

Current standard protocols of RT-PCR of nasopharyngeal swab in neonatal patient is not sensitive enough for determination of vertical transmission

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Short Report

Keywords: Infectious Disease Transmission Vertical, SARS-CoV-2, COVID-19

Posted Date: February 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-228645/v2>

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Abstract

The pandemic experienced in recent months has raised questions that should be investigated in the clinical practice. Transplacental transmission of SARS-CoV-2 and the consequences to the fetus and newborn have called attention due to the increasing number of infections, contradicting previous evidences that there was no possibility of coronavirus transmission from the mother to the fetus. We presented three cases of pregnant women with positive SARS-CoV-2 antibodies serology on admission in Naval Hospital Marcílio Dias (HNMD), Rio de Janeiro, Brazil. Samples of umbilical cord blood was double positive (IgM and IgG) for one patient, double negative for one patient and positive for IgG and negative for IgM for third patient. Maternal and neonatal nasopharyngeal swab samples analyzed by PCR for SARS-CoV-2 was positive for two maternal patients and negative for all newborns tested. It was possible to detect the SARS-CoV-2 in amniotic fluid and umbilical cord blood using the *nested*-PCR technics, thus being successfully evidenced transplacental transmission. We suggested that nasopharyngeal swab PCR test of neonates does not have a correlation with vertical transmission and thus, this molecular test is not useful for investigation of transplacental infection.

Introduction

At the end of 2019, a novel coronavirus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread around the world from China and consolidated coronavirus disease 2019 (COVID-19) as one of the most striking pandemic disease so far. Transmission of SARS-CoV-2 occurs primarily through respiratory droplets and the virus replicates in the upper respiratory tract [1,2], entering the 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, extra-pulmonary repercussions began to be observed such as gastrointestinal and neurologic symptoms [4,5].

Special concern has been raised about pregnant women and the possible vertical infection of the fetus and neonate. 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]. Due to these previous findings, it was theoretically concluded that SARS-CoV-2 would also not be transmissible from mother to fetus through the placenta [5,6]. In fact, first case reports of pregnant women infected with SARS-CoV-2 showed no evidence of intrauterine infection of COVID-19 [7]. However, the increase number of SARS-CoV-2-positive pregnant women allowed data collect of different diagnostic methods performed in different site samples in order to determine the possibility of vertical transmission of SARS-CoV-2 [8]. But the number of studies is still limited which makes it difficult to attest the evidence for the vertical transmission of SARS-CoV-2 [9].

Materials And Methods

Population and Sample

The following report was previously approved by the Ethics Committee on Human Research of Naval Hospital Marcílio Dias (HNMD), Rio de Janeiro, Brazil (CAAE 36066820.8.0000.5256).

All pregnant women admitted to the HNMD for delivery had blood tested for detection of anti-SARS-CoV-2 IgG/IgM antibodies as a screening test for detection of COVID-19 diseased pregnant patients. Serological testing was performed using Leccurate Rapid Test (Lepu Medical), according to the manufacturer recommendations.

Expectant mothers with positive results on at least one antibody and whose delivery would occurred by cesarean section were invited to participate in the research by donating nasopharyngeal swabs of the mother and the newborn as well as the placenta, amniotic fluid and umbilical cord blood samples.

Nasopharyngeal swabs was collected following the Centers for Disease Control and Prevention (CDC) instruction guidelines. RT-PCR for detection of SARS-CoV-2 was performed in the Molecular Biology Laboratory of Biomedical Research Institute, located at HNMD. The protocol used was in accordance to the CDC's Emergency Use Authorization (USA).

Amniotic fluid was sterile collected during cesarean section shortly before the amniotic sac rupture, and was tested for detection for molecular detection of SARS-CoV-2.

Umbilical cord blood was sterile collected with a needle after clamping and cleaning of the cord for both detection of anti-SARS-CoV-2 IgG/IgM antibodies and molecular detection of SARS-CoV-2.

