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Research Article

JOURNAL OF PEDIATRICS, PERINATOLOGY AND CHILD HEALTH

ISSN: 2687-8046

Assessment of SARS-CoV-2 Vertical Transmission through Nested RT-PCR Testing of Neonatal Samples: Three Case Reports

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Abstract

The novel Coronavirus Disease 2019 (COVID-19) pandemic has drawn attention to the possible transplacental transmission of SARS-CoV-2 and the consequences to the fetus and newborn, despite initial assumption that there was no possibility of coronavirus transmission from the mother to the fetus. More recently, cases of newborns infected with the novel coronavirus have been reported in the scientific community. We present three cases of pregnant women with positive SARS-CoV-2 antibody serology on admission to the Marcílio Dias Naval Hospital, Rio de Janeiro, Brazil, and the diagnostic tests performed on the newborns. RT-PCR tests were negative for all neonatal nasopharyngeal swab samples tested, although SARS-CoV-2 was detected in amniotic fluid and umbilical cord blood using nested PCR techniques, thus successfully demonstrating transplacental transmission. We suggest that nasopharyngeal swab PCR tests of neonates may have some limitations for the investigation of transplacental infection, therefore, this molecular test needs more attention for this kind of investigation.

Keywords: SARS-CoV-2; COVID-19; Vertical Transmission; Nested RT-PCR Technique; Case Report

Introduction

At the end of 2019, a novel coronavirus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in China and spread around the world, consolidating coronavirus disease 2019 (COVID-19) as one of the most striking pandemic diseases to date. The transmission of SARS-CoV-2 occurs primarily through respiratory droplets, and the virus replicates in the upper respiratory tract of the host [1, 2] entering pulmonary cells via the ACE-2 receptor [3]. The first pneumonia cases reported at the beginning of the outbreak showed that COVID-19 was a respiratory infectious disease with mainly pulmonary involvement, but with the increase in cases, extrapulmonary repercussions began to be observed, such as gastrointestinal and neurological symptoms [4, 5].

Special concern has been raised about pregnant women and the possible infection of the fetus and neonate via vertical transmission. Epidemiological features of other coronaviruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), showed no evidence of vertical transmission [5]. Neonatal

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Citation: Maria Alice Fusco, Vinícius Mantini, Jarba de Souza Salmont Júnior, Raphael Guimarães de Carvalho Gomes, Andressa Rangel de Oliveira Lima, Helena Loschi de Oliveira Clarim, Erika Caminha Ferreira, Simone Saraiva de Abreu Almeida, Camilla Graça Salmont, Vanessa de Sousa Rizzo-Valente, Antônio Carlos Almeida de Oliveira, Felipe Vale Fortes Rodrigues, Gabriele Zanirati, Nicole Becker, João Pedro Galina, Jaderson Costa da Costa, Daniel Rodrigo Marinowic. Assessment of SARS-CoV-2 Vertical Transmission through Nested RT-PCR Testing of Neonatal Samples: Three Case Reports. Journal of Pediatrics, Perinatology and Child Health 6 (2022): 370-376.

Received: July 11, 2022
Accepted: July 18, 2022
Published: August 04, 2022

Fusco MA et al., J Pediatr Perinatol Child Health 2022
DOI:10.26502/jppch.74050120

SARS-CoV-2 infections are extremely rare and, to date, there is no consensus on the possibility of the occurrence of intrauterine infection caused by vertical transmission [6, 7]. Due to these previous findings, it was theoretically concluded that SARS-CoV-2 would also not be transmissible from the mother to the fetus through the placenta [5, 8]. In fact, the first case reports of pregnant women infected with SARS-CoV-2 showed no evidence of intrauterine infection with SARS-CoV-2 [9]. However, the increased number of SARS-CoV-2-positive pregnant women allowed data collection from various sites, using different diagnostic methods to determine the possibility of vertical transmission of SARS-CoV-2 [10].

As described in some case reports, amniotic fluid, umbilical cord blood, smears from oropharyngeal mucosa, and colostrum samples collected from infected mothers were negative for SARS-CoV-2 as determined by RT-PCR [11, 12]. However, some controversies have arisen, as IgM antibodies were found in newborns of mothers affected by COVID-19 [13]. We decided to investigate, through serological and molecular tests, a variety of biological samples obtained from three mothers and their newborns to observe the occurrence of vertical transmission of SARS-CoV-2.

