ISSN 1669-9106 MEDICINA (Buenos Aires) 2023: 83: 185-189

SHEDDING OF INFECTIOUS SARS-COV-2 IN TWO ASYMPTOMATIC CHILDREN

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Received: 7-III-2023
Accepted: 22-III-2023

Abstract

Asymptomatic infections with SARS-CoV-2 are associated with viral transmission and have a key role in the propagation of the pandemic. Understanding viral shedding during asymptomatic infections is critical. Unfortunately, data on asymptomatic SARS-CoV-2 infection in children is extremely limited. To determine the presence of viral viable shedding, we prospectively followed two healthy children of a family where both parents developed mild COVID-19 (April 2021). SARS-CoV-2 detection was made by RT-PCR and virus isolation by cell culture from saliva samples. Positive samples were sequenced to identify variants of SARS-CoV-2. Serum samples were evaluated to determine the presence of antibodies using a single enzyme-linked immunosorbent assay (ELISA, COVIDAR IgG). Both children were SARS-CoV-2 positive and asymptomatic. In addition, the virus grew in cell culture from saliva samples. Furthermore, one child showed viable SARS-CoV-2 for at least 17 days after the onset symptoms from his father. The recommended isolation period for asymptomatic contacts during the acquisition of data had been established for 10 days; however, this child remained with viable virus beyond that period. The positive samples from both children were consistent with B.1.1.28.1 lineage (Gamma). In both asymptomatic children, anti-Spike IgG was detected. Asymptomatic children may represent a source of infection that should not be underestimated during this pandemic.

Key words: asymptomatic, SARS-CoV-2, COVID-19, pediatric, viral shedding

Resumen

Excreción de SARS-CoV-2 infeccioso en dos niños asintomáticos

Las infecciones asintomáticas por SARS-CoV-2 están asociadas a la transmisión viral y tienen un papel clave en la propagación de la pandemia. Comprender la excreción viral durante las infecciones asintomáticas es fundamental. Desafortunadamente, los datos sobre la infección asintomática por SARS-CoV-2 en niños son extremadamente limitados. Para determinar la presencia de excreción de virus viable, se siguió prospectivamente a dos niños sanos de una familia en la que ambos padres desarrollaron COVID-19 leve (abril 2021). La detección de SARS-CoV-2 se realizó por RT-PCR y el aislamiento del virus por cultivo celular a partir de muestras de saliva. Las muestras positivas se secuenciaron para identificar variantes de SARS-CoV-2. En las muestras de suero se determinó la presencia de anticuerpos utilizando un

ensayo de ELISA (COVIDAR IgG). Ambos niños fueron positivos para SARS-CoV-2 y asintomáticos. Además, el virus creció en cultivos celulares a partir de muestras de saliva. Uno de los niños mantuvo SARS-CoV-2 viables durante al menos 17 días después de la aparición de los síntomas de su padre. El período de aislamiento recomendado para contactos asintomáticos durante la adquisición de datos se había establecido en 10 días, sin embargo, este niño permaneció con virus viable más allá de ese período. Las muestras positivas de estos niños correspondieron al linaje B.1.1.28.1 (Gamma). En ambos niños asintomáticos se detectó anticuerpos IgG anti-Spike. Concluimos que los niños asintomáticos pueden representar una fuente de infección que no debe subestimarse durante esta pandemia.

Palabras clave: asintomático, SARS-CoV-2, COVID-19, pediátrico, diseminación viral

Asymptomatic infections with SARS-CoV-2 are associated with viral transmission and have a key role in the propagation of the pandemic[1]. Understanding viral shedding during asymptomatic infections is critical. Unfortunately, data on asymptomatic SARS-CoV-2 infection in children is extremely limited[2].

In adult patients with SARS-CoV-2 infection RT-PCR in respiratory samples can be positive 3 days up to several weeks from symptoms onset. Furthermore, some patients can become positive again after a period of negative testing[3]. In children, the mean time of positive RT-PCR is 11.1 days in symptomatic and 9.4 days in asymptomatic subjects[4]. However, a positive RT-RCR test does not necessarily reflect shedding of viable virus.

Infectiousness of SARS-CoV-2 can begin 2-3 days prior to symptoms onset and declines 7 days from symptoms onset. Viable SARS-CoV-2 detected in cell culture virus is isolated in asymptomatic adults mostly within 7 days after the initial positive RT-PCR[5]. In symptomatic children, viable virus was detected in cell culture up to 5 days after onset of symptoms[6]. Longer viral shedding has been described in very few pediatric cases, either from critical patients or from children with oncohematologic diseases. Specifically, viable SARS-CoV-2 was isolated up to day 54 in a critical pediatric patient and up to 139 days in an immunocompromised child with severe COVID-19[2]. To our knowledge, there is a lack of data on isolation of SARS-CoV-2 in asymptomatic and otherwise healthy children.