For detection of SARS-CoV-2 in the amniotic fluid and umbilical cord blood samples, we used a *nested* PCR technic. The total RNA was extracted by using a SV-Total RNA kit (PROMEGA) according to the manufacturer recommendations. The reverse-transcriptase first-strand DNA synthesis was performed by 3' primer technique using M-MLV reverse transcriptase (Thermo Fisher Scientific) with two reverse primers (hCOVassay1 R: 5'AGCAGCATCACCGCCATTG 3' and hCOVassay2 R: 5' CCGCCATTGCCAGCCATTG 3'). After the transcription reaction, the product generated was quantified in a NanoDrop fluorometer (Thermo Fisher Scientific). The samples were amplified from the 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). The primers sequences used were hCOVassay1 – F 5'GCCTCTTCTCGTTCTCATCAC 3' / R 5'AGCAGCATCACCGCCATTG 3' and hCOVassay2– F 5'AGCCTCTTCTCGTTCTCATCAC 3' / R 5'CCGCCATTGCCAGCCATTG 3'. After the first reaction of 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 a sample.

Placentas were collected at birth for hystopatological analysis by an experienced placental pathologist.

Results

The following are reported the ocurrence of distinct cenarios regarding the results of the analysis of sorological, molecular and histopathological analysys of assorted samples of the mother and the neonate,

as well as clinical presentation of 3 pregnant women. A summary of the cases can be seen in table 1.

Case 1:

A 26 years-old pregnant woman (second pregnancy, no comorbidities) was admitted to the hospital for elective labor at 40 gestational weeks on 30 June 2020. She reported headache, nausea, myalgia, malaise, joint pain started two days before hospitalization. She underwent cesarean section due to refusal of induction. Serological screening test showed IgM positive and IgG negative results. Nasopharyngeal swab samples was negative for SARS-CoV-2 detection. RT-PCR tests of the nasopharyngeal neonate swab sample was also negative for SARS-CoV-2 detection. Serological test of cord blood was negative for IgG/IgM novel coronavirus antibodies. However, *nested* RT-PCR analysis was able to detect SARS-CoV-2 in the amniotic fluid and umbilical cord blood samples. Histopathological study of the placenta showed moderate circulatory changes suggestive of maternal vascular malperfusion. Area of old infarction and retroplacental haemorrhage was noticed, as well as mild multifocal *delayed villous maturation*. *In the intervillous space was detect mild/moderate fibrin deposition, hemorrhage and calcification foci. Subchorionic thrombosis was present.*

Case 2:

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

Case 3:

A 32-year-old woman (second pregnancy, no comorbidities) was admitted to HNMD at 39⁺² gestational weeks on 10 July 2020. During anamnesis she referred 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 sample was analysed by RT-PCR and the result was also positive for SARS-CoV-2. Cesarean surgery was indicated for the following day, after progression of the delivery was no longer detected. Nasopharyngeal swab sample of the neonate

was 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 on the amniotic fluid and cord blood samples. Placental histopathological analysis demonstrated mild nonspecific vascular changes. *Intervillous space with mild fibrin deposition, hemorrhage and calcification foci. It was observed mild multifocal delayed villous maturation and mild fibrin deposition in the decidua. In the umbilical cord was observed fibrin in one vascular lumen and mild hemopoiesis.*

		Case 1	Case 2	Case 3
Age (years)		26	31	32
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	Assymptomatic	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 <i>nested</i> PCR		positive	positive	positive
Umbilical cord blood <i>nested</i> PCR		positive	positive	positive

Table 1: Clinical presentation (mother) and laboratory tests results (mother and newborn) for SARS-CoV-2 / COVID-19.

Discussion

As a new disease, many questions still need to be clarified about COVID-19 and one of these refers to the possible different methods of transmission, besides the respiratory route.

Later case of clinical manifestation of COVID-19 in a neonate [12] called the attention to the possible vertical transmission of SARS-CoV-2, even in a small percentage of cases compared to the number of

infected mothers.

In order to determine a possible vertical transmission of SARS-CoV-2 from the mother to the fetus during pregnancy, we selected 3 pregnant women with confirmed laboratory diagnosis for COVID-19. It is considered to be vertical transmission not only during pregnancy, with the invasion of virus in placenta through hematogenous route, but also during delivery through transcervical route and postpartum infection through environmental exposure [5,12]. In order to exclude contamination by hematogenous or transcervical routes, we selected three cases of pregnant women whose delivery occurred by cesarean section to ensure that a possible transmission has occurred via transplacental route.