Methods

The following report was previously approved by the Ethics Committee on Human Research of the Marcílio Dias Naval Hospital (HNMD) with approval number 36066820.8.0000.5256. The participants provided written informed consent for the collection of their clinical data, their own biological samples, as well as samples of their newborns. Since April 2020, all pregnant women admitted to the HNMD for delivery had their blood tested for the detection of anti-SARS-CoV-2 IgG/IgM antibodies as a screening test for the diagnosis of COVID-19 in pregnant patients. Serological testing was performed using the Leccurate Rapid Test (Lepu Medical), according to the manufacturer´s recommendations. Expectant mothers with positive results for at least one antibody in the serological test and whose delivery would occur by cesarean section were invited to participate in the research by donating nasopharyngeal swabs of the mother and the newborn as well as placenta, amniotic fluid and umbilical cord blood samples.

Nasopharyngeal swabs were collected following the Centers for Disease Control and Prevention (CDC) [14] instruction guidelines. RT-PCR for the detection of SARS-CoV-2 was performed in the Molecular Biology Laboratory of Biomedical Research Institute, located at Marcílio Dias Naval Hospital (HNMD). The protocol used was in accordance with the CDC's Emergency Use Authorization (USA). Also, amniotic fluid was sterilely collected during cesarean section shortly before amniotic sac rupture and umbilical cord blood was sterilely collected with a needle after the clamping and cleaning of the cord. Both amniotic fluid and cord blood were tested for molecular detection of SARS-CoV-2, using two different protocols, CDC and Charité. Umbilical cord blood was also tested for the detection of anti-SARS-CoV-2 IgG/IgM antibodies.

Subsequent nested RT-PCR technique was performed for SARS-CoV-2 detection in the amniotic fluid and umbilical cord blood samples at the Brain Institute of Pontifical Catholic University of Rio Grande do Sul (PUCRS), accredited for the diagnosis of COVID-19. Total RNA was extracted by using an SV-Total RNA kit (Promega, Madison, Wisconsin, USA) according to the manufacturer´s recommendations. Reverse-transcriptase first-strand DNA synthesis was performed by the 3' primer technique using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) with two reverse primers (hCOVassay1 R: 5'AGCAGCATCACCGCCATTG 3' and hCOVassay2 R: 5' CCGCCATTGCCAGCCATTC 3'). After the transcription reaction, the product generated was quantified by a NanoDrop fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were amplified from an initial amount of 200 ng of ssDNA for each sample using the PowerUp SYBR Green Master Mix kit with uracil DNA glycosylase (UDG) activation (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used were hCOVassay1 – F 5'GCCTCTTCTCGTTCCTCATCAC 3'/R 5'AGCAGCATCACCGCCATTG 3' and hCOVassay2–F 5'AGCCTCTTCTCGTTCCTCATCAC 3'/R 5'CCGCCATTGCCAGCCATTC 3'. After the first RT-PCR, a new amplification was performed using the same primer set as the first PCR. For this new reaction (nested RT-PCR), the PCR product generated in the initial amplification was used as the template. Placentas were collected at birth for histopathological analysis by an experienced placental pathologist.

Case Description

Case 1

A 26-year-old pregnant woman (second pregnancy, no comorbidities) was admitted to the HNMD for elective induction of labor at 40 gestational weeks on 30 June 2020. She reported headache, nausea, myalgia, malaise, and joint pain that had started two days before hospitalization. She underwent cesarean section due to her refusal of induction. Serological screening test showed IgM-positive and IgG-negative results. Nasopharyngeal swab samples were negative for SARS-CoV-2 detection. RT-PCR test of the nasopharyngeal neonatal swab samples were also negative for SARS-CoV-2 detection. Serological tests of cord blood were negative for IgG/IgM novel coronavirus antibodies. However, nested RT-PCR analysis was able to detect SARS-CoV-2 in amniotic fluid and umbilical cord blood samples. Histopathological study of the placenta showed moderate circulatory changes suggestive of maternal vascular malperfusion. An area of old

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Fusco MA et al., J Pediatr Perinatol Child Health 2022
DOI:10.26502/jppch.74050120

infarction and retroplacental hemorrhage was noticed, as well as mild multifocal delayed villous maturation. Mild/moderate fibrin deposition, hemorrhage and calcification foci were detected in the intervillous space. Subchorionic thrombosis was present.

