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SARS-CoV-2 is not found in human semen during mild COVID-19 acute stage

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Abstract

The aim of this study was to verify the presence of SARS-CoV-2 in the seminal sample of men during the acute phase of COVID-19. A prospective study was performed with inclusion of twenty-two men diagnosed with COVID-19 through RT-PCR from pharyngeal smear samples and who were in the acute phase of infection. These men were evaluated regarding medical history and physical examination. Furthermore, seminal samples of each men were collected 7, 14 and 21 days after the infection was confirmed. The sample were used for seminal analysis, as well as for the presence of SARS-CoV-2 using RT-PCR technique. In addition, cell culture was performed with subsequent repetition of the analysis of viral presence. None of the semen samples collected was positive for the detection of the virus that causes COVID-19. Most of the men evaluated had a mild condition and the loss of smell was the most frequent symptom. There were no significant changes in seminal parameters within the period of study. Based on our pilot data, patients with a mild form of COVID-19 in the acute stage of the disease are unlikely to have SARS-CoV-2 in semen.

KEYWORDS
COVID-19, SARS-CoV-2, semen, viruses

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a viral disease that mainly affects the respiratory system of those infected (Carvalho et al., 2021). People with this condition can be classified into five categories of severity, namely: *asymptomatic*, individuals with a positive diagnosis for the disease but without symptoms; *mild*—individuals with symptoms such as fever, cough, sore throat but not shortness of breath, dyspnoea or abnormal chest images; *moderate*—patients who, during clinical or imaging examination, present characteristics of lower respiratory disease and oxygen saturation ≥94%; *severe*—those who had oxygen saturation <94%, a ratio between arterial oxygen partial pressure and the fraction of inspired oxygen <300 mm/Hg, respiratory rate >30 breaths/minute or pulmonary infiltrates >50%; *critical*—those individuals who have respiratory failure, septic shock and/or multiple organ dysfunction (NIH, 2021). In addition, COVID-19 seems to go through 3 different phases: early infection phase that lasts for 5–7 days, pulmonary phase and hyperinflammation phase, and these last two phases can be observed between the 7th and 15th day after the onset of the disease (Siddiqi & Mehra, 2020).

The infectious agent responsible for this new disease is Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Carvalho et al., 2021). This virus has a spherical morphology and it is believed that, to infect the cell, it uses mechanisms similar to SARS-CoV (Xu et al., 2020). In a canonical pathway, the viral spike (S) protein binds to the cellular receptor angiotensin-converting enzyme 2 (ACE 2) to promote the fusion of the viral and cellular

membranes; in order for the whole process to happen, it is necessary the activation (priming) of the S, which is given by the transmembrane protease serine 2 (TMPRSS2) (Hoffmann et al., 2020). In a noncanonical mechanism, human coronaviruses can use other host proteins with or without the presence of ACE2 to aid the viral entry (Colaco et al., 2021).

These cellular components (ACE2 and TMPRSS2) have been found in considerable expression in lung cells (Lukassen et al., 2020) and nasal epithelial cells (Sungnak et al., 2020); such characteristic may be the justification for the main symptoms of COVID-19 to be fever, cough, shortness of breath and fatigue (Centers for Disease Control and Prevention (CDC), 2020). Because this disease also generates symptoms that go beyond the respiratory system, such as vomiting and diarrhoea (Centers for Disease Control and Prevention (CDC), 2020; Gupta et al., 2020), other tissues were analysed and receptors that facilitate viral interaction were also found in them (Zou et al., 2020).

Thus, the male reproductive system is one of the places of vulnerability for this virus. The prostate, an organ with great expression of the TMPRSS2 gene in its epithelial cells, has a small percentage of cells that coexpress TMPRSS2 and ACE2, indicating that there is a fragility to the COVID-19 virus, but with a low risk (Song et al., 2020). Still, there are reports that one of the symptoms of this infection may be orchialgia (Pan et al., 2020), which could indicate some action of this virus on the testicles. It is known that the cell receptor used by SARS-CoV-2 is present in the somatic and germ cells of the testicles, and the protein responsible for the priming of the viral S appears only in the germ cells (Wang & Xu, 2020). However, when analysing the coexpression of the cell receptor (ACE2) and the virus activation protein (TMPRSS2), a rare presence of the two genes was observed together (Pan et al., 2020; Stanley et al., 2020). These results raise doubts about the interference of SARS-CoV-2 in male fertility and its presence in the seminal sample.