Serological antibody-based test for SARS-CoV-2 was performed as a screening test in the totality of pregnant women admitted to the HNMD for delivery to identify those one who had COVID-19 or was in the course of the disease. Thus, it was identified one case with positive results for both IgM and IgG (case 3), indicating current or recent infection for COVID-19 and two cases of positive IgM and negative IgG (case 1 and 2), indicating current infection [13]. Antibodies seroconversion can occur between less than 1 week and more than 6 weeks after the emergence of symptoms [13]. In fact, obstetric patient case 3 related to had symptoms of headache, runny nose, nausea, myalgia, malaise, joint pain 2 months before the hospitalization date. In one case, serological test was positive for IgM even with no symptoms related by the patient (case 2). This finding corroborates with another study that identified 13.7% asymptomatic obstetric patients through RT-PCR screening test for SARS-CoV-2, pointing out to the importance of carrying out screening diagnostic tests in order to identify infected patients and monitor them more carefully, as well as their infants [14].

Following, RT-PCR tests of the maternal nasopharyngeal swabs identified two positive cases. In case 2, positive result was in the selected pregnant women. Positive results for SARS-CoV-2 was identified in case 2 and 3, indicating the presence of RNA of SARS-CoV-2 virus. Positive RT-PCR test can be observed in the incubation period prior to the beginning of COVID-19 symptoms. (as seen in case 2), (in case 2, for example) and last until the resolution of symptoms (in case 3, for example) [13]. SARS-CoV-2 was not detected in case 1, being compatible with positive IgM serological test and the onset of symptoms two months before testing.

The probability of occurrence of infectious vertical transmission through transplacental route increases with increased gestational age [8] and the results of serological and molecular tests performed in the three cases showed that they were infected in the third trimester of pregnancy. In 5 cases of fetal death, SARS-CoV-2 was detected in the amniotic fluid or placenta and vertical transmission was attested to have occurred during the third trimester of gestation [15].

To verify the presence of SARS-CoV-2 in the neonates of our study, RT-PCR was performed in the nasopharyngeal swabs collected shortly after labor and before contact with their mothers and the results turned out to be negative. Although RT-PCR is considered the gold standard for diagnoses of SARS-CoV-2 infection, diagnostic efficiency of this test in newborns has not been established yet [16]. This can be

explained by the fact that the airways are not functional during intrauterine life and that proliferation of SARS-CoV-2 in the upper respiratory tract seems to be irrelevant for the infection of the fetus [17].

Because the hematogenous route is the most probably mechanism for vertical viral infection [18], serological test of the 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 2 and IgG was detected in the newborn of the case 1, probably of maternal origin that was transferred to the fetus by the placenta. In case 3, positive results for both IgM and IgG in the cord blood immediately after birth is a highly indication that vertical infection occurred, since IgM is a macroglobulin that can not cross the placenta from mother to the fetus [8]. The presence of IgM in the cord blood with negative result for the detection of SARS-CoV-2 in swab samples of the newborn is rare but can be observed [19], which confirms that RT-PCR analysis of nasopharyngeal swab samples may not be a gold standard for diagnosis of COVID-19 in neonates [5].

Detecting of SARS-CoV-2 was also performed in amniotic fluid and umbilical cord blood samples of the 3 cases. A systematic review identified 51 amniotic fluid samples tested but none of them was positive for SARS-CoV-2 RNA [8]. In our study, first RT-PCR was also not able to detect the virus, then *nested* RT-PCR was considered for these types of samples. In fact, CDC guideline attests RT-PCR analysis to be performed solely in upper respiratory swab specimens. We could successfully detect SARS-CoV-2 in all of the amniotic fluid and cord blood samples by *nested* RT-PCR. This

This technique could nested polymerase chain reaction (*nested* PCR) normally used in situations in which it is necessary to increase the sensitivity and/or specificity of PCR [20]. The product of the first amplification reaction (all negative for SARS-CoV-2) was used as the template for the second PCR. Additionally, we used the reverse primers for reverse transcription, generating a specific ssDNA for the region of interest. The use of nested PCR methodology allows to increase the sensitivity of the test up to 100 folds [21], thus allowing a differentiated research potential for viral infection with a low number of copies of initial genetic material.