The objective of this study was to determine the presence of viable virus by cell culture in saliva samples from two asymptomatic and otherwise healthy children infected with SARS-CoV-2.

A prospective study in a family group infected with SARS-CoV-2 was conducted. Family members were prospectively followed for up to 28 days (during April, 2021). Demographic and clinical data were collected. This study was approved by the Ethics Committee of CEMIC (Protocol: 1298/20).

Sequential saliva and fecal samples were obtained every 3 days. Nucleic acid was extracted from 100µl and eluted in 15µl using manual columns (Quick-RNA TM Viral Kit, Zymo Research CORP.), following manufacturer's recommendation.

Detection of SARS-CoV-2 was performed with an in-house one-step real time RT-PCR multiplex assay targeting the E gene of SARS-CoV-2 and the human RNAsa P gene as an internal control, in a CFX 96 Deep Well™ Real Time System (BioRad). A positive result was considered when the human RNAse gene or the internal amplification control were positive and the cycle threshold (Ct) value was less than 40[7].

To analyze the signature amino acid mutations on the Spike protein of the variants of SARS-CoV-2, Sanger sequencing of segment 29 of the CDC amplification protocol that includes amino acids 428 to 750 was performed[8].

SARS-CoV-2 isolation was performed in a BSL3 facility at *Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), Universidad de Buenos Aires*. Vero cell monolayers were inoculated with 300 µl of pre-filtered infectious saliva sample diluted in 300ul of DMEM (Sigma) supplemented with Fetal Bovine Serum (4%), streptomycin (50 µg/ml), penicillin (50 U/ml) and amphotericin B (125 ng/ml). Cells were monitored for virus-associated cytopathic effect (CPE) for 96 hours. Positive supernatants were confirmed by RT-qPCR.

SARS-CoV-2 serology was evaluated in both children using COVIDAR IgG assay, which uses a combination of a trimer stabilized spike protein and the receptor binding domain (RBD) in a single enzyme-linked immunosorbent assay

(ELISA) plate (*Fundación Instituto Leloir- CONICET-Laboratorio Lemos, Argentina*)[9].

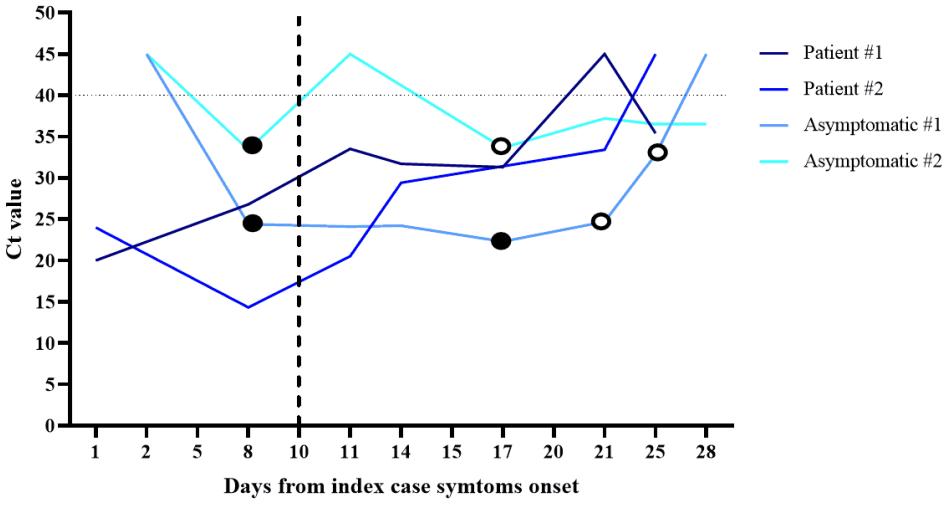
A breakthrough male case with COVID-19 was identified on April 6, 2021 (Patient#1). His family included his wife (43 years old, Patient#2), a 9 years old boy (Asymptomatic#1) and a 12 years old girl (Asymptomatic#2). Both parents were healthcare workers who had completed the Sputnik V vaccine scheme (2 doses) in February 2021. All subjects were previously healthy. The family returned from a short holiday trip on April 4, 2021. On the same day, Asymptomatic#1 developed pharyngitis. A rapid pharyngeal test obtained the following day was positive for *Streptococcus pyogenes*, and he received antibiotics. His nasopharyngeal swab (NPS) for SARS-CoV-2 was negative.

