of face mucosa with contaminated hands. For this reason, the main recommendations to avoid infection are the wearing of masks and frequent hand washing. However, mounting evidence suggests that this virus may also be an enteric virus. First, gastrointestinal symptoms have been described in patients with COVID-19 since the onset of the pandemic and a recent meta-analysis of >6600 patients with COVID-19 described that up to 15% had gastrointestinal symptoms, with the three most common symptoms being nausea or vomiting, diarrhea and loss of appetite [3]. Second, the pooled estimate of SARS-CoV-2 viral RNA positivity in fecal samples was 54%, with positivity persisting for up to 47 days after symptom onset [3]. SARS-CoV-2 RNA detection, but also intracellular staining of viral nucleocapsid protein in gastric, duodenal and rectal epithelia demonstrated that SARS-CoV-2 infects these gastrointestinal epithelial cells [4]. Finally, we were able to observe that SARS-CoV-2 can grow in Caco-2 cells, polarized cells derivate from colorectal cancer [5]. In a recent work carried out in our institute, we have identified a cluster of cases associated with a specific clone called genotype Marseille 1 [6]. The index case was imported from Tunisia, and the first cases subsequently diagnosed were associated with ships connecting North Africa to Marseilles, in travelers, but also in several crewmembers exposed to a ruptured sewage pipe. Travelers and crewmembers were infected with the same virus without direct contact with each other. We therefore raised the possibility

of fecal-oral or fecal-respiratory transmission of SARS-CoV-2. Despite several attempts, viable SARS-CoV-2 was reported in stool of only six different patients [7–10]. In the present work, all SARS-CoV-2 PCR-positive samples from stools obtained in our laboratory were inoculated in order to evaluate the presence of a viable virus.

2. Study design

In this study, we attempted to isolate by cell culture SARS-CoV-2 from stool samples received in our laboratory at the IHU Méditerranée Infection for patient suffering from COVID-19. All samples were collected as part of the diagnosis and follow-up of patients for Covid-19 and the study was approved by the ethical committee of the University Hospital Institute Méditerranée Infection (N°: 2020-021).

3. Materials and methods

From March 4, 2020 to April 29, 2020, 128 stool samples (0.2 g in 1 ml of buffer, Sigma *Virocult*®, Elitech, Puteaux, France) from 54 patients were tested positive for SARS-CoV-2 by PCR targeting E gene [11]. Of these, 106 frozen samples from 46 patients and stored at -80◦C were available for viral isolation. After thawing, a 500 µL diluted sample was mixed with 150µl of HBSS buffer and then filtered using a 0.22-µm pore-sized centrifugal filter (Merck Millipore, Darmstadt, Germany). Four wells of Vero E6 cells were each inoculated with 50µl of the filtrate, as previously described [12]. The only change from the original protocol is that after the first week of subculture, instead of two blind subcultures each week, we performed 5. Once a cytopathic effect was detected in the well, the content of the well was collected. 600 µL was frozen to conserve the virus, and 200µl was used to perform the SARS-CoV-2 qPCR for confirmation of presumptive identification, then whole genome sequencing [13]. The E gene of SARS-CoV-2 was amplified through RT-PCR (upstream primer: ACAGGTACGTTAATAGTTAATAGCGT; downstream primer:

ATATTGCAGCAGTACGCACACA; probe: FAM-ACACTAGCCATCCTTACTGCGCTTCG-TAMRA). Sequencing was performed on Miseq Instrument with the Illumina Nextera XT Paired-end strategy. Genome consensus sequences were obtained by mapping reads with CLC Genomics workbench v7 against the genome Wuhan-Hu-1 (MN908947) with length fraction at 0.8 and similarity fraction at 0.9. The consensus sequence was analyzed with Nextclade web interface (https://clades.nextstrain.org/).

4. Results

Four weeks after inoculation, i.e at the third subculture, two samples showed cytopathic effects that appeared as a group of rounded cells forming aggregates comparing with the negative control, as shown in figure 1. All other inoculations remained negative after the 5th sub-culture. RT-PCR performed on the two supernatants confirmed that the cytopathic effect was due to active SARS-CoV-2 proliferation, with cycle threshold (Ct) values of 17.29 and 16.22. Whole genome sequencing and analysis of isolate showed that the two strains had slightly different sequences of type 20A/8371T and 20B/19818T-28845T, respectively [14] (Figure 2). These two stool samples at a PCR Ct of 33.2 (2.585 copies/mL) and 33.4 (2.250 copies/mL), respectively with viable SARS-CoV-2, were collected on April 14th and 15th from the same patient. This patient was a 62-year-old man who had undergone a kidney transplant 21 years ago. He also had diabetes, hypertension and overweight. He consulted in the emergency department on April 13th because for the past 10 days he had been experiencing asthenia, loss of appetite, diarrhea and weight loss, without respiratory symptoms (Figure 3). COVID-19 pneumonia was diagnosed on chest CT. SARS-CoV-2 PCR performed on nasopharyngeal swab two times per day on April 13th and 14th was negative. It was positive once on nasopharyngeal swab on April 15th at a Ct of 33.5 (2.099 copies/mL). The culture

