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Detection of Herpes Simplex Virus Infection in Patients With Ongoing Miscarriage Using Serological Tests and Real-Time Polymerase Chain Reaction

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Abstract

Background: Herpes simplex virus (HSV) is one of the most frequent viruses affecting females' sexual and reproductive health.
Objectives: The current study aimed to determine the HSV serostatus and viral shedding in patients with ongoing miscarriage.
Methods: Two hundred and eight females were included in the study; IgM antibodies against HSV1/2 were detected in serum samples; the real-time polymerase chain reaction (PCR) quantification of viral DNA was performed on cervicovaginal samples. Positive females were tested for IgG anti-HSV-2.
Results: The results indicated 12.5% IgM-positive and 2.9% real-time PCR positive samples. None of the patients was positive for the both analyses, simultaneously. Among IgM-positives cases, 16.6% were also IgG-positive; whilst in PCR-positives samples, 20% were also IgG-positive. The presence of viral DNA without detectable IgM or IgG antibodies could indicate a recent infection or a reactivation with low copy numbers.
Conclusions: IgM alone is not a marker for viral shedding in genital tract. Molecular testing in conjunction of IgG test should be evaluated as an option to determine HSV status, and applied for research on HSV genital infections records.

Keywords: Herpes, Diagnosis, Viral Shedding

1. Background

Spontaneous abortion defined as the termination of a pregnancy prior to completion of 20 weeks of gestation, is one of the most frequent adverse pregnancy outcomes, estimated in 12% - 76% of all pregnancies (1-3). The most frequent causes of pregnancy loss in the first trimester are of genetic origin (4). Other important risk factors are maternal age, placental inflammation and infection (5, 6); however, the etiology is often uncertain.

It is believed that some of the infectious agents involving in pregnancy loss and congenital malformations are included in the TORCH screening: *Toxoplasma gondi*i, cytomegalovirus (CMV), rubella and herpes simplex virus (HSV) amongst others. HSV is one of the most frequent sexually transmitted viruses, and the cause of significant morbidity in the affected populations; recent estimates indicate a large burden of genital herpes worldwide (7). Asymptomatic shedding is important in HSV transmission dynamics. The most devastating consequence is neonatal herpes; 90% of neonatal infections are transmitted intrapartum; neonates born from mothers recently infected or without detectable antibodies at the time of delivery are at higher risk of disease and death (8, 9). As mentioned, HSV is traditionally considered amongst the risk factors for spontaneous and recurrent abortion, and the serological tests show utility in some settings; however it is suggested to discontinue the routine use in others (10). As recently reviewed by Giakoumelou et al. (11), HSV role in spontaneous abortion is still controversial and further studies are required. Studies by el-Sayed Zaki and Goda (12) reported 40% IgM positivity in females with pregnancy loss; while Sebastian et al. (13) showed statistically significant relationship between pregnancy loss and anti-HSV IgM positivity (P = 0.022). Additional methods employed to diagnose HSV infection include nucleic acid amplification techniques; although they are powerful tools, the molecular detection should not always correspond to the presence of symptoms or lesions, or to the serological diagnosis (14, 15).

2. Objectives

Therefore, the clinical significance of the tests can vary. The current study aimed to gain knowledge on the frequency of HSV shedding in patients with ongoing spontaneous miscarriage and to discuss the results that can be obtained when screening the same females with serological and viral DNA tests.

3. Methods

The current cross-sectional descriptive study included patients with up to 20 weeks of gestation, attending hospital regional Ignacio Garcia Tellez in Merida, Yucatan, presenting gestational vaginal bleeding; further defined as acute miscarriage and missed or incomplete miscarriage diagnosed by ultrasound examinations. All participants signed an informed consent. The project was approved by the corresponding bioethical committee (reference number: CEI-CIR-14-09). On the same examination, cervicovaginal samples were collected by cytobrush and deposited in 5 mL of phosphate-buffered saline (PBS) with penicillin 500 U/mL, streptomycin 500 mg/mL and gentamicin 4 mg/mL. Immediately, blood-serum samples were obtained from venous punctuation (approximately 5 mL). Samples were kept at 4°C during transportation to the virology laboratory of the regional research center for processing.

