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EPIDEMIOLOGY OF HERPES SIMPLEX VIRUS INFECTION IN PREGNANCY: A PILOT STUDY

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Herpes simplex virus (HSV) infection is one of the most common sexually transmitted viral diseases worldwide. HSV type 2 causes most genital herpes and HSV type 1 is usually transmitted via non-sexual contacts. We studied 109 pregnant women between January 2007 and December 2008, in relation to their age, condom use, number of sexual partners, age at first intercourse, parity and smoking habits. The aim of this study is to evaluate the prevalence of HSV cervical infection and HSV co-infection with other genital microorganisms associated with poor neonatal outcome. Our results show that of the 109 outpatients enrolled, 30% were HSV1 and/or HSV2 positive, of whom 30% were infected with both HSV1 and HSV2, 18% were infected with HSV1 alone and 52% with HSV2 alone. A significant association between HSV1 and HSV2 infection was found, and the prevalence of HSV2 infection in women infected with HSV1 was 63%. The prevalence of HSV1/2 varied in the presence of other vaginal microorganisms but a statistical significant association was not found. This pilot study is probably too small to obtain statistically significant results. Nevertheless, using these observed results, we calculated that about 530 patients with comparable features should be enrolled to detect an increase of 50% in HSV infection due to the presence of other genital infections and potential risk factors.

Herpes simplex virus (HSV) infection is one of the most common sexually transmitted viral diseases worldwide (1). Herpes simplex virus type 2 (HSV-2) and Herpes simplex virus type 1 (HSV-1), belonging to the *Herpesviridae* family, are transmitted through epithelial mucosal cells and skin wounds, and migrate to nerve tissues where they persist in a latent state. HSV-1 is found predominantly in the trigeminal ganglia, and HSV-2 in the lumbosacral ganglia. Nevertheless, these viruses can infect both orofacial areas and the genital tract (2).

These viruses are highly contagious and are not generally associated with long-term deterioration in health. Diagnosis is often associated with stress and concerns about both further sexual relationships and the safety of child-bearing. The greatest risk of disease in the newborn comes with late-pregnancy acquisition of genital infection in the mother and is probably due to her lack of type-specific antibodies.

In the general population, HSV seroprevalence is lower and HSV-2 rates are higher in women than in men (1). In pregnant women, the seroprevalence

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was found to be 72% in the United States (3). The seroprevalence of HSV-1 and HSV-2 varies with race and ethnicity.

Since newborns from populations whose HSV seroprevalence is low appear to be at greater risk for neonatal herpes, a more extensive knowledge of the prevalence in different ethnic groups worldwide is needed to optimize prevention strategies of neonatal disease.

Several co-factors have been associated with the risk of HSV-2 infection: age, gender, race, serologic status, condom use, number of sexual partners, duration of HSV-2 in the source partner, duration of relationship. Some of these cases are likely to relate to episodes of viral shedding, while others are less clear (4-7). In this study, age, condom use, number of sexual partners, age at first intercourse, parity and smoking habits were taken into consideration..

The aim of this cross-sectional pilot study is to evaluate the prevalence of HSV cervical infection and HSV-co-infection with other genital microorganisms associated with acute vaginal discomfort and/or poor outcome in pregnant women.

MATERIALS AND METHODS

Study population

Pregnant women attending for routine gynaecologic care in the "Cervico-vaginal Pathology Unit" of the Gynaecology and Obstetrics Department of Policlinic "Umberto I" in Rome, between January 2007 and December 2008 were eligible for inclusion.

Samples were taken first by an Ayre's spatula from fornixes for wet mount preparation and then by a cotton-tipped swab from the endocervix or from fornixes for the detection of HSV1 and/or HSV2 DNA and bacteriological tests. Women were eligible for the study if they were pregnant, answered a structured questionnaire on socio-demographic characteristics, sexual behaviour, reproductive history and smoking habits, and provided samples adequate for PCR assays. One hundred and nine women, who satisfied these criteria, were enrolled.

Office and laboratory procedures

On a scheduled visit, during the physical examination, after observation of the vulva and vestibule, an unmoistened sterile speculum was inserted into vagina, so that the vaginal walls, fornixes and cervix could be evaluated for eventual erythema, color and viscosity of discharge. pH was measured on vaginal walls and in fornixes by colorimetric paper with 10 comparison colors for pH values between 4.0 and 7.0. Vaginal fluor samples were collected from lateral fornixes by a wooden Ayre's spatula, mixed with saline and 10% potassium hydroxide on two different slides and then immediately observed under the Ph microscope (8-9).

