Full title of manuscript Vertical transmission of COVID-19: SARS-CoV-2 RNA on the fetal side of the placenta in pregnancies with COVID-19 positive mothers and neonates at birth

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Introduction

Vertical transmission of SARS-CoV-2, the virus responsible for COVID-19 infection, is still a controversial issue and studies on placental correlations are still limited. We report our experience with placental SARS-COV2 markers of infection in a series of mothers affected by COVID-19 in the third trimester of pregnancy.

Methods

Patients

All pregnant women diagnosed with COVID-19 infection who delivered at Papa Giovanni XXIII Hospital in Bergamo between March 5, 2020 and April 21, 2020 were included in the study. Maternal and neonatal charts were retrospectively reviewed. Institutional Review Board approved the study and informed consent were obtained from the patients.

Placentas

All the placentas were collected at birth, and sampled and analyzed at Papa Giovanni XXIII Hospital.

Paraffin-embedded formalin-fixed placentas sections were incubated with hematoxylin and eosin (DAKO) and anti-CD68 antibody (mouse origin , Clone KP1, DAKO) that stains macrophages.

Real time RT-PCR

We collected a nasopharyngeal swab (NP) (*FLOQSwab, Copan, Italia*) in UTM (*Universal Transport Medium, Copan, Italia*) respectively from mother and newborn and a sample of placental biopsy that was stored at -80°C in Biobank after treatment with RNA*later*-ICE (*ThermoFisher Scientific*). Subsequently a small piece of placenta (about 3 mm^3) was digested with 50 µl of proteinase K (*QIAGEN, Germany*) and 200 µl of Tris -EDTA buffer solution (Sigma-Aldrich, Germany) for an hour.

Single-molecule RNA in situ hybridization

SARS-CoV-2 (COVID-19) virus has been detected applying the RNAscope® technology (ACD, Advanced Cell Diagnostics), an RNA in situ hybridization technique described previously.[1] Paired double Z oligonucleotide probes were designed against target RNA using custom software. The following probe was used: V-nCoV2019-S, 848568, NC_045512.2, 20 pairs, nt 21631-23303. The RNAscope 2.5 LSX Reagent Kit-Brown IVD Automation (Leica BOND III) was used according to the manufacturer's instructions. FFPE tissue section samples were prepared according to

manufacturer's recommendations. Each sample was quality controlled for RNA integrity with a probe specific to the housekeeping genes UBC (Ubiquitin C) and PPIB (Cyclophilin B). Negative control background staining was evaluated using a probe specific to the bacterial dapB gene. Each punctate dot signal representing a single target RNA molecule could be detected with standard light microscopic analysis.

Results

Between March 5, 2020 and April 21, 2020 twenty-two women affected by COVID-19 infection delivered at Papa Giovanni XXIII Hospital, Bergamo, Italy.

Two of the 22 neonates, born from COVID-19 mothers, resulted positive for PCR of NP swab.

Case 1: The first neonate was vaginally delivered on March 27, after spontaneous labor of a mother with fever, cough and positive COVID-19 NP at 37.6 weeks of gestation. Neonatal weight was 2,660 grams, Apgar scores were 9/10 respectively at 1 and 5 minute, umbilical artery pH was 7.28. The mother wore surgical mask in labor and at the delivery, skin to skin contact wasn't permitted, rooming–in and breast-feeding with mask were allowed. The newborn had positive NP swabs immediately at birth, after 24 hours, and after 7 days; he remained asymptomatic, except for mild initial feeding difficulties and was discharged from the hospital at ten days of life just for observation as this was the first positive neonatal case encountered.

Case 2: The second newborn was delivered by cesarean section at 35.1 weeks from a mother with fever, cough and positive COVID-19 NP swab; the cesarean section was performed for non-reassuring fetal status. The neonate was female, weighted 2686 grams, Apgar scores were 9/10 respectively at 1 and 5 minute, umbilical artery pH was 7.32, and upon birth she was immediately separated from the mother and admitted to the neonatal intensive care unit. Neonatal NP swab was negative at birth and turned positive at day-7 day, with no contact between mother and neonate during that period. No neonatal complication were observed, only some feeding difficulties were reported in the first days of life; she was discharged on day of life 20 mainly due to routine late preterm care.

The placentas of these two women who delivered neonates with SARS-CoV-2 positive NP swabs (cases 1 and 2) showed chronic intervillositis, with presence of macrophages, both in the intervillous and the villous space. The immunohistochemical study demonstrated chronic intervillositis with macrophages CD68 + infiltration (Figure 1a-b and 2a-b).