was negative for this swab, but direct amplification and sequencing on the sample allowed us to determine it was a 20A/8371T sequence type as in the first stool sample. Laboratory results on the day of admission revealed acute kidney injury and mild inflammation. Maintenance immunosuppressive treatment consisted of tacrolimus 6.5 mg/day and prednisone 5 mg/day. Treatment with azithromycin was administered for five days, hydroxychloroquine for ten days and ceftriaxone for seven days from April 14th. The dose of tacrolimus was temporarily halved. Acute functional renal failure secondary to diarrhea corrected after refilling and discontinuing diuretics and ACE inhibitors. C reactive protein normalized on April 18th. Nasopharyngeal SARS-CoV-2 PCR was positive only once on April 15th and was negative on April 21st, 22th and 28th. In stool, SARS-CoV-2 PCR was negative on April 28th. It has not been controlled since April 15, when the diarrhea stopped.

5. Conclusions

In this work, we sought to determine whether the SARS-CoV-2 RNA positive stool contained infectious virus and then whether the stool could be a source of transmission. We succeeded in isolating viable SARS-CoV-2 only in 2/106 (1.9%) stool samples from 1/46 (2.2%) patients with COVID-19. To date, viable cases of SARS-CoV-2 have been reported in the feces of only six different patients, despite the common detection of viral RNA [7–10]. All of these patients were Chinese and contracted COVID-19 during the first trimester of 2020. One patient developed diarrhea [10] while two others did not [7]. No information about gastrointestinal symptoms was available for the last three patients. The viral load was considered high in 2 patients, but copy number was not provided [7], reported to be at Ct between 20 and 24 in 2 patients [8] and at 33.6 in one patient [10], as in our case. It was not indicated for the 6th patient [9].

Despite relatively high Ct, we succeeded in isolating viable SARS-CoV-2 in two different stools sampled one day apart from the same patient. COVID-19 presented in this patient as enteric infection, evolving for 10 days at the time of diagnosis and severe enough to cause acute kidney injury. Although pneumonia was detected by CT scan, respiratory symptoms were absent throughout the illness. This observation suggests that gastrointestinal infections can occur before respiratory symptoms [15,16], but also without them. Interestingly, our patient had also low viral excretion in the upper respiratory sample. The fact that two culture-positive stool samples have a viral load comparable to that of culture-negative nasopharyngeal sample suggests that there was continued viral multiplication in the digestive tract of this patient. Several studies suggested that SARS-CoV-2 may be actively replicating in the gastrointestinal tract [10], even after viral clearance in the respiratory tract [17]. The viral excretion from the digestive tract may last longer than that from the respiratory tract since fecal samples may remain positive for SARS-CoV-2 RNA for approximately five weeks after respiratory tract samples become negative for SARS-CoV-2 RNA [17].

We cannot exclude that viral load in the stool could be high but appear low due to PCR inhibitors present in stools. However, as isolation occurred at the third subculture, the viral load was likely low. We identified two different SARS-CoV-2 strains in two stools sampled one day apart from the same patient. Genome sequence differences were probably not related to subcultures as the comparison of more than 50 SARS-CoV-2 full-length genomes sequenced in our laboratory showed no significant differences between the sequences from isolates or samples. Genome sequences of the two strains were sufficiently different to hypothesize a dual SARS-CoV-2 infection as it has already been reported [18,19], rather than genome variants related to ongoing evolution of SARS-CoV-2 in the human body [20].

We believe that isolation of viable SARS-CoV-2 only in stools of an immunocompromised kidney transplant recipient in this work is not by chance. In a large French nationwide cohort of 279 kidney transplant recipients with COVID-19, diarrhea was the third most frequent symptom on admission (43.5%) after fever (80%) and cough (63.6%)[21]. Gastrointestinal symptoms were significantly more frequent than those reported in the general population [3]. It remains uncertain whether the immunosuppression state contributes to the high proportion of gastrointestinal signs in the kidney transplant recipients. However, higher COVID-19-related mortality compared to non-transplant hospitalized patients has been reported despite a similar occurrence of severe disease [22]. SARS-CoV-2 plasma load was reported to be associated with COVID-19 severity and mortality, and respiratory shedding to be prolonged [22]. Moreover, patients receiving profound immunosuppression following hematopoietic stem-cell transplantation or receiving cellular therapies may excrete viable SARS-CoV-2 for at least 2 months in respiratory samples [23]. As in other viral infections in kidney transplant recipients, SARS-CoV-2 will probably more fully display its potential dangerousness than in immune-competent [24].