On April 6, (day 1), Patient#1 (the index case) developed fever and myalgia and his NPS was SARS-CoV-2 positive. On the same day, Patient#2, who was asymptomatic, also tested positive for SARS-CoV-2. The following day, both asymptomatic children were RT-PCR negative for SARS-CoV-2 in saliva and NPS samples. On day 7, Patient#2 developed COVID-19 symptoms including fever, myalgia, arthralgia and headache. On day 8, both asymptomatic children became SARS-CoV-2 positive. Both adults developed mild COVID-19 and remained RT-PCR positive for 21 and 25 days. Children remained asymptomatic throughout the study period and they had RT-PCR positive in saliva for 25 and 28 days. Viable viruses were detected in children by cell culture on days 8 and 17 (Fig 1). Positive cell culture samples correlated with RT-PCR Ct values ranging from 22.3 to 33.4. In addition, stool samples were SARS-CoV-2 positive in both children for up to 21 and 28 days.

Viral sequencing in Patient#2 and both children showed four mutations corresponding to E484K, N501Y, D614G and H655Y, consistent with B.1.1.28.1 lineage (Variant Gamma or Variant P.1). Both asymptomatic children seroconverted and showed detectable SARS-CoV-2 anti-Spike IgG levels (65 and 227 UI/ml).

SARS-CoV-2 pandemic affects mostly adult patients and shedding time of viable SARS-CoV-2 has been well established. However, data on viable shedding in asymptomatic healthy children is lacking. In this study, we describe the presence of viable SARS-CoV-2 from saliva samples in two asymptomatic healthy children.

Figure 1 | SARS-CoV-2 isolation and detection by RT-PCR in saliva and NPS samples
Sequential samples of Patient #1, #2, Asymptomatic #1 and #2. Filled circle: positive culture. Empty circle: negative culture. Dotted vertical line: Theoretical de-isolation date. Ct value ≥ 40 are RT-PCR negative results



Ct value: cycle threshold value; NPS: nasopharyngeal swab

Given the low rate of infections in pediatrics, asymptomatic children, even those with close contacts to positive cases, are usually not screened for SARS-CoV-2. In this study, two asymptomatic children living with their infected parents, who were prospectively followed, showed RT-PCR positivity and viable virus. This observation underscores the potential role of asymptomatic children in the spread of the virus, especially considering that most children remain asymptomatic[10].

Interestingly, the asymptomatic child with viable viral shedding for at least 17 days from index case's symptoms onset, would have been potentially contagious beyond the isolation period that was suggested, in this moment, by the Ministry of Health in the region. The isolation period for asymptomatic close contacts of a positive case had been determined for 10 days from the case's symptoms onset. Other works have shown that most of the children with COVID-19 have silent disease, but SARS-CoV-2 RNA can still be detected in the respiratory tract for a prolonged period[11].

Successful cell culture isolation was associated with Ct values lower than 23[12]. In our study, isolation was successful even on samples with higher Ct values. This finding suggests that at least in children Ct value >23 cannot rule out the presence of viable virus. Murata et al. found similar results, from nasopharyngeal swab samples from an older adult who became infected with SARS-CoV-2 on a cruise ship[5]. In this study, sequence analysis demonstrated the presence of Gamma variant (lineage P1), which was circulating in Argentina in 2021, but was later displaced by Omicron variant[13]. Whether this variant remains contagious for longer periods or has a different kinetic in children is still to be determined.

RT-PCR in saliva samples was shown to be convenient and successful in detecting SARS-CoV-2 in symptomatic adult patients[7]. In our study, saliva samples were also useful in detecting SARS-CoV-2 in asymptomatic children. Furthermore, these samples were also useful for successful viral isolation in cell culture. As nasopharyngeal swabs can be painful and bothersome, particularly in children, saliva samples represent a more convenient, non-invasive and painless option[14, 15]. Confirming the presence of true infections, both children were found to have anti-S IgG for SARS-CoV-2 in subsequent serum samples.

The main limitation of this study is that only two children were evaluated. Despite this limitation, our observation showed that the presence of viable virus in saliva samples from asymptomatic children can last for at least 10 days from the initial PCR positivity and can represent a source for spreading.

In summary, our observation underscores the importance of testing asymptomatic children since they can also shed viable virus for several days. Given the difficulties for obtaining nasal swabs in children, saliva samples can provide a reasonable alternative for detection of SARS-CoV-2.

Acknowledgment: We thank the study volunteers for providing the analyzed samples.

Funding statement: This study was partially supported by a grant from *Fondo para la Investigación Científica y Tecnológica* (FONCYT) [IP-COVID 19: 0938] awarded to Dr. Marcela Echavarría and an internal fund from CEMIC and *Fundación Norberto Quirno* N° 01/20.

Conflicts of interest: MS is a consultant to Basilea, a speaker for Pfizer and the principal investigator in Argentina for NIH grant UM1AI104681. The rest of the authors have no conflict of interest.

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