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Herpes Simplex Virus: Prevalence in Placental Tissue and Incidence in Neonatal Cord Blood Samples

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Herpes simplex virus (HSV) infection is one of the most prevalent infectious diseases worldwide, with HSV-2 being primarily associated with genital infections. HSV-2 is believed to account for the majority of cases of neonatal herpes, which may cause diverse of complications in infected newborns. The present study sought to estimate the prevalence of HSV-2 in placental tissue samples and the incidence of HSV-2 in the umbilical cord blood of newborn infants. Placental tissue samples from 201 women (maternal-side and fetal-side = 402 specimens) and 184 neonatal cord blood samples, all collected at the obstetric ward of a University hospital were studied. HSV-2 was detected by means of nested PCR. The prevalence of HSV-2 in placental samples was 9.0% (n = 18), and the incidence of neonatal HSV-2 infection was 1.1% (n = 2). All HSV-2-positive patients were asymptomatic at the time of delivery and none reported genital herpes. Women with a time between rupture of membranes and delivery of ≥360 min had an approximately fourfold risk of HSV-2 infection in the placental tissue (95% CI 0.93–5.66, $P = 0.01$). These results suggest that HSV-2 is present in the placenta of asymptomatic women and that a risk of transmission to the neonate exists. New strategies must be implemented for the management of asymptomatic patients who are capable of transmitting the virus to the newborn. ***J. Med. Virol. 86:519–524, 2014***.

KEY WORDS: polymerase chain reaction (PCR); neonatal herpes; HSV-2; placenta

INTRODUCTION

Herpes simplex virus (HSV), the causative agent of oral and genital herpes, is classified into two serotypes, HSV-1 and HSV-2 [Nahmias and Dowdle, 1968]. HSV-2 is the most common cause of genital HSV infection [Fleming et al., 1997; Smith and Robinson, 2002; Xu et al., 2006; Straface et al., 2012] and it is believed to be the leading cause of neonatal herpes [O'Riordan et al., 2006; Whitley et al., 2007; Straface et al., 2012].

Transmission of HSV-2 to the newborn can occur by transplacental hematogenous spread, during delivery or in the postnatal period [Kimberlin et al., 2001a,b; Lamounier et al., 2004], even despite suppressive antiviral therapy [Pinninti et al., 2012]. This transmission can cause ocular and cutaneous lesions, meningoencephalitis, fetal malformations [Kimberlin, 2007; Straface et al., 2012], or disseminated infection [O'Riordan et al., 2006; Kimberlin, 2007; Arai et al., 2012; Straface et al., 2012]. Both primary and recurrent maternal infections may cause congenital disease, although the risk is lower in the lattter [Straface et al., 2012; Jaiyeoba et al., 2012].

In studies carried out in the U.S. [McDonagh et al., 2004; Satosar et al., 2004] and Greece [Syridou et al., 2008] designed to quantify the occurrence of HSV in placental tissue samples, prevalence rates

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from 2.6% to 9.0% have been reported. In another U.-S. study that tested several types of specimens (including cord blood samples) collected from 303 pregnant women at high risk of viral infection, HSV was detected in nine samples (3.0%) [Van den Veyver et al., 1998]. Al-Buhtori et al. [2011] investigated the occurrence of viral infection in samples of placenta and other tissues from 73 cases of fetal death in England and Australia, with 6.8% being positive for HSV.

There are little data on neonatal herpes in developing countries such as Brazil [WHO, 2011]. The highest incidence of HSV infection is found among women of reproductive age [Kriebs, 2008], and the high rate of HSV-2 infection in this population also accounts for an increased risk of neonatal herpes [Clemens and Farhat, 2010], making HSV a public health concern [Cusini and Ghislanzoni, 2001; Clemens and Farhat, 2010]. In this scenario, the prevalence of HSV-2 in placental samples from parturient women and the incidence of HSV-2 in neonatal cord blood in a developing country were estimated in the present study, thus contributing to the state of knowledge on occurrence of HSV-2 infections, a key aspect of prevention and treatment.

MATERIALS AND METHODS

Ethical Aspects

The study was approved by the Universidade Federal do Rio Grande (FURG) Health Research Ethics Committee (CEPAS No. 54/2011). All participants (or their legal guardians when appropriate) provided written informed consent.