A "whiff test" by 10% potassium hydroxide was performed for each sample.

Wet mount examination detected *Lactobacilli*, Candida blastospores and branching/budding hyphae, clue cells, motile *Trichomonas vaginalis* and white blood cells.

Bacterial vaginosis

The presence of bacterial vaginosis was evaluated microscopically on samples collected from lateral vaginal fornixes. A bacterial vaginosis diagnosis was made when three of the four clinical and microscopic findings standardized by Amsel et al (10) were detected: vaginal pH greater than 4.5; presence of clue cells; grey homogenous vaginal discharge; and positive "whiff test".

Bacterial vaginitis

The presence of bacterial vaginitis was evaluated microscopically on samples collected from lateral vaginal fornixes. Bacterial vaginitis was suspected if a depletion of Lactobacilli was detected in the presence of vaginal phlogosis and bacteria. Using cultural methods, such as the finding of *Streptococcus spp* or *Enterococcus*, diagnosis of bacterial vaginitis was confirmed.

Trichomonas vaginalis

Wet mount in sterile physiologic saline was examined microscopically (400×) within 15 min for motile trichomonads (11). The culture was on Oxoid's Trichomonas Medium n.2 for the recovery of *T. vaginalis* and incubated aerobically a 37°C. The broth was examined microscopically for motile trichomonads on days 1, 3 and 5 (12-13).

Detection of microorganisms

Streptococcus agalactiae (group B) strains were identified from clinical samples by being weakly beta-haemolytic on Columbia CNA Agar A (Oxoid). The suspected colonies were serologically confirmed (Lancefield's classification) (Slidex Strepto-Kit, bio Mèrieux, S.p.A, Italy) (12-13).

Chlamydia trachomatis.

Endocervical swabs were tested for the presence of *C. trachomatis* using the BD ProbeTec ET Assays (BDPT, Becton Dickinson and Company, Franklin Lakes, NJ) according to the manufacturer's instructions. This method amplifies DNA from *C. trachomatis* in separate wells and optionally can monitor inhibition of amplification for each

Table I. *Characteristics of studied groups: univariate analysis of age, sexual, reproductive and behavioral history.*

	HSV(+)	HSV(-)	Total	HSV(+)%	Comparison[§]	95% CI	Significance
TOTAL: frequency	**33**	**76**	**109**	**30%**			
Age							
mean age (years) (sd)	**31.97 (5.29)**	**32.17 (5.27)**	**32.11 (5.26)**		**diff. - 0.20**	**-2.38 to 1.94**	**P=0.86**
Frequency:							
<25 years	**4**	**6**	**10**	**40%**			
25- years	**6**	**16**	**22**	**27%**	**OR = 0.56**	**0.12 to 2.71**	**P=0.47**
30- years	**11**	**27**	**38**	**29%**	**OR = 0.61**	**0.14 to 2.62**	**P=0.50**
35- years	**11**	**20**	**31**	**35%**	**OR = 0.83**	**0.20 to 3.38**	**P=0.80**
40+ years	**1**	**7**	**8**	**13%**	**OR = 0.21**	**0.02 to 2.25**	**P=0.19**
Age at 1st intercourse							
mean age (years) (sd)	**20.48 (4.99)**	**19.51 (3.44)**	**19.81 (3.97)**		**diff. 0.97**	**-0.70 to 2.61**	**P=0.24**
Frequency:							
≤16 years	**5**	**10**	**15**	**33%**			
17-18 years	**9**	**28**	**37**	**24%**	**OR = 0.64**	**0.17 to 2.39**	**P=0.51**
19-20 years	**8**	**16**	**24**	**33%**	**OR = 1.00**	**0.25 to 3.93**	**P=1**
≥21 years	**11**	**22**	**33**	**33%**	**OR = 1.00**	**0.27 to 3.65**	**P=1**
Number of partners							
mean (sd)	**2.58 (3.40)**	**3.46 (3.51)**	**3.19 (3.48)**		**diff. -0.88**		**P=0.22**
Frequency:							
1 partner	**15**	**29**	**44**	**34%**			
2 or more partners	**18**	**47**	**65**	**28%**	**OR = 0.74**	**0.32 to 1.69**	**P=0.47**
Parity							
Nulliparous	**16**	**40**	**56**	**29%**			
Parous	**15**	**28**	**43**	**35%**	**OR=1.34**	**0.57 to 3.15**	**P=0.50**
NR*	**2**	**8**	**10**	**20%**			
Spontaneous abortion							
No	**22**	**57**	**79**	**28%**			
Yes	**8**	**13**	**21**	**38%**	**OR=1.59**	**0.59 to 4.31**	**P=0.36**
NR*	**3**	**6**	**9**	**33%**			
Induced abortion							
No	**24**	**56**	**80**	**28%**			
Yes	**4**	**10**	**14**	**30%**	**OR=0.93**	**0.25 to 3.41**	**P=0.91**
NR*	**5**	**10**	**15**	**33%**			
Contraceptives							
never used any type	**27**	**68**	**95**	**28%**			
used at least one type	**6**	**8**	**14**	**36%**	**OR=1.89**	**0.61 to 5.87**	**P=0.27**
Smoking							
No	**26**	**62**	**88**	**30%**			
Yes	**3**	**10**	**13**	**23%**	**OR=0.72**	**0.19 to 2.74**	**P=0.63**
NR*	**4**	**4**	**8**	**50%**			