Case 2

A 31-year-old pregnant woman (first pregnancy, no comorbidities) was admitted for labor at 41^{+1} gestational weeks on 08 July 2020. The interruption of pregnancy by cesarean surgery was indicated due to the refusal of drug induced labor. She denied having had symptoms of COVID-19 and was asymptomatic at the time of hospitalization. However, a serological screening test revealed the presence of IgM antibodies for SARS-CoV-2. Her nasopharyngeal swab samples analysis also detected the virus through RT-PCR. Serological test of umbilical cord blood revealed she was positive for IgG antibodies. RT-PCR analysis of the neonatal nasopharyngeal swab samples was negative for SARS-CoV-2. Subsequent nested RT-PCR analysis detected SARS-CoV-2 in umbilical cord blood and amniotic fluid samples. The placenta was small-for-gestational age (SGA) on gross examination. Vascular alteration findings in the histopathological analysis were suggestive of maternal vascular disease. Delayed villous maturation was also observed. The decidua and subchorionic space presented with moderate fibrin deposition in a lamellar pattern. In the intervillous space, extensive thrombus and mild fibrin deposition, hemorrhage, calcification foci and mild acute local inflammatory infiltrate were detected. Chorangiosis was present. In the umbilical cord, extensive hemorrhage in the jelly and extramedullar mild hemopoiesis was observed.

Case 3

A 30-year-old woman (second pregnancy, no comorbidities) was admitted to the hospital at 39^{+2} gestational weeks on 10 July 2020. During anamnesis she reported having had headache, loss of appetite, myalgia, fatigue, anosmia and ageusia two months before the date of hospitalization. The screening serological test for SARS-CoV-2 was positive for IgG/IgM antibodies. Nasopharyngeal swab samples were analyzed by RT-PCR, and the result was also positive for SARS-CoV-2. Cesarean surgery was indicated for the following day, after the progression of the delivery was no longer detected. Nasopharyngeal swab samples of the neonate were also collected immediately after delivery and before maternal contact, and RT-PCR analysis of this specimen was negative for the detection of the virus. Umbilical cord blood was tested for SARS-CoV-2 antibodies, and the result was positive for both IgG and IgM. Nested RT-PCR was able to detect SARS-CoV-2 in amniotic fluid and cord blood samples. Placental histopathological analysis demonstrated mild nonspecific vascular changes. The intervillous space had mild fibrin deposition, hemorrhage and calcification foci. Mild multifocal delayed villous maturation and mild fibrin deposition were observed in the decidua. In the umbilical cord, fibrin was observed in one vascular lumen, and mild hemopoiesis was observed.

Table 1: Clinical presentation and laboratory test results for SARS-CoV-2 of mothers and newborn infants.

Case / Presentation		1	2	3
Age (years)		26	31	30
Gestational weeks on admission		40	41^{+1}	39^{+2}
History of symptoms reported by the patient		Headache, nausea, myalgia, malaise, joint pain 2 days before hospitalization	Asymptomatic	headache, loss of appetite, myalgia, fatigue, anosmia and ageusia 2 months before the date of hospitalization
Maternal Serology	IgM	positive	positive	positive
	IgG	negative	negative	positive
Maternal nasopharyngeal PCR		negative	positive	positive
Newborn nasopharyngeal PCR		negative	negative	negative
Umbilical cord blood serology	IgM	negative	negative	positive
	IgG	negative	positive	positive
Amniotic fluid qRT-PCR		negative	negative	negative
Umbilical cord blood qRT-PCR		negative	negative	negative
Amniotic fluid nested PCR		positive	positive	positive
Umbilical cord blood nested PCR		positive	positive	positive
Gestational age (days)		280	288	275
Birthweight (g)		3085	3120	3585
1 min Apgar score		8	8	9
5 min Apgar score		10	9	9

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Diagnostic assessment

The three cases showed positive results for nested RT-PCR in the neonatal samples (amniotic fluid and umbilical cord blood), confirming the vertical transmission of SARS-CoV-2 virus, despite the occurrence of distinct scenarios regarding the results of serological and molecular analysis of assorted samples of the pregnant women, as well as the clinical presentation of them. A summary of the cases can be seen in Table 1. The screening test performed in the day of hospitalization for delivery showed detected result for IgM serological test. However, the results of IgG serology and RT-PCR of nasopharyngeal swab samples showed different combination of results, which reflects the distinct periods of maternal infection by the COVID virus (illness, decline or convalescence periods) at the time of delivery. Despite the period of the mother´s infection or the combination of COVID tests results, the fetuses were exposed to SARS-CoV-2 through vertical transmission.