Study Design and Patients

This was a cross-sectional study designed to assess the prevalence of HSV-2 in placental samples from parturient women and the incidence of HSV-2 in cord blood samples from their newborns. Specimens were collected from September 2011 through September 2012, using a convenience sampling strategy. Sample size was calculated in the Epi-Info software environment, on the basis of a presumed HSV-2 prevalence of 3.3–9.0% [McDonagh et al., 2004; Syridou et al., 2008; Al-Buhtori et al., 2011], with a 95% confidence interval. The sample consisted of 201 women seen at the obstetric ward of Hospital Universitário Dr. Miguel Riet Corrêa Jr. (HU/FURG), a University hospital in Rio Grande, RS, Brazil. Only 184 neonatal blood samples were processed, as the volume of blood collected was insufficient for testing in 17 cases. Clinical examination was performed in all women when they were admitted to the obstetric ward. Review of medical charts of mothers and neonates were also conducted to seek information on patients' clinical characteristics. All participants completed a self-report questionnaire for data collection on sociodemographic, obstetrical, and gynecological factors.

Specimens

Overall, 586 specimens were collected: 201 of maternal-side placental tissues (decidua); 201 of fetal-side placental tissues; and 184 cord blood samples. Placental samples were obtained by means of biopsy of the placental disk (maternal side and fetal side). Biopsy fragments were placed individually into microcentrifuge tubes containing 300 μl of TE buffer (Tris–HCl 10 mM pH 8.0; EDTA 1 mM) [Rombaldi et al., 2008], further used for DNA extraction. After umbilical cord clamping and complete delivery of the placenta and fetal membranes, cord blood (approximately 20 ml) was collected into EDTA-containing tubes [Rombaldi et al., 2008] and stored at 4°C until DNA extraction.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from placental tissue specimens with the Purelink Genomic DNA kit (Life Technologies, Carlsbad, CA) according to the manufacturer's specifications. DNA was extracted from cord blood samples using the GFX PCR DNA and Genomic Blood Purification Kit (GE Healthcare, Little Chalfont, UK), in accordance with the manufacturer protocol for DNA extraction from blood.

For detection of HSV-2 in placental tissue and blood samples, PCR were performed with two primer pairs in nested assays, using an adapted version of the protocol described by Aurelius et al. [1993, 1993] and Schmutzhard et al. [2004]. The following primers were used for amplification of an HSV-2 *gpG* gene fragment: HSV21F 5′-TCAGCCCATCCTCCTTCGGCAGTA-3′ and HSV21R 5′-GATCTGGTACTCGAATGTCTCCG-3′ (first round); and HSV22F 5′-AGACGTGCGGGTCGTACACG-3′ and HSV22R 5′CGCGCGGTCCCAGATCGGCA3′ (second round), yielding a DNA fragment of 100 bp. Consecutive PCR were performed with 10 μl of extracted DNA and 10 μl of the first-round product, respectively, 1× PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTP, 1 U of recombinant *Taq* DNA polymerase (Life Technologies, São Paulo, Brazil), 0.5 μM of primers HSV21F and HSV21R or HSV22F and HSV22R, and H_2O Mili-Q to a final volume of 50 μl. Cycling conditions were the same for the first and second rounds of amplification: 94°C/5 min (1 cycle); 94°C/30 sec, 57°C/30 sec, 72°C/1 min (30 cycles); and 72°C/7 min (1 cycle). Second-round PCR products were visualized under UV after electrophoresis in a 2.5% agarose gel and staining with ethidium bromide.

Direct DNA sequencing was performed to confirm HSV-2 positivity for each case. The resulting sequences were compared with reference sequences deposited in the GenBank database, based on BLAST searches.

Statistical Analysis

Data on sociodemographic, gynecological, clinical, and laboratory variables were analyzed. The chi-square test was used for comparing categorical

variables. Prevalence ratios, potential risk factors and protective factors were calculated, and frequency distributions and percentages were determined. Differences were considered statistically significant when $P < 0.05$. Multivariate analysis with Poisson regression was also performed, followed by construction of a hierarchical linear model, which incorporated variables with $P \leq 0.20$ on crude analysis. The first level comprised demographic and socioeconomic variables, whereas the second included variables that constituted risk factors for HSV-2 infection. All analyses were carried out in SPSS for Windows v.12 and Epi-info v.3.5.2.