Automated DNA extraction of cervicovaginal samples was performed using MagNa Pure LC total nucleic acid isolation kit (Roche). Extracted DNAs were tested for HSV using real-time polymerase chain reaction (PCR) amplification of the UL30 gene which is common for both HSV types 1 and 2; according to the previously described protocol (16), using FAM/TAMRA-labelled TaqMan probe, the amplification was performed on a 7500-Fast Thermocyler (Applied Biosystems). For quantitative determinations, standard curves were performed using serial log10 dilutions of cloning vectors containing the corresponding HSV genome fragment from HSV types 1 and 2 reference strains (McIntyre and G respectively, kindly donated by BL Barron Romero). As an internal control for real-time PCR, β-globin gene was amplified from human genome (16) and the results of viral quantification were corrected to be expressed in a total volume of one millilitre of reaction buffer.

Serum samples were analysed by an enzyme immunoassay (EIA) to detect IgM antibodies against HSV1/2 (DRG, Germany) following the manufacturer's instructions. IgG EIA test against HSV-2 (DRG, Germany) was further performed to all positives to any of the previous tests. Each assay included the appropriate positive and negative controls. The clinical records were transferred to a database in SPSS software ver. 21.

4. Results

The mean age of the 208 females in the study was 28.37 years, ranging from 14 to 43. The majority of the subjects (n = 185, 88.9%) were in their first trimester of gestation and 22 (10.6%) subjects in the second trimester (up to week 20 were included exclusively); one patient had missing data. The described miscarriage categories were: 145 subjects with acute miscarriage (includes complete miscarriage); 30 subjects with incomplete miscarriage (as confirmed by ultrasound examination); 25 subjects with missed miscarriage (as confirmed by ultrasound); 8 subjects with other/not defined.

None of them presented ulcers or lesions compatible to genital HSV at the time of sampling; none referred genital HSV episodes in the past. It was considered a low-risk population as the majority were monogamous (78.4% were married, 98.6% had only one sexual partner for the last six months), had never presented sexually transmitted diseases (STDs) (92.8%), had high school diploma or higher (87%) and the mean age of sexual onset was 20.1 years (13 - 39 years). In IgM serological analysis, 12.5% (26/208) were positive; while 2.9% (6/208) were positive in real-time PCR analysis. None of the patients was positive for both PCR and IgM analyses, simultaneously. The results are summarized in Table 1 (only the positive samples in either test are presented).

The ranges of viral loads in PCR-positives were estimated from 800 to 178 and 350 copies/mL, with a mean of 32,933 copies/mL. Among the IgM positives, 16% were positive for HSV-2 IgG (4/25, one not tested). Among real-time PCR positives, 20% were also positive for HSV-2 IgG (1/5, one not tested).

5. Discussion

There were 12.5% IgM seropositives and 2.9% positives for viral DNA detection in cervicovaginal samples. Previously, the utility of IgM serology test for HSV-2 was discussed; a study by Choudhry et al. (17) suggested a poor diagnostic value of this test in sexual transmitted infections (STIs) clinics, while other studies by el-Sayed Zaki and Goda (12) recommended the specific HVS-2 IgM screening to diagnose spontaneous and repetitive abortion cases. Moreover, results of another study recommended HSV1/2 IgM combined test to estimate recent infection burden (18). The current study used combined HSV1/2 IgM and real-time PCR tests because of the changing epidemiology of HSV types; HSV-1 was increasingly detected as the cause of genital herpes worldwide (19, 20). As indicated, a real-time PCR positive sample did not correspond to IgM detection in the

Table 1. Results of Serological and Real-Time PCR Testing to Determine HSV Infection in the Study Subjects[a,b]

Sample Code	IgM HSV1/2	IgG HSV-2	Real-time PCR for HSV1/2	Viral Load in Copies/mL	Viral Load in Copies per 10,000 cells
287	+	-	-	0	0
290	+	-	-	0	0
291	+	-	-	0	0
292	+	+	-	0	0
304	+	+	-	0	0
322	+	Grey zone	-	0	0
332	+	-	-	0	0
336	+	-	-	0	0
337	+	-	-	0	0
338	+	-	-	0	0
345	+	-	-	0	0
352	+	-	-	0	0
374	+	+	-	0	0
392	+	-	-	0	0
395	+	-	-	0	0
397	+	Grey zone	-	0	0
401	+	-	-	0	0
416	+	-	-	0	0
442	+	NA	-	0	0
621	+	-	-	0	0
682	+	-	-	0	0
684	+	-	-	0	0
715	-	-	+	3400	75
721	+	+	-	0	0
724	+	-	-	0	0
768	-	-	+	7750	119
779	-	+	+	178350	2833
788	-	-	+	800	17
819	-	-	+	850	3
822	-	NA	+	6450	146
827	+	-	-	0	0