There are a few studies in the literature that sought to analyse whether SARS-CoV-2 is or is not present in the seminal sample of infected men; however, these assessments were made on samples from individuals already in the recovery phase, or in the acute phase of the disease without identifying the period of infection (days of diagnosis) (Guo et al., 2021; Holtmann et al., 2020; Pan et al., 2020; Song, Wang, et al., 2020). Thus, an early and evolutionary assessment of the presence and interference of the virus that causes COVID-19 in semen is important.

This study aimed to verify the presence of SARS-CoV-2 in the seminal sample of men during the acute phase of COVID-19.

2 | MATERIAL AND METHODS

This prospective study was conducted from May to November 2020 and involved twenty-two male volunteers aged between 23 and 31 years old who sought the university hospital with suspicion of COVID-19. After positive results (RT-PCR) in pharyngeal smear samples for SARS-CoV-2, they were invited to participate in the study.

The informed consent form was obtained from each participant and was approved by the Sao Paulo Federal University (UNIFESP) research ethics committee (CAAE 31916820.8.0000.5505). All men answered a routine questionnaire to obtain the clinical history and underwent a physical examination, performed by a urologist specialised in infertility. They also performed semen collection for seminal analysis and detection of SARS-CoV-2.

2.1 | Seminal analysis and preparation

Semen samples were obtained by masturbation during the acute and recovery phase of COVID-19. Each volunteer performed three collections 7, 14 and 21 days after initial diagnosis. After seminal liquefaction, semen analysis was performed according World Health Organization (WHO) manual of 2010 (World Health Organization, 2010).

For viral detection (RT-PCR), an aliquot of semen was transferred to a tube containing lactate ringer in a 1:1 ratio. The remaining sample was then centrifuged at 2,000 rpm for 30 min and the seminal plasma was frozen at −80°C for subsequent cell culture.

2.2 | Detection of SARS-CoV-2 in semen

The detection of SARS-CoV-2 RNA by RT-PCR was performed according to the IFU for the GeneFinder™ COVID-19 Plus RealAmp Kit (OSANG Healthcare Co., Ltd.) (GeneFinder, 2020; Zymo Reserch, 2020). RNA was isolated from the subject's semen using the Quick-RNA Viral Kit (Zymo Research), according to the manufacturer's protocol. After extraction, the RNA was used immediately, and the remaining RNA was stored at −80°C. The Master Mixture was prepared by mixing 10 μl of COVID-19 Plus Reaction Mixture and 5 μl of COVID-19 Plus Probe Mixture per sample; a sufficient amount of Master Mixture was prepared for all the samples and controls that were tested. Then, 15 μl of the Master Mixture was transferred to a 96-well plate to which either (a) 5 μl RNA sample, (b) 5 μl negative control (DEPC-treated water) or (c) 5 μl positive control (DNA plasmids encoding the SARS-CoV-2 RdRP, E, and N genes, and human *RNASE P* gene), were added. The plate was sealed and centrifuged at 2,000 rpm for 10 s. The thermal cycling conditions were as follows: (a) 1 cycle at 50°C for 20 min, (b) 1 cycle at 95°C for 5 min and (c) 45 cycles at 95°C for 15 s and 58°C for 60 s. CT values are considered positive by RT-PCR if Ct values <40.

A value of up to 40 cycles was defined as a positive test, and a value of 40 cycles or more was defined as a negative test (value defined according to the manufacturer).

2.3 | Cell culture

Seminal plasma samples from two patients were subjected to cell culture to check for viral replicative capacity. For the direct isolation of SARS-CoV-2, a cell line of VERO E6 (African green monkey kidney) was

used, as described by Matsuyama et al., 2020 (Matsuyama et al., 2020). The seminal plasma was diluted with culture medium and filtered through a disposable filter (0.22 nm) for inoculation. Subsequently, viral amplification was performed on VERO E6 cell line as previously described by Hoffman et al, 2020 (Hoffmann et al., 2020).