RESULTS

Maternal and fetal samples were obtained from 201 women. The prevalence of HSV-2 in the maternal-side placental tissue samples was 9.0% (n = 18). Seven of these (3.5%) were also positive on the fetal side. As shown in Table I, the only sociodemographic variable significantly associated with HSV-2 infection was household income. Women whose income was equivalent to two Brazilian minimum wages had 81.0% protection against acquisition of HSV-2 infection (95% CI 0.04–0.84, $P = 0.04$).

With respect to gynecological history, 90% of participants (n = 181) reported no history of sexually transmitted diseases. However, 15 of those were positive for HSV-2. There were no significant associations between the presence of HSV-2 and gynecological variables. Regarding obstetric variables, the only significant association with HSV-2 infection was time between rupture of membranes and delivery: women with a time of $\geq$360 min were at an approximately fourfold higher risk of HSV-2 positivity in placental tissues (95% CI 0.93–5.66, $P = 0.01$; Table I).

The overall incidence of HSV-2 DNA in cord blood samples was 1.1% (2/184); in one case, both placental tissue samples (maternal and fetal) were negative for HSV-2. Four infants were stillborn, but both maternal and fetal sides of the placenta were negative for HSV-2 in all those cases.

The women participating in the study did not present with any characteristic lesion of genital herpes at the time of admission to the obstetric ward. No HSV-2-positive patient was symptomatic at delivery. Both cases of HSV-2-positive newborns were primiparous, and their mothers had no history of pregnancy loss, but had hypertensive disorders of pregnancy. The review of the medical records showed that neither child had presented clinical changes suggestive of herpes upon physical examination at the delivery room and at the sixth hour after birth. Both newborns also had normal neurological examination and the 5-min Apgar score was 9. However, one patient presented toxic erythema on the face and lower limbs on the fourth day after birth, but as no other symptoms were reported, and no specific treatment was provided.

In the multivariate analysis performed (Table II), the following variables were identified as independent factors providing protection against HSV-2 infection: income equivalent to two minimum Brazilian wages (95% CI 0.01–0.66, $P = 0.02$) and time between rupture of membranes and delivery <360 min (95% CI 0.07–0.86, $P = 0.02$). The remaining variables included in the model had no significant effect.

DISCUSSION

In the present study, the prevalence of HSV-2 in the maternal-side placental tissue (decidua) samples was 9.0% (n = 18), with seven also positive on the fetal side (3.5%). This prevalence was similar to that reported by McDonagh et al. [2004], who investigated the incidence of pathogenic bacteria and concurrent infections with human cytomegalovirus (CMV) and HSV type 1 and 2 in placental tissue biopsy samples. McDonagh et al. [2004] also reported that HSV infection was more focal, usually found in the maternal side of the placenta (decidua), similar to the findings of the present study. However, on comparison with other studies which have reported prevalence rates of 2.6–6.8% [Satosar et al., 2004; Syridou et al., 2008; Al-Buhtori et al., 2011], the prevalence in the current study was higher.

The overall incidence of HSV-2 in cord blood was 1.1%; in one of the two cases. HSV-2 was not found in the placental specimens (both maternal and fetal). During pregnancy, the virus may ascend from the lower genital tract [Hain et al., 1980; Jeffries, 1991; Nigro et al., 2011] and cause neonatal infection, which could explain the detection of HSV-2 in the cord blood sample despite a negative placental biopsy found in this study. In the other case, both mother and neonate tested positive for HSV-2, confirming transplacental transmission. Hoppen et al. [2001] also found HSV-2 in a cord blood sample from a neonate with herpes infection diagnosed in utero, although the presumed route of transmission was not described.