After the purification of viral RNA from 200 µl of clinical samples, the detection of RdRp, E and N viral genes was obtained by Real time PCR (GeneFinder^{TM} COVID-19 Plus Real*Amp* Kit (Platform

ELITe InGenius®, ELITech Group, France) according to WHO protocol.[2] We performed an ISH (in situ hybridization) with RNAscope technology, a method that enables the detection through the V-nCov2019-S probe the SARS-CoV-2 spike protein mRNA . We tested not only case 1 and 2, i.e. the positive COVID-19 mothers with positive COVID-19 neonates, but also two negative controls: a positive COVID-19 mother with negative COVID-19 neonate (case 3) as well as a negative COVID mother and neonate dyad (case 4). Individual and clustered brown chromogenic dots using a standard bright field microscope were observed in the syncytiocytotrophoblast of both placentas of mothers of positive COVID-19 neonates (Figure 1c and 2c). No evidence of positive dots were seen in the positive COVID-19 mother with negative COVID-19 neonate (Figure 3c; case 3), as well as in case 4. Positive control probes were well expressed in all tissues tested and the negative control probe ensured that there was no background staining related to the assay and that tissue specimens were appropriately prepared. No significant alterations were detected in the other placental histologic examinations of all women COVID 19 positive who delivered infants with negative swabs.

Discussion

The possibility of SARS-CoV-2 vertical transmission is still controversial. Literature reporting evidence of vertical transmission is limited.[3] Two reports described presence of elevated SARS-CoV-2 IgM antibodies in three newborns, but repeated NP samples in the infants were negative.[4] Wang et al[5] reported one case with positive qRT-PCR in both the mother and the neonate. The neonate was delivered by cesarean section, transferred to the neonatology, the baby had no contact with the mother and neonate's NP swab turned out to be positive 36 hours after birth; in this case swabs from placenta were negative, but a possible mother-to-child transmission of SARS-CoV-2 cannot be excluded. Penfield et al. reported the presence of SARS-CoV-2 RNA in 3/11 placental samples from COVID 19 positive women. None of the infants tested positive or demonstrated symptoms.[6]

To our knowledge, ours is the first report of cases of positive PCR for SARS-CoV-2 in mother, neonate and placental tissues. The RNA ISH assay gave us the possibility of direct visualization of the virus, evaluating the molecular target SARS-CoV-2 spike protein mRNA while retaining tissue morphology, a feature that is lost in other methods such as PCR. The RNAscope probe detected positive staining for COVID-19 viral RNA in the infected tissues but not in the uninfected placentas demonstrating the specificity of RNAscope probes. The presence of SARS-CoV-2 RNA in the syncytiothrophoblast signifies presence of the virus on the fetal side.

Conclusions

This is the first study describing SARS-CoV-2 RNA on the fetal side of the placenta in two cases of mothers infected with COVID-19 and with neonates also positive for the virus at birth. These findings support the possibility of vertical transmission of SARS-CoV-2, the virus responsible for COVID-19 infection, from the mother to the baby in utero.

Moreover, the direct visualization of SARS-CoV-2 RNA in the infected placentas raise the possibility of estimating the viral load in cells with morphological context. Further studies are required to confirm our results.

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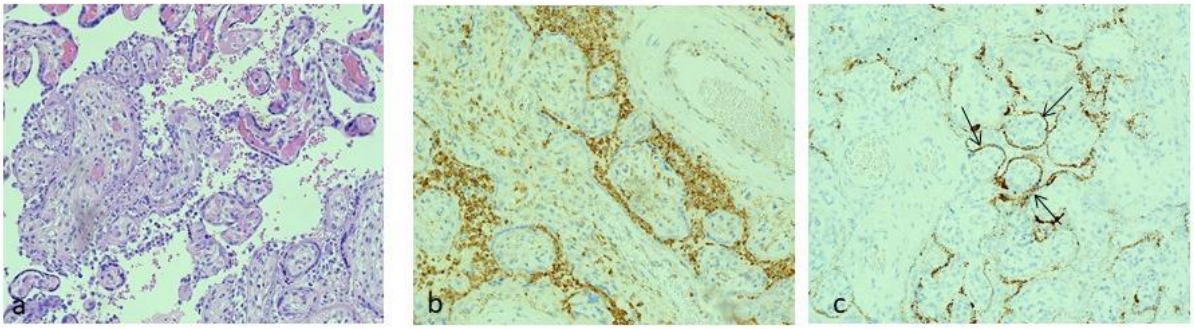

Figure 1. Case 1: Covid positive mother and neonate, with SARS-CoV-2 antigen seen in villous syncytiotrophoblasts, i.e. fetal side of the placenta.