After demonstrating viral replication by checking the cytopathic effect formation within 96 hr of culture, the viral titre was determined by the amount of infectious viral particles capable of forming plaque (PFU/ml). Subsequently, molecular detection of SARS-CoV-2 in the culture supernatant was performed using the RT-PCR test.

2.4 | Data analysis

Initially, the procedures of descriptive statistics were applied. The symptoms were described and evaluated according to the frequency they were developed by the volunteers. For seminal evaluation, a graph was used to show the amount of motile morphologically normal spermatozoa (concentration × volume × progressive motility × morphology) for each patient in each collection period. Moreover, statistical analysis was performed with these parameters (SPSS software). Firstly, the Shapiro–Wilk test was applied to verify the normality of the data; those variables that did not show normal distribution were transformed by logarithm. In addition, the Mauchly teste was used to analyse sphericity. Then, the General Linear Model test for repeated measures was applied; p was considered significant when it was <0.05.

3 | RESULTS

Of the 22 volunteers, 20 were classified as having mild infection and underwent home treatment with symptomatic drugs and oximetry control, and two required hospitalisation, one with a moderate condition who sought medical care complaining of asthenia, myalgia, cough, anosmia and haemoptysis, being admitted to the ICU due to hemodynamic instability and another with a severe form of the disease with an imaging exam suggestive of a bacterial process and an oxygen saturation of 90% at the time of hospitalisation but without the need for O_2 support. All patients collected at least one seminal sample that was used to verify the presence of the virus.

The descriptions of age, symptom classification, time interval between first symptoms and diagnosis, symptoms and first collection of seminal samples and between diagnosis and first collection, and presence of the virus in the semen of each patient are shown in Table 1. The mean age of the patients was 28.23 years. The average number of days between the diagnosis of COVID-19 and the first

TABLE 1 Description of individual patient clinical symptoms, time-course and PCR results

Patient	Age	Symptoms' classification	Time between symptoms onset and diagnosis (days)	Time between symptoms onset and first semen sample collection (days)	Time between diagnosis and first semen sample collection (days)	Presence of viruses on semen sample
1	27	Mild	4	10	6	-
2	30	Mild	2	8	6	-
3	30	Mild	3	9	6	-
4	27	Mild	5	12	7	-
5	24	Mild	5	11	6	-
6	27	Mild	3	9	6	-
7	26	Mild	4	10	6	-
8	26	Mild	6	13	7	-
9	30	Mild	2	9	7	-
10	30	Mild	1	8	7	-
11	30	Mild	1	6	5	-
12	29	Severe	3	11	8	-
13	27	Mild	3	8	5	-
14	29	Mild	2	7	5	-
15	28	Mild	4	10	6	-
16	30	Mild	3	10	7	-
17	33	Mild	4	12	8	-
18	31	Moderate	10	18	8	-
19	31	Mild	5	11	6	-
20	23	Mild	11	17	6	-
21	24	Mild	2	9	7	-
22	29	Mild	1	8	7	-

TABLE 2 Frequencies of symptoms, treatment and hospitalisation ($n = 22$)

	Count	Frequency (%)
Symptoms		
Fever	10	45.5
Tremors	2	9.1
Cough	12	54.5
Dyspnoea	2	9.1
Coryza	8	36.4
Sore throat	8	36.4
Abdominal pain	3	13.6
Diarrhoea	3	13.6
Loss of smell	16	72.7
Loss of taste	9	40.9
Dizziness	1	4.5
Myalgia	11	50.0
Asthenia	6	27.3
Headache	11	50.0
Body ache	2	9.1
Nasal obstruction	2	9.1
Testicular pain		
Yes	2	9.1
No	20	90.9
Treatment		
Yes	6	27.28
No	16	72.72
Hospitalisation		
Yes	2	9.1
No	20	90.9

collection of seminal samples was 6.45 days. None of the samples collected for this study was positive for the presence of SARS-CoV-2. In samples that underwent cell culture with subsequent application of RT-PCR, the virus was not detected either.