Women whose income was equivalent to two Brazilian minimum wages had 81% protection against HSV-2 infection (95% CI 0.04–0.84, $P = 0.04$) as compared with those whose earnings were equivalent to three or more minimum wages. This finding held true after multivariate analysis ($P = 0.02$) and conflicts with the results of previous studies, which suggest that infection is associated with lower household income [Fleming et al., 1997; Xu et al., 2006]. In a study conducted by Clemens and Farhat [2010], participants whose income was equivalent to three or more minimum wages exhibited a higher prevalence of HSV-2 positivity (13.5%) as compared with their lower-income counterparts (11.2%), which corroborates the present research findings.

Time between rupture of membranes and delivery was the only obstetric factor significantly associated

TABLE I. Sociodemographic, Obstetrical and Gynecological Profile of Parturient Women Analyzed in the Study, Stratified by HSV-2 Positivity in Placental Tissue Samples

Variable/Category	n (%)[d]	HSV-2$^+$, n (%)[d]	Prevalence ratio	95%CI	p[a]
Age (years)					
31–40	40 (19.9)	4 (22.2)	1.0		0.45
21–30	100 (49.8)	11 (61.1)	1.10	0.37–3.25	
≤20	58 (28.9)	3 (16.7)	0.52	0.12–2.19	
Educational attainment (years)					
≥9	104 (51.7)	10 (55.6)	1.0		0.92
≤8	87 (43.3)	8 (44.4)	0.96	0.39–0.32	
Marital status					
Stable partner	144 (71.6)	13 (72.2)	1.0		0.90
No stable partner	52 (25.9)	5 (27.8)	1.07	0.40–2.84	
Income (in minimum wages)[b]					
≥3	58 (28.9)	9 (52.9)	1.0		**0.04**
2	68 (33.8)	2 (11.8)	0.19	0.04–0.84	
≤1	56 (27.9)	6 (35.3)	0.69	0.26–1.81	
Skin color (self-reported)					
White	124 (61.7)	10 (55.6)	1.0		0.68
Non-white	79 (34.8)	8 (44.4)	1.42	0.59–3.42	
Age at onset of sexual intercourse (years)					
≥16	96 (47.8)	12 (66.7)	1.0		0.14
≤15	95 (47.3)	6 (33.3)	0.51	0.20–1.29	
Number of lifetime partners					
1	56 (27.9)	4 (22.2)	1.0		0.50
2–4	88 (43.8)	8 (44.4)	1.27	0.40–4.03	
≥5	43 (21.4)	6 (33.3)	1.95	0.59–6.49	
Number of sexual partners in the 6 months preceding study					
0	4 (2.0)	1 (6.3)	1.0		0.23
1	185 (92.0)	15 (93.8)	0.32	0.06–1.90	
Condom use					
Yes	82 (40.8)	5 (27.8)	1.0		0.19
No	112 (55.7)	13 (72.2)	1.90	0.71–5.13	
Contraception					
Condom + other methods	55 (27.4)	4 (22.2)	1.0		0.64
Hormonal contraceptives	128 (63.7)	12 (66.7)	1.29	0.43–3.82	
Other methods	4 (2.0)	1 (5.6)	3.44	0.49–23.97	
None	7 (3.5)	1 (5.6)	1.96	0.25–15.19	
Comorbid STDs					
No	188 (93.5)	15 (88.2)	1.0		0.38
Yes	13 (6.5)	2 (11.8)	1.86	0.47–7.26	
Awareness of herpes					
Aware	23 (11.4)	3 (16.7)	1.0		0.80
Somewhat aware	53 (26.4)	5 (27.8)	0.72	0.19–2.78	
Unaware	116 (57.7)	10 (55.6)	0.66	0.20–2.22	
Gravidity					
First pregnancy	80 (39.8)	7 (41.2)	1.0		0.07
Second pregnancy	49 (24.4)	1 (5.9)	0.23	0.03–1.84	
≥3 pregnancies	63 (31.3)	9 (52.9)	1.63	0.64–4.14	
History of abortion					
No	150 (74.6)	13 (81.3)	1.0		0.98
Yes	35 (17.4)	3 18.8)	0.99	0.30–3.28	
Mode of delivery					
Cesarean	118 (58.7)	7 (38.9)	1.0		0.64
Vaginal	81 (40.3)	11 (61.1)	2.29	0.93–5.66	
Time between rupture of membranes and delivery					
≤360 min	105 (52.2)	5 (38.5)	1.0		**0.01**
≥360 min	46 (22.9)	8 (61.5)	3.65	1.26–10.57	
Gestational time[c]					
≥38 weeks (full-term)	145 (72.1)	14 (93.3)	1.0		0.26
≤37 weeks (preterm)	30 (14.9)	1 (6.7)	0.35	0.05–2.53	