Regarding the analysis of the placentas, there was no evidence of abnormality that could be observed macroscopically, except the small size of the placenta of case 2. Microscopic analysis revealed maternal and fetal vascular malperfusion of the placentas, corroborating with a systematic review of histopathological lesions observed in third semester placentas of COVID-positive mothers [22]. 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 [22,23]. The observed lesions associated with coagulation in the placentas of this study may be related to any inflammatory disease, and SARS-CoV-2 can not be excluded. Hypertension and preeclampsia can also be associated with vascular thrombotic disease [24], but we can exclude these hypotheses in the three cases presented.

Histopathological analysis of the placentas showed some extension of old infarction and thrombi, lesions associated with coagulation. Vascular thrombotic disease may be associated with hypertension and preeclampsia [24], pathologies not observed in the mothers of this study. Another possibility is an

associated inflammatory disease to be the cause of the vascular lesions, and SARS-Cov-2 can not be excluded. Also, the placentas from the three cases presented a mild fibrin deposit as well as foci of hemorrhages and calcifications in the intervillous space. It is worth mentioning that only in the placenta of case 2 it was noticed a mild focal acute inflammatory infiltrate (mild acute intervillositis), which may be related to a viral infection. Regarding the umbilical cord, in case 2 it was also observed extensive foci of hemorrhage in the jelly and hemopoiesis. It is important to notice that so far, there is no specific pathology characteristic of SARS-CoV-2 that could be observed in the placenta [25].

We are still facing the uncertainties that follows the emergence of a novel virus. As the pandemic spreads around the world, more case reports of COVID-19 accumulate, which allow us to conclude that SARS-CoV-2 is a virus capable of cross the placenta and infect the fetus, even at low rates. We conducted different diagnostic tests that add value information in order to confirm vertical virus transmission via the transplacental route. Also, the results of serological and PCR tests obtained of maternal and neonatal samples indicated that RT-PCR of infant nasopharyngeal swab samples is not an option for detection of SARS-CoV-2 vertical infection. Instead, amniotic fluid or umbilical cord blood are non invasive samples that can be analyzed by serological or virological tests.

So far, there is no standard protocol that states the best sample to collect or diagnostic test to run in order to determine the occurrence of vertical transmission [26]. The results here presented show that a single analysis test may not be enough for a diagnostic conclusion and we strongly suggest to conduct different analysis in order to confirm a possible infection of the neonate.

Conclusion

Combination of maternal serological and molecular results and an alternative molecular technique (*nested* RT-PCR) of fetal samples allowed us to determine the occurrence of 3 cases of vertical transmission of SARS-CoV-2 in pregnant women infected on the third trimester of gestation. Analysis of the test results showed that RT-PCR of nasopharyngeal swab samples of neonate should not be considered to evaluate a transplacental transmission.

Declarations

Author Contributions: Conceptualization, M.A.F. and J.S.S.J.; Data curation, V.M, R.C.G.C. and C.G.S.; Formal analysis, M.A.F., V.M. and D.R.M.; Funding acquisition, D.R.M.; Investigation, M.A.F., J.S.S.J., V.M. and D.R.M.; Methodology, M.A.F., J.S.S.J., V.M. and D.R.M.; Project administration, M.A.F.; Resources, V.M., J.S.S.J., R.G.C.G., C.A., A.R.O.L., H.L.O.C., S.S.A.A., F.V.F.R., G.Z., N.B., J.P.G., J.C.C. and D.R.M.; Supervision, M.A.F.; Visualization, M.A.F.and D.R.M.; writing - original draft preparation, M.A.F. and D.R.M.; writing – review and editing, D.R.M.

Funding: This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Naval Hospital Marcílio Dias (protocol code 36066820.8.0000.5256, september 22 2020)

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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