The number of times SARS-CoV-2 may have developed in the stool is very low. Performing cell culture with cytotoxic fecal specimens is technically challenging. However, the present work and others have shown that the virus can survive in the digestive tract [7–10]. Jeong *et al.*, although they failed to directly demonstrate the presence of viable virus in stools using cell culture isolation, were able to isolate SARS-CoV-2 from ferrets that were inoculated with a stool sample from a COVID-19 patient [25]. Thus, this demonstrates the presence of viable virus in the stool of this COVID-19 patient. These results suggest that, as SARS-CoV and Middle Eastern Respiratory Syndrome (MERS-CoV), SARS-CoV-2 can be transmitted through fecal-oral contact.

In conclusion, SARS-CoV-2 is also an enteric virus that is important to detect in the stools in cases of diarrhea, particularly after kidney transplantation. SARS-CoV-2 can be transmitted through the stool. Since its culture is difficult in the stool but a fecal-oral transmission has been proven, the control of viral RNA excretion in the stool can be proposed to judge the disappearance of contagiousness.

Figure 1. Cytopathic effect of SARS-CoV-2 on Vero E6 cells (a) uninfected cells as negative control and (b) infected cells with the stool samples. The images were captured simultaneously at 4 dpi (days post-infection) at sub-culture 3 using ZEISS Zen Microsoft software with x10 magnification scale.

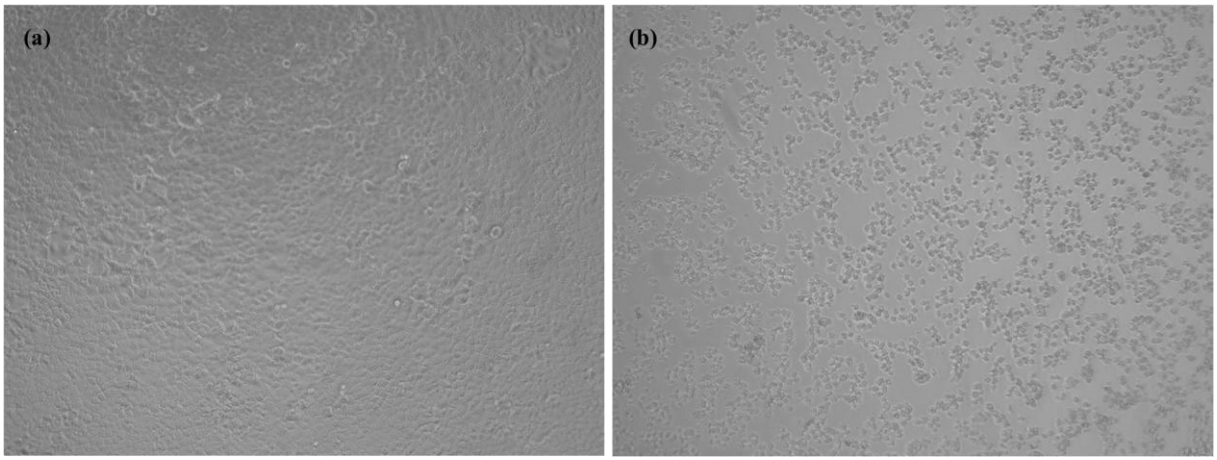


Figure 2. Phylogenetic tree showing the positions of the two SARS-CoV-2 strains isolated from stools relative to other phylogenetically close neighbors. Stool isolate 1 and 2 represent the two isolated strains from clinical samples of a kidney transplant patient. Nomenclature was based on Nextstrain. Genomic sequences of isolates 1 and 2 are available on GYSAID under accession numbers EPI_ISL_860093 and EPI_ISL_860094, respectively.