The frequency of symptoms, presence of testicular pain, need for treatment and need for hospitalisation is shown in Table 2.

Regarding the seminal analysis, 8 patients were excluded, of which 6 did not return for the subsequent analyses, 1 was azoospermic, and 1 had extremely low-sperm concentration, making it impossible to evaluate all semen variables. No significant difference was found between the seminal parameters when analysed on days 7, 14 and 21 after the initial diagnosis (Table S1). The evolution of the total number of motile morphologically normal spermatozoa in each patient can be followed in Figure S1.

4 | DISCUSSION

The concern about the involvement of the male genital system in relation with COVID-19 by SARS-CoV-2 has been based on several aspects previously mentioned.

Initial studies (Pan et al., 2020; Song, Wang, et al., 2020) evaluated semen in recovered patients, with no virus found in the sample analysed. Although other more recent studies reaffirm this finding (Guo et al., 2021; Holtmann et al., 2020; Paoli et al., 2020), suspicion of the presence and possible transmissibility through semen was maintained during the acute phase of the disease.

A study (Li et al., 2020) evaluated a total of 38 patients, 15 of whom were in the acute phase of the disease and found the presence of the virus in the semen in 4 (26%) of those analysed during the initial phase, and in another 2 (8%) of those already recovered, (with 2 and 3 days of recovery). It is important to mention that this study did not describe the severity of the patients, but we can predict that, because they are hospitalised, they would potentially be classified between moderate to critical cases. Another important issue is the lack of description of the seminal collection process, which may pose risks of contamination of the sample by viruses not present in the semen. A recent research evaluated 15 men and the virus was present in 1 subject (Machado et al., 2021). Another study (Rawlings et al., 2020) evaluated 6 patients, with 6–14 days of symptoms, without finding viruses in the semen, despite maintaining viruses in salivary and nasal analysis.

The severity of the condition, in addition to the time of onset of symptoms, may be related to viraemia (Walsh et al., 2020), and to the possibility of the presence of the virus in other secretions such as the seminal. It is hypothesised that patients of greater severity and with shorter duration of the disease could have a greater chance of presenting SARS-CoV-2 in semen.

Our study was designed to try to answer this question; we evaluated patients during the acute phase with three serial collections, one each week after the onset of symptoms and diagnosis. Notwithstanding, almost all of our cases were of patients with mild symptoms, since moderate, severe and critical patients are, for the most part, in hospital and oxygen therapy, which makes seminal collection for masturbation impossible for ethical reasons, hospitality of service and risks of worsening of the clinical picture even momentarily. In all samples analysed, SARS-CoV-2 was not detected. In one of the samples, the result was indeterminate (CT value of 37 for gene E and negative for the other genes [N and RdRP]). In this and another random sample, we performed additional cell culture, aiming to find low viral concentrations not previously detected by RT-PCR. But even after culture, the new RT-PCR of these samples was negative.

Clinically, some studies have reported complaints of orchialgia in up to 19% of the patients analysed (Holtmann et al., 2020; Pan et al., 2020). During clinical evaluation, there was a complaint of mild bilateral orchialgia in only 2 patients with no clinical evidence of orchitis.

Seminal evaluation of these patients was also performed weekly during the 3-week follow-up. There was no difference between the total number of motile morphologically normal spermatozoa analysed in this period. Another article also evaluated the seminal pattern of patients after 26–34 days of symptoms, finding no evidence of seminal change in these patients (Guo et al., 2021). It is known that a complete spermatogenesis cycle occurs around 60 days (Misell et al., 2006) and that the presence of any acute febrile syndrome in this period can interfere with sperm production (Carlsen

et al., 2003), thus, a longer-term follow-up can determine whether COVID-19 interferes with the fertile potential of these men.

We consider the limitations of this study to be the relatively low number of patients evaluated and the representativeness of only mild cases; however, we can also consider that the inclusion of only patients with mild symptoms is also an advantage as it allowed us to characterise a more homogeneous population. Therefore, based on our pilot data, patients with a mild form of COVID-19 in the acute stage of the disease are unlikely to have SARS-CoV-2 in semen.

DATA AVAILABILITY STATEMENT

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

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