Discussion

As a new disease, many questions remain about COVID-19 and one of these questions refers to the possible methods of transmission, in addition to the respiratory route. A case report of clinical manifestation of COVID-19 in a neonate [15] called attention to the possible vertical transmission of SARS-CoV-2, even if occurring only in a small percentage of cases compared to the number of infected mothers. To determine a possible vertical transmission route of SARS-CoV-2 from the mother to the fetus during pregnancy, we selected three pregnant women with confirmed laboratory diagnosis of COVID-19. We were careful to select pregnant women whose delivery occurred by cesarean section to ensure that possible transmission occurred via the transplacental route, to exclude contamination by hematogenous or transcervical routes [5, 15]. A serological antibody-based test for SARS-CoV-2 was performed as a screening test in all pregnant women admitted to the HNMD for delivery to identify those who were infected with COVID-19. Antibody seroconversion can occur less than 1 week to more than 6 weeks after the emergence of symptoms [16]. Thus, one case was identified with positive results for both IgM and IgG (case 3), and two cases of positive IgM and negative IgG (cases 1 and 2). Next, RT-PCR tests of the maternal nasopharyngeal swabs identified positive results in cases 2 and 3, indicating the presence of SARS-CoV-2 RNA. A positive RT-PCR test can be observed in the incubation period prior to the beginning of COVID-19 symptoms (as seen in case 2), and last until the resolution of symptoms (in case 3, for example) [16].

In one case, a serological test was positive for IgM and RT-PCR tests even though the patient had no symptoms related to COVID-19 (case 2). This finding corroborates another study that identified asymptomatic COVID-19 infected obstetric patients through RT-PCR for SARS-CoV-2, indicating the importance of performing diagnostic screening tests to identify infected patients and monitor them, as well as their infants, more carefully [17]. The probability of the occurrence of vertical transmission through the transplacental route increases with increased gestational age [9], and the results of serological and molecular tests, combined with the evolution of symptoms in the three cases showed that the patients were infected in the third trimester of pregnancy. In a case series of 5 fetal deaths, SARS-CoV-2 was detected in the amniotic fluid or placenta, and vertical transmission occurred during the third trimester of gestation [18].

To verify the presence of SARS-CoV-2 in the neonates of our study, RT-PCR was performed on nasopharyngeal swab samples collected shortly after labor and before contact with the mothers, and the results were negative. Although RT-PCR is considered the gold standard for diagnosing SARS-CoV-2 infection, the diagnostic efficiency of this test in newborns has not yet been stablished [7]. This can be explained by the fact that the airways are not functional during intrauterine life and that the proliferation of SARS-CoV-2 in the upper respiratory tract seems to be irrelevant for fetal infection [19]. Because the hematogenous route is the most likely mechanism for vertical viral transmission [7], serological testing of umbilical cord blood was performed, and the results were different in the three cases. IgM and IgG antibodies for SARS-CoV-2 were negative in case 1 and IgG was detected in the newborn of case 2, which was probably of maternal origin that was transferred to the fetus by the placenta. However, positive results for both IgM and IgG in the cord blood of case 3 immediately after birth are a high indication that vertical transmission occurred, since IgM is a macroglobulin that cannot cross the placenta from the mother to the fetus [10]. The presence of IgM in cord blood with a negative result for the detection of SARS-CoV-2 in swab samples of newborns is rare but can be observed [13, 20], which confirms that RT-PCR analysis of nasopharyngeal swab samples may not be the gold standard neither for the detection of vertical transmission nor the diagnosis of COVID-19 in neonates [5, 20].

SARS-CoV-2 detection was also performed in amniotic fluid and umbilical cord blood samples of the three cases. A systematic review identified 51 amniotic fluid samples tested for SARS-CoV-2, none of which were positive for SARS-CoV-2 RNA [10]. However, a case report presented the results of a transplacental infection of SARS-CoV-2 in different organs of a 4-day-old child who died due to vascular complications related to poor placental perfusion. The detection of SARS-CoV-2 in the brain, heart, and lung was possible only after the application of the nested PCR technique, and infection was proven by genetic sequencing [21]. In our study, the initial RT-PCR analysis was also not able to detect the virus, and further nested RT-PCR was considered for these types of samples. In fact, CDC

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guidelines recommend that RT-PCR analysis should be performed solely in upper respiratory swab specimens (CDC, 2022), but there are alternative protocols available to meet diagnostic demands [22]. We successfully detected SARS-CoV-2 in all amniotic fluid and cord blood samples by nested RT-PCR. This technique could be used in situations in which it is necessary to increase the sensitivity and/or specificity of the PCR [23]. The product of the first amplification reaction (all negative for SARS-CoV-2) was used as the template for the second nested RT-PCR. Additionally, we used reverse primers for reverse transcription, generating a specific ssDNA for the region of interest. The use of nested PCR methodology allows us to increase the sensitivity of the test up to 100-fold [24], thus allowing differentiated research potential for determining viral infection in instances of a low copy number of initial genetic material.