* *NR: not recorded data;*

[§] *diff. is the difference between two mean values and OR is the odds ratio compared with the first category*

Table II. *Different microorganisms infection in HSV positive and negative women.*

	HSV(+)	HSV(-)	Total	HSV(+)%	odds ratio	95% CI	significance
Candida albicans							
negative	22	58	80	28%			
positive	9	13	22	41%	OR=1.83	0.69 to 4.86	P=0.23
Total (% positive)	31 (29.0%)	71 (18.3%)	102 (21.6%)				
NR*	2	5	7	29%			
Mycoplasma spp							
negative	31	58	89	35%			
positive	1	16	17	6%	OR=0.12	0.02 to 0.69	P=0.02
Total (% positive)	32 (3.1%)	74 (21.6%)	106 (16.0%)				
NR*	1	2	3	33%			
Wet mount examination			67				
NR*			42				
***Doderlein bacillus* (normal)**							
negative	8	17	25	32%			
positive	13	29	42	31%	OR=0.95	0.31 to 2.92	P=0.93
Total (% positive)	21 (62.0%)	46 (63.0%)	67 (62.0%)				
NR*	12	30	42	29%			
Aerobic bacterial vaginitis							
negative	18	42	60	30%			
positive	3	4	7	43%	OR=1.75	0.36 to 8.52	P=0.49
Total (% positive)	21 (14.3%)	46 (8.7%)	67 (10.5%)				
NR*	12	30	42	29%			
Vaginal mycosis							
negative	16	36	52	31%			
positive	5	10	15	33%	OR=1.13	0.32 to 3.99	P=0.85
Total (% positive)	21 (24.0%)	46 (21.8%)	67 (22.4%)				
NR*	12	30	42	29%			
Bacterial vaginosis							
negative	21	45	66	32%			
positive	0	1	1	0%			
Total (% positive)	21	46 (2.2%)	67 (1.5%)				
NR*	12	30	42	29%			
Trichomonas vaginalis							
negative	21	44	65	32%			
positive	0	2	2	0%			
Total (% positive)	21	46 (4.4%)	67 (3.0%)				
NR*	2	30	42	29%			
Aerobic common germs (cultural assay)							
negative	30	66	96	31%			
positive	3	9	12	25%	OR=0.73	0.18 to 2.90	P=0.66
Total (% positive)	33 (9.1%)	75 (12.0%)	108 (11.1%)				
NR*	0	1	1	0%			
Streptococcus agalactiae							
negative	32	69	101	32%			
positive	1	7	8	13%	OR=0.31	0.04 to 2.32	P=0.26
Total (% positive)	33 (3.0%)	76 (9.2%)	109 (7.3%)				

**NR: not recorded data*

specimen using strand displacement amplification (SDA) and detection by fluorescent energy transfer (FRET) probes, producing a method-other-than-acceleration (MOTA) score for each specimen. Specimen processing and BDPT performance followed the manufacturer's instructions. The original algorithm involved retesting specimens with MOTA scores between 2000 and 9999. A negative repeat result (MOTA score <2000) was