A. Chorionic villi showing chronic intervillositis with macrophages. Paraffin-embedded formalin-fixed placenta sections at standard brightfield microscope 20X ; Tissue sections stained with hematoxylin and eosin (H&E, 20x).

B. Macrophages in intervillous spaces highlighted by anti-CD68 immunohistochemistry.

C. In-situ hybridisation for SARS-CoV-2 highlighting the presence of SARS-CoV-2 spike antigen in villous syncytiotrophoblasts. Black arrows show brown dots positive signals of COVID-19 inside syncytiotrophoblast of chorionic villi cross section.

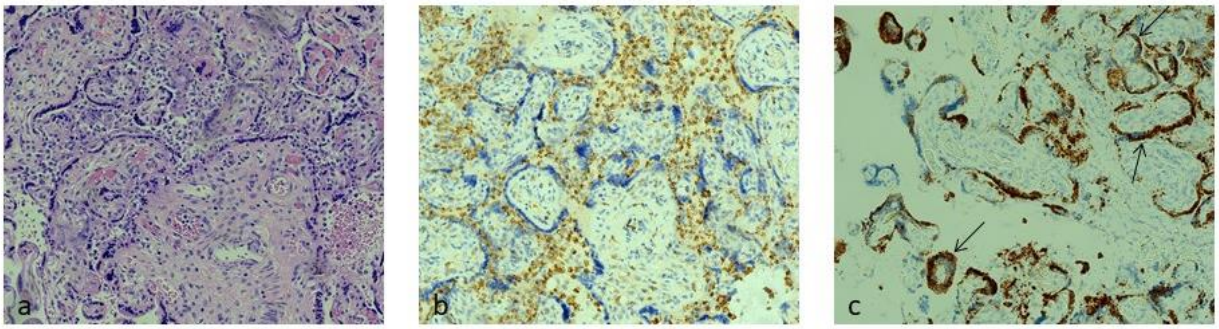

Figure 2: Case 2: Covid positive mother and neonate. Covid positive mother and neonate, with SARS-CoV-2 antigen seen in villous syncytiotrophoblasts, i.e. fetal side of the placenta.

A. Chorionic villi showing chronic intervillositis with macrophages. Paraffin-embedded formalin-fixed placenta sections at standard brightfield microscope 20X; Tissue sections stained with hematoxylin and eosin (H&E, 20x).

B. Macrophages in intervillous spaces highlighted by anti-CD68 immunohistochemistry.

C. In-situ hybridisation for SARS-CoV-2 highlighting the presence of SARS-CoV-2 spike antigen in villous syncytiotrophoblasts. Black arrows show brown dots positive signals of COVID-19 inside syncytiotrophoblast of chorionic villi cross section.

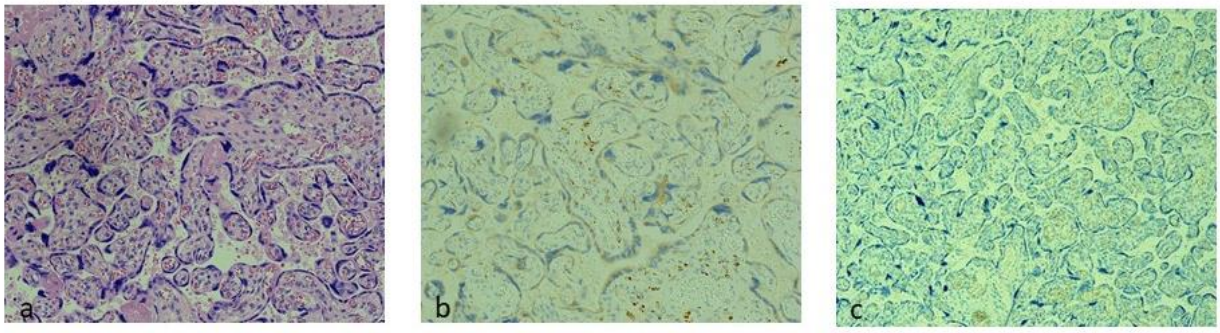

Figure 3: Case 3: COVID positive mother but COVID negative neonate, i.e. control placenta with virus not visualized.

A. Normal chorionic villi. Paraffin-embedded formalin-fixed placenta sections at standard brightfield microscope 20X ; Tissue sections stained with hematoxylin and eosin (H&E, 20x)

B. Normal placental tissues incubated with anti-CD68 antibody.

C. In-situ hybridisation for SARS-CoV-2 with absence of SARS-CoV-2 spike antigen in villous syncytiotrophoblasts.