Risk factors associated with vertical transmission of CMV in Southern Brazil: a cross-sectional study based on the molecular prevalence of the virus in placental tissue and umbilical blood cord

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Abstract

Objective: This study estimated CMV molecular prevalence in placental biopsies as well as in umbilical blood cord and its correlation with infection of pregnant women and their newborns to determinate vertical transmission risks. **Design:** A cross-sectional study **Setting:** Obstetric Center of the University Hospital in Rio Grande, Brazil **Population:** 496 pregnant women and their newborns **Methods**: Biopsies were collected from peripheral and central portions of each placenta, separated on fetal and maternal interfaces, matched with cord blood, totaling 1488 samples. PCR technique and sequencing were used to investigate the prevalence. **Main Outcome Measures:** Bivariate and multivariate analysis were performed to determinate sociodemographic, clinical and gynecological data associated to CMV vertical transmission. **Results:** CMV DNA was found in 5.2% of placental maternal interface and 5.4% of fetal interface with a positive result for CMV in 3.6% in cord blood. In more than 90% of the cases, there was no match between positive CMV DNA cord blood and positive placentas, indicating vertical transmission ascending from genital tract. The income factor (less than 1 minimum wage) was significantly associated with prevalence of CMV in placentas ($p = 0.03$). In cord blood samples, non-white skin color and early age at the onset of sexual intercourse were risk factors associated with the infection ($p = 0.04$). **Conclusions:** The occurrence of CMV DNA found in cord blood suggests the pattern observed appears to be ascending from genital tract of asymptomatic mothers. Economical and environmental factors present a negative impact on fetal-maternal transmission of cytomegalovirus.

Introduction

The placenta is a transient organ and although it is a physical barrier between maternal blood and fetal circulation, several low molecular weight substances are carried or passively cross it ensuring fetal growth and development. Viral infection in the placenta indicates a great risk of vertical transmission, which is dependent on specific maternal immune response and specific viral receptor expression in placenta or decidual cells and might result in pregnancy complications such as preterm birth, intrauterine growth restriction and miscarriage[1,2,3].

In this scenario, Human Cytomegalovirus (CMV), a member of*Herpesviridae* family, has ability to remain, to cross the placenta and infect the fetus, like only few pathogens are able to do. This virus is a major cause of congenital infections in humans and maternal infection may result in symptomatic disease or asymptomatic infection in the neonate. CMV may be present in both the maternal interface and in the villi which belong to the fetal tissue, indicating that it may persist in the placenta independent of fetal infection[1, 4]. The

placenta is a reservoir for CMV and plays an important role in vertical transmission[4]. Cervical reactivation and shedding are important routes for prenatal transmission[5,6].

The virus can be transmitted to the fetus during primary maternal infection, causing malformation and other severe symptoms and, to a lesser extent in latent reactivation or re-infection by different strains, problems in fetal development and sensorineural hearingloss. Cell permittivity is due to CMV interaction with heparin surface cell receptors expressed in many cell types, including fibroblastos, trophoblasts, macrophages and monocytes, epitelial cells, endothelial cells and muscle cells [1,7]. Maternal immunity function influences viral infection but the route of CMV viral transmission includes various permissive cell types present in the placenta[6-8].

The present cross-sectional study estimated CMV molecular prevalence in placenta biopsies and determined CMV DNA prevalence in the placenta and cord blood, also correlating variables and suggesting possible parameters that can assemble a more elaborate picture about the expected outcomes of a pregnancy under this condition.

Methods

Study design and Ethical Approval

Sampling was composed by 496 women attended at the Obstetric Center of the University Hospital Dr. Miguel Riet Corrêa Jr. in the city of Rio Grande, Southern Brazil, between . All subjects (parturients) provided a written informed consent for participation in the study. Sociodemographic, obstetrical and gynecological data were obtained by applying a self reported questionnaire. The present work was approved by the Research Ethics Committee at the Health Area (CEPAS) of the Federal University of Rio Grande-FURG (protocol number 54/2011), RS-Brazil.

Patients and specimens

Biopsies of peripheral and central portions were collected from 496 placentas between March 2011 and March 2014, immediately after each woman delivered the newborn, and subsequently separated on fetal and maternal interfaces. The collection of placental tissue was performed using different sterile surgical materials for each face of the placenta and the collected fragments were placed into microtubes containing 300µL of TE buffer (10 mmol/L Tris-HCL pH 8,0; 1mmol/L EDTA).