[a] Coding: +, positive; -, negative; grey zone as established by the test manufacturer; NA, not available.
[b] Viral load per 10,000 cells is adjusted to β-globin copies.

studied group. The presence of viral DNA without IgM detection could indicate a reactivation that may occur without IgM rises (8). An example of this situation was the sample no. 779 (Table 1), which was positive for HSV-2 IgG and PCR, but negative for IgM.

On the contrary, an HSV1/2 IgM positive test with a negative PCR could indicate one of the following possibilities: a, infection of the oral mucosa, overestimating the rates of genital infection; b, genital infection without detectable viral shedding at the moment of sampling. About the last scenario, it is said that shedding episodes are highly variable, and the detection depends on the applied methods, duration of the study and anatomical site of sampling (21). However, since the current study was cross-sectional sur-

vey, viral shedding was tested only once.

It is clear that patients with positive HSV-2 IgG are infected with HSV-2; however, an IgM1/2 positive result could reflect a reactivation of either types not necessarily HSV-2. It was the case of samples no. 292, 304, 374, and 721 (Table 1). The grey-zone could indicate that seroconversion is in progress and might represent recent infection, as was the samples no. 322 and 397.

HSV-2 IgG negative with IgM HSV1/2 positive could indicate: a, a recent infection with either types; b, a reactivation of HSV-1.

Finally, the study found PCR positive samples with negative HSV1/2 IgM and negative HSV-2 IgG tests; it was the case of samples no. 715, 768, 788 and 819 (Table 1; no. 822 was not available for IgG testing). Although asymptomatic shedding represents an important part of HSV epidemiology, the clinical relevance of the obtained results is not known; presumably, they may represent very recent exposures to the virus. It is reported a higher risk for neonates born to viral-positive seronegative mothers (8). However, the miscarriage event cannot be linked to the viral shedding, or to the presence of antibodies.

It is noteworthy that viral shedding quantification in all samples was relatively low (800 - 7750 copies/mL). It is unknown which viral levels may result in transmission, but Gardella et al. (14, 15) reported transmission from PCR low-positives even of 500 - 1,000 copies of DNA.

EIA for detection of IgG remains the most commonly used tests, however, this test alone is not a marker of baby exposure to mother's infection at labour or sexual partner exposure. IgM testing or periodic screening for transmissibility is suggested by some authors; despite its low diagnostic value (17, 18). According to the current study observations, IgM HSV1/2 serology was not useful to estimate the risk of exposure to viral shedding in the patients.

HSV infection dynamics is difficult to study. IgM and real-time PCR testing could aid to identify different stages of infection, in conjunction to IgG test. The presence of anti-HSV1/2 IgM antibodies in the studied group did not correspond to actual viral shedding in the genital tract. Since the etiology of HSV in spontaneous abortion is still not fully defined, patients with a positive real-time PCR result, high viral loads and negative serology are of clinical interest and for further research. The available studies show contradictory results about the etiological role of HSV in spontaneous abortion (13, 22-24); therefore, more research is needed. A limitation of the current study was that abortive tissues were not available to test the presence of HSV DNA, and that it was not a case-control study. Another limitation was that data on oral herpetic lesions were not available.

The current observations might be extrapolated for the third trimester pregnancies, in which a positive PCR result with negative serology would represent higher risk for transmission to the product, or in other cases higher risk for transmission to the sexual partner, or for the future development of genital ulcers; in agreement to what was previously suggested (8, 15).

It is important to consider the interpretation of each available test, either for research, diagnosis or epidemiology purposes. Cost-effectiveness studies in low resources settings are needed to determine if molecular tests will be a choice to prevent complications such as neonatal herpes in high-risk populations and concealed serodiscordant partner. Nevertheless, molecular techniques rather than serology applications are useful to investigate the effect of HSV shedding during gestation and delivery.

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