[a]Chi-square test.
[b]Calculated as equivalence to the Brazilian minimum wage at the time of the study (approximately US$ 350.00).
[c]Calculated by means of the Capurro's method.
[d]All respondents.
Bold indicates statistically significant results.

TABLE II. Multivariate Analysis of Characteristics Associated With HSV-2 Prevalence in Parturient Women Analyzed in the Stud‘

Variable	Prevalence ratio	95% CI	*P*
Income[a]			
≥3× minimum wage	1.0		
=2× minimum wage	0.07	0.01–0.66	**0.02**
≤1× minimum wage	0.35	0.09–1.38	0.13
Time between rupture of membranes and delivery			
≥360 min	1.0		
≤360 min	0.02	0.07–0.86	**0.02**

[a]Calculated as equivalence to the Brazilian minimum wage at the time of the study (approximately US$ 350.00).
Bold indicates statistically significant results.

with HSV-2 positivity in this study. Women with a time of ≥360 min had an approximately fourfold higher risk of detection of HSV-2 in placental tissue (95% CI 0.93–5.66, $P = 0.01$). On multivariate analysis, a time between rupture of membranes and delivery of <360 min was identified as an independent protective factor against infection (95% CI 0.07–0.86, $P = 0.02$). Microbial invasion of the amniotic cavity occurs in approximately one-third of all patients with premature rupture of membranes [Romero et al., 1992], and is associated with perinatal infection [Mercer, 2003; Jaiyeoba et al., 2012]. In a U.S. study of 91 neonatal herpes cases, premature rupture of membranes was also associated with neonatal infection [Mark et al., 2006]. These studies corroborate the findings of this investigation, providing evidence of the major role of membrane rupture in HSV-2 infection of the placenta and potential vertical transmission of the virus.

All HSV-2-positive patients were asymptomatic at the time of delivery and none reported genital herpes. This is consistent with the findings that 60–80% of women who deliver an HSV-infected newborn have asymptomatic genital HSV infection at the time of delivery [Whitley et al., 1980, 1988; Yeager and Arvin, 1984], and both symptomatic and asymptomatic infection can be transmitted to the newborn [Kimberlin and Baley, 2013].

Of the women who had HSV-2-positive newborns (two cases), both presented with hypertensive disorders of pregnancy. Gibson et al. [2008] found that fetal exposure to herpes virus infection was associated with pregnancy-induced hypertensive disorders. Another study conducted by Sun et al. [2004] indicated that HSV-2 infection might be an independent risk factor for essential hypertension. This suggests that HSV-2 can be correlated to hypertension, which according to these studies may indicate changes in the efficacy of placental barrier, allowing the transmission of this virus.

None of the HSV-2-positive newborns presented with clinical changes suggestive of herpes, but one of them had toxic erythema on the face and lower limbs at day four after birth. According to Kimberlin [2007], other conditions can mimic neonatal HSV infection, which include noninfectious cutaneous disorders like toxic erythema of the newborn. While the first newborn was released on the following day, the second stayed in the hospital for 10 days, but did not return for further follow-up. Therefore, additional, late HSV infection manifestations could not be evaluated for the studied children.

HSV-2 infection is ubiquitous. The risk of fetal transmission makes the virus a global public health issue, particularly because HSV-2 infection is most common among women of childbearing age. In the studied sample, there was evidence of HSV-2 infection in some asymptomatic women, demonstrating the potential for neonatal infection. In all cases, infection was asymptomatic. New strategies must be implemented for the management of patients without symptoms of infection who might transmit HSV to their baby. HSV antibody testing and typing in antenatal booking blood samples may help identify women at risk, together with offering an opportunity to highlight symptoms during pregnancy that might be mistaken for candida infection.

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