The collection of umbilical cord blood for molecular assays was performed after cord clamping. Blood from the umbilical cord was collected using a syringe (27/5 needle) by puncturing 3ml of blood.

Placental DNA extraction

DNA extraction from placental tissue was performed according to an adapted protocol[9] from Purelink Genomic DNA commercial kit (Life Technologies, Carlsbad, CA). DNA quality was estimated by PCR of human CCR2 and HLAG genes. PCR products were assessed by electrophoresis on 1.5% agarose gels stained with Blue Green Loading Dye (LGC Biotecnologia®, São Paulo, Brazil) and then visualized by UV light.

Cord Blood DNA extraction

DNA extraction from the cord blood samples was performed using the Mammalian Extraction DNA kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Enzimes Proteinase k and RNAse A were added to 200 µl of sample and briefily vortexed. Protein digestion was realized by adding Purelink Genomic Lysis Binding Buffer and then the mix was vortexed and incubated at 55°C for 10 minutes. Ethanol was added and mix was vortexed for 5 seconds. Supernatant was column filtered and Wash Buffer 1 was added and centrifuged for 1 min at 10.000 rpm. The latter step was repeated once added by Wash Buffer 2. Thefiltrate was discharged and centrifugation repeated. Elution Buffer at a volume of 100 µl was added to the filter and centrifuged for 2 minutes at 15.000 rpm.

The DNA product was maintained at -4 ° C, at the DNA Bank from Molecular Biology Laboratory . After extraction, electrophoresis in 0.8% agarose gel stained with Blue Green was performed and DNA

fragments were visualized by UV light through the transilluminator, to ensure the method was successfully accomplished.

CMV Polimerase Chain Reaction

CMV DNA in the samples was detected by PCR method, using an adaptated protocol from Kumazaki and colleagues (2002)[4]. Two different CMV regions were amplified: part of the early gene (IE) and the phosphoprotein 71 gene (pp71)[4]. Standard precautions, such as negative controls (using water as a template) and two positive controls, were taken in order to avoid sample contamination. PCR reaction was composed by the following reagents: by 5 µL of DNA template, 1.5 mM MgCl 2, 0.5 mM primers, 0.2 mM dNTP, 1x Buffer, 1 U Taq DNA polymerase enzyme and milique water. PCR assays were performed in a thermocycler with the following cycling conditions: initial denaturation step at 94 ° C for 4 minutes, followed by 35 cycles under the following conditions: 94 ° C for 30 seconds, 64 ° C for 60 seconds, 72 ° C for 60 seconds and 72 ° C for 5 minutes, followed by final extension at 10 ° C.

Positive samples were repeated at a new reaction with positive and negative controls for confirmation. PCR products were assessed by electrophoresis in a 2% agarose gel stained with Ethidium Bromide and verified by UV illumination. The positive cord blood samples were sequenced using an ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems(r), Foster City, CA, USA) in an automated ABI 3130XL analyzer (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Clinical data, gynecological, laboratory and demographic variables of each participant were collected from their medical records. Independent variables associated to risk factors were: age, educational attainment, skin color, marital status, income, age at onset of sexual intercourse, number and previous abortion history. Data were analyzed by SPSS Version 12.0. The Chi-square and Poisson regression was also performed. Significant differences were considered when P values were lower than 0.05.

Results

CMV DNA prevalence in placenta tissue and sociodemographic risk factors

Placenta biopsies from 496 women were analyzed and divided into maternal and fetal interfaces. The molecular prevalence of CMV in maternal interface of placentas was 5.2% and in fetal interface was 5.4%. Parturients had an average of 25 years (SD +/- 6 years). Among these, 42.6% were in the first pregnancy and 19% had previous abortion.

Table I.

Some sociodemographic factors have shown a tendency in bivariate analysis: women who studied less than 9 years and nonwhite skin color were factors associated with the molecular presence of the virus in placentas. The income factor (shown as minimum wage) was significantly associated with the CMV molecular prevalence in placentas. Obstetrical and gynecology variables were not associated with the outcome (data not shown).