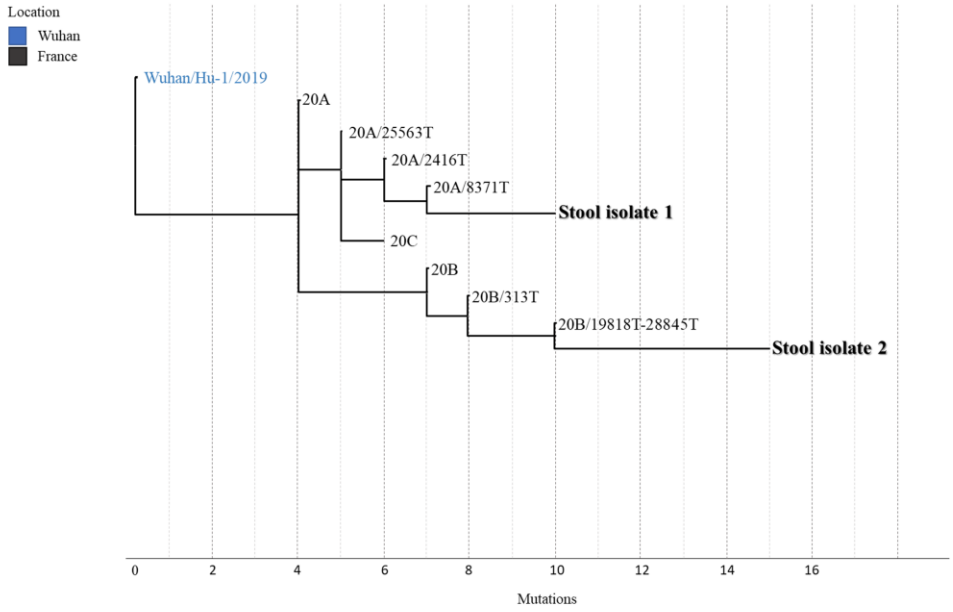
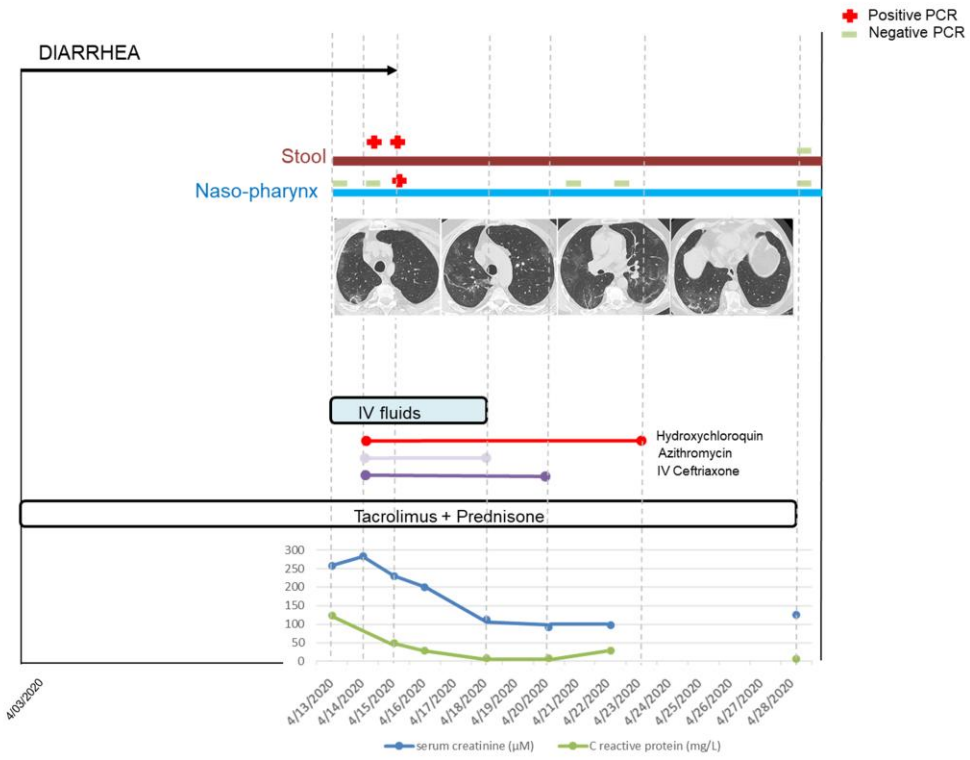


Figure 3. Clinical, biological, virological and treatment timeline during the course of COVID-19.

IV: intravenous

Gastroenteritis began ten days before hospitalization. Diarrhea ceased on April 15th. SARS-CoV-2 PCR was positive first in the stool and then in the pharynx. Typical COVID-19 pneumonia existed on the CT-scan, while the patient presented no respiratory symptom. Acute functional renal failure was corrected after refilling and discontinuing diuretics and ACE inhibitors. The dose of tacrolimus was temporarily halved. Treatment with azithromycin was administered for five days, hydroxychloroquine for ten days and ceftriaxone for seven days. C reactive protein normalized on April 18th. The two consecutive fecal samples were positive for SARS-CoV-2 by RT-PCR and culture.



Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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