Regarding the pathology analysis of the placentas, there was no evidence of abnormality that could be observed macroscopically, except for the small size of the placenta in case 2. Microscopic analysis revealed maternal and fetal vascular malperfusion of the placentas, corroborating a systematic review of histopathological lesions observed in third trimester placentas of SARS-CoV-2-positive mothers [25]. Maternal vascular malperfusion can be observed in placentas of SARS-CoV-2-infected mothers, even though the virus itself was not identified by RNA in situ hybridization [24, 25]. The observed lesions associated with coagulation in the placentas of this study may be related to any inflammatory disease, and SARS-CoV-2 cannot be excluded. Hypertension and preeclampsia can also be associated with vascular thrombotic disease [26], but we can exclude these hypotheses in the three cases presented. It is important to note that so far there is no specific pathological characteristic of SARS-CoV-2 infection that could be observed in the placenta [26].

At the beginning of the pandemic the possibility of transmission was considered to be a very rare or even impossible event to occur [7, 8]. However, we are still facing the uncertainties that follow the emergence of this novel virus, and more case reports of COVID-19 have accumulated as the pandemic has spread worldwide, which has allowed us to conclude that SARS-CoV-2 is a virus capable of crossing the placenta and infecting the fetus, even at low rates [27]. Even so, there is still no consensus regarding the transplacental route of transmission of SARS-CoV-2. This is due to the heterogeneity and sensitivity of diagnostic methods, and the conditions for obtaining the newborn samples [10]. We conducted different diagnostic tests that add crucial information to confirm vertical virus transmission via the transplacental route. Additionally, the results of PCR and serological tests obtained from maternal and neonatal samples indicated that RT-PCR of infant nasopharyngeal swab samples is not an option for the detection of SARS-CoV-2 resulting from vertical transmission, corroborating Konstantinidou [28]. Instead, amniotic fluid or umbilical cord blood are samples that can be obtained noninvasively and analyzed by serological or virological tests for the detection of SARS-CoV-2.

Despite the evidence of vertical transmission, the 3 infants related were born with appropriate weight and gestational age, normal Apgar and had no clinical signs of COVID-19. In fact, most newborns infected with SARS-CoV-2 at that time were asymptomatic for COVID-19 [29]. However, the emerging reality of hospitalizations of infant and children mainly associated with the new variants of the novel coronavirus [30, 31] reinforces the need for the development of more sensitive molecular detection techniques capable of detecting the presence of the novel coronavirus in samples from newborns or extraembryonic tissues. The underreporting of novel coronavirus infections in neonatal medical care settings puts other at risk for infection, and can generates major public health problems, such as disease outbreaks and the collapse of these care units.

Conclusion

To date, there is no standard protocol that states the best sample to collect or diagnostic test to run to determine the occurrence of SARS-CoV-2 vertical transmission. The results presented here show that a single analysis test may not be sufficient for a diagnostic conclusion, and we strongly suggest conducting different analyses to confirm a possible infection of the neonate, including nested RT-PCR, regardless the infectious status of the mother at the time of delivery.

Competing interests

The authors declare that they have no competing interests

Author Contributions

MAF and JSSJ were responsible for the conceptualization and design of the study. AROL, HLOC, ECF and SSAA were responsible for the acquisition of the biological samples. ACAO performed the histopathological examination of the placentas. VM, RCGC. and CGS were responsible for data curation. FVFR, GZ, NB, JPG, JCC and DRM performed RT-PCR and nested RT-PCR tests. DRM was responsible for funding acquisition. MAF and DRM wrote the text. DRM, VSRV, GZ and FVFR were responsible for text review and edit. All authors were responsible for the final content.

Funding

This study was financed in part (fellowship grant) by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001.

Availability of Data and Materials

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

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Citation: Maria Alice Fusco, Vinícius Mantini, Jarba de Souza Salmont Júnior, Raphael Guimarães de Carvalho Gomes, Andressa Rangel de Oliveira Lima, Helena Loschi de Oliveira Clarim, Erika Caminha Ferreira, Simone Saraiva de Abreu Almeida, Camilla Graça Salmont, Vanessa de Sousa Rizzo-Valente, Antônio Carlos Almeida de Oliveira, Felipe Vale Fortes Rodrigues, Gabriele Zanirati, Nicole Becker, João Pedro Galina, Jaderson Costa da Costa, Daniel Rodrigo Marinowic. Assessment of SARS-CoV-2 Vertical Transmission through Nested RT-PCR Testing of Neonatal Samples: Three Case Reports. Journal of Pediatrics, Perinatology and Child Health 6 (2022): 361-376.