Table II shows Poisson multivariate analysis of the previously mentioned factors. Again, the prevalence of CMV in maternal interface of placentas was associated with an income lower than 1 minimum wage ($p = 0.03$).

Table II.

CMV DNA prevalence in umbilical cord blood and risk factors involved in the transmission

Molecular analysis of cord blood samples was performed. A total of 496 cord blood samples were analyzed, and the molecular prevalence of CMV DNA was 3.6%. Except for a single case, all the remaining positive cord blood samples were not associated with CMV DNA positive placentas after analysis by PCR reaction. Table III demonstrates the bivariate and multivariate analysis for the evaluated risk factors.

Table III.

Risk factors such as skin color, income, previous abortion history and age onset of sexual intercourse, which were apparently associated with cord blood infection in bivariate analysis were also submitted to multivariate analysis aiming to indentify independent factors. White skin color (self reported) and age at onset of sexual intercourse higher than sixteen years old remained as factors providing protection against CMV vertical transmission as shown in Table IV (p=0.04).

Table IV.

Main clinical characteristics of CMV DNA positive umbilical cord blood subjects (mother and newborn)

Eighteen newborns were positive for CMV in the cord blood, strogly suggesting the virus vertical transmission. Data were obtained from 16 medical records available which considered data concerning the mother and clinical aspects of newborns including birth relevant information. The main characteristics are shown in Table V.

Table V.

Discussion

Main findings

CMV DNA was found in 5.2% of maternal and 5.4% of fetal interface. Many studies have reported that the presence of CMV in the amniotic fluid and placenta may be transient, but it is known that various cell types present in placenta have receptors for the virus and, therefore, are permissive to the infection [1, 2,10]. Avanzi et al. (2013) demonstrated the total cellular permittivity for CMV infection using *in vitro* cultures of placental mesenchymal cells, raising evidence that the placenta is an important fetus contamination route by the virus [11], and this correlation was previously reported by other authors[1,12-15]. Kumazaki and co-workers (2002) investigated biopsies from placental parenchyma and membrane and were able to determine by PCR that CMV DNA may be present on both maternal interface and villi, indicating that the virus may persist in placental tissue independent of fetal infection[4]. The CMV prevalence is significant according to studies that investigated CMV DNA presence by PCR in placentas associated with fetal death, low birth weight and fetal hydrops, resulting in a prevalence of 4% to 27.4% [2, 14 - 18]. Previously conflicting CMV prevalence data have been currently observed along the last decades [4, 14 - 17].

Considering the high antibody prevalence in Brazilian population, with 90 to 95% women in fertile age, it was expected that a reactivation of infection would indeed occur induced by virus replication and permanence in placental tissue with or without infected cord blood, suggesting that placental infection is more common than neonatal disease[14, 17]. Theiler and co-workers (2006) have found the presence CMV DNA in the cord blood from two out of 433 neonates by PCR assay, and one of those was asymptomatic for the disease. Both were newborns from mothers presenting a profile of viral reactivation (IgM negative/IgG positive) [22]. Another study compared samples from 983 women resulting in a risk profile of 9% (maternal IgM seropositive) and CMV DNA prevalence of 0.4% in cord blood [23]. More recently, Uematsu and colleagues (2016) investigated suspicious congenital asymptomatic CMV cases in 182 patients presenting neurological symptoms, 32,4"% (54) were positive for the infection at different levels of severity, demonstrating that the infection effects may be latter observed, once that at the moment of birth the newborns were not diagnosed [23]. Albano and co-workers (2017) reported CMV DNA prevalence in cord blood of donors and revealed 59 congenital positive infection asymptomatic cases from which only three mothers were diagnosed with CMV during gestation period[25].

In this study, low weight and prematurity occurred in some newborns. Was evident that some of the women presented infections during gestation period, such as tuberculosis or urinary infection, and even there was a case of HIV seropositive. The correlation between polimicrobiane infections during gestation and maternal response modulation was previously described, therefore demonstrating the course of infection and closure of such situation[26]. There is the possibility of CMV reactivation at any site once it is a classic opportunist agent [21].

Strengths and limitations

Regarding the limitations, in this study, 19% of parturients reported previous spontaneous abortion, 19% of these were CMV positive in the placenta. In almost 50% of parturients CMV positive, virus was detected in cord blood had abortion history. Although there was statistical association between CMV and abortion history in bivariate analysis, it was not possible to correlate no previous episodes of poor obstetric history as a protection factor to vertical transmission or CMV presence in the last pregnancy. However, this information may be relevant in the investigation of patients at risk of vertical transmission. This result might be caused as a consequence of the sample size in the studied population.

Interpretation

Ascendant transmission from genital tract appears to be an important transmission route[1]. According to Rocha and colleagues (2012), after detection CMV in cervical samples of asymptomatic women at reproductive age, this could be a great method of pregnant women accompaniment that may reactivate the infection or have primo-infection under asymptomatic way[27]. Thus, it could be suggested that this might be the transmission pathway, once in the present study the placenta is apparently involved only in a single case. One-third of parturients with intrauterine infected newborns did not have a fixed partner, and besides the viral reactivation hypothesis due to a concomitant infection[24], it might have happened reinfection by other CMV subtypes during the gestation period caused by sexual intercourse exposure.

Women at risk of transmitting CMV to the fetus should perform noninvasive tests, such as ultrasonographic monitoring of the fetus (that is able to detect malformations and signs of fetal infection) and even invasive tests such as cordocentesis and amniocentesis[2,16]. Among those neonates presenting the virus in cord blood only one presented ears malformation and micrognatia, besides prematurity and low weight at birth. Concerning this specific case, as observed in at least three more cases, mother realized no accompaniment examns during gestation period. Therefore, it is not possible to affirm for sure that the virus prevalence and this characteristic are correlated, once the mother was carrying other conditions that might have caused the proper scenario for this closure. However, this possibility should be strongly considered.

Relevant socio-economic factors such as malnutrition, inappropriate health care services and bad hygiene contribute to maternal-fetal infection increase in developing coutries[17]. Concerning the present study, the sociodemographic factors such as skin color and schooling have demonstrated a tendency in mother infection by CMV. Besides, the exposure time to the virus might be important, principally in reactivation cases. Women's early onset of sexual intercourse, before 15 years old, was a risk factor to the virus presence in the umbilical cord blood. Therefore, the exposure certainly occurs and women carrying CMV can probably go under reactivation and transmit the virus to fetus. Simultaneously to the income factor, which was statistically significant, these data indicate that the lack of knowledge concerning the disease and preventive measures, as well as lack of sanitary conditions, commonly observed in poor populations, are strongly connected in this scenario.

Conclusion

The present work demonstrated the presence of CMV DNA in placental tissue from asymptomatic parturients, evincing permittivity of this tissue to the virus. Also, CMV presence was investigated in the umbilical cord blood, which is responsible for nourishing the fetus along development, accusing a possible vertical transmission of the pathogen. There was no involvement of the placenta in more than 90% of the evaluated cases, and transmission apparently occurred by an ascendant pathway of the genital tract, once the virus can be released this way. This result reinforces that the placental barrier is efficient against viral infection, even when the virus present the ability to replicate in this site.

Considering the presented research scenario of a university hospital attending people under vulnerable social conditions at Southern Brazil, this study showed that familiar income lower than a minimum wage was a risk factor concerning the virus presence in the mother, consequently resulting in risk of transmission to the newborn. Skin color, was an independent risk factor, once mothers who were not self declared white

presented at least 3 times more chances of CMV transmission to fetus during gestation. Classic economical and environmental factors from developing countries present a negative impact on maternal-fetal transmission of CMV, despite no consensus was still found concerning the tracking of the infection.

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Disclosure of interest

The authors have no conflicts of interest to disclose.

Contribution to authorship

AMMB and ECA, conceived the study. All authors planned the study. AMMB, MAS, VPH and CGV obtained the funding for the study. ECA, FF-J, TM, MJ, CR and RCL collected and processed the samples. ECA and FF-J analyzed the data and ECA and RCL drafted the manuscript. AMMB and ECA contributed to data interpretation. All authors reviewed the manuscript, and gave input at all stages of the study. All authors have approved the final version of the manuscript for submission.

Details of ethics approval

This study was approved by the Research Ethics Committee at the Health Area (CEPAS) of the Federal University of Rio Grande (FURG) (CEPAS Ndeg 54/2011). All participants (or their legal guardians when appropriate) provided written informed consent for participating in the study

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