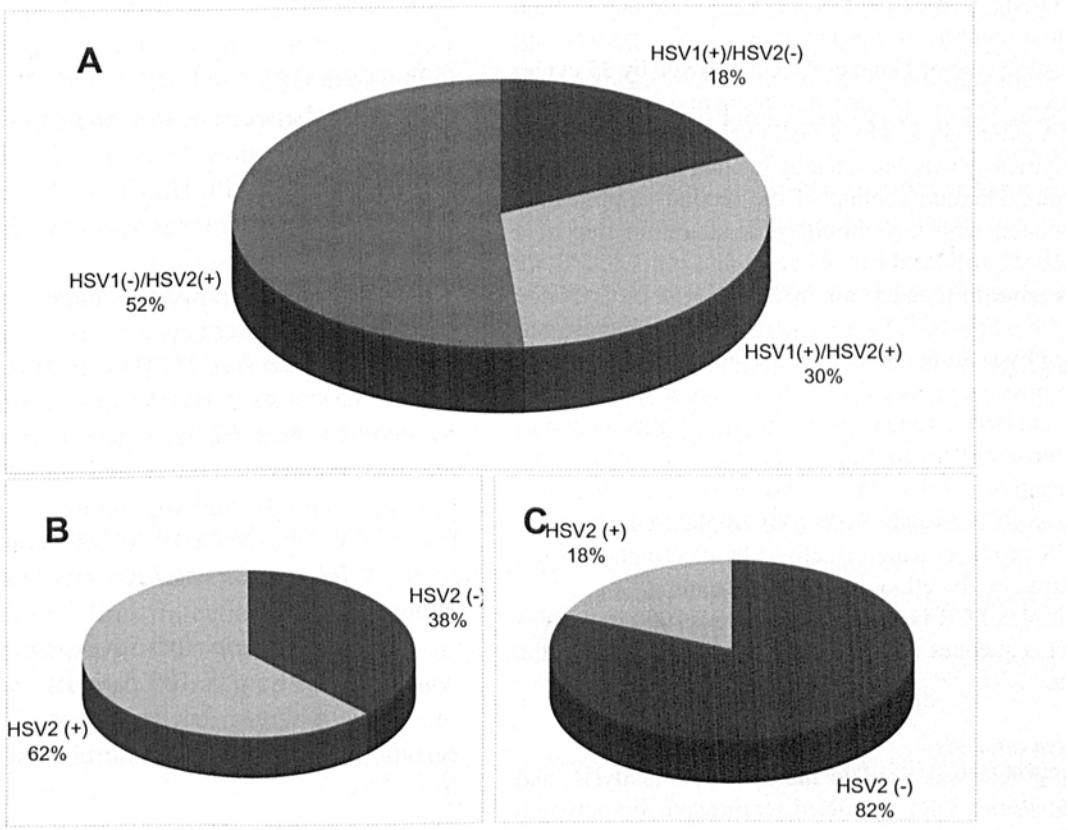

Fig. 1. *Distribution of HSV1 and HSV2 infection. A) Distribution of HSV1 and HSV2 infection in 33/109 HSV1 and/or HSV2 positive pregnant women. B) The risk of HSV2 infection in the 16 women infected with HSV1 is 63% vs C) only 18% of the 93 women who are HSV1 negative. There is a strong association between HSV1 and HSV2 infection (p=0.0002).*

considered indeterminate.

Mycoplasma spp.

Mycoplasma IST 2 (bioMérieux) was used for the isolation of *Mycoplasma hominis* and *Ureaplasma urealyticum* according to the manufacturer's instructions. The diagnostic kit provided information regarding the presence or absence of *M. hominis* and *U. urealyticum* and an estimate of the density of each organism (cut-off 10^4-colour-changing units- CCU/ml) together with its antimicrobial susceptibilities to doxycycline, josamycin, ofloxacin, erythromycin, tetracycline, pristinamycin, azithromycin, clarythromycin, ciprofloxacin.

Yeasts. For each patient, a vaginal sample was cultured on Sabouraud dextrose agar (Oxoid) aerobically at 37°C for 24-48 h (12-13). The identity of the clinical isolates was confirmed by conventional mycological methods (14-15), such as the germ tube induction test in serum (*C. albicans* or *C. dubliniensis*), microscopic morphology, and growth on Oxoid Chromogenic Candida Agar (Oxoid) (14). The species of non-*Candida albicans* yeast isolates were identified with API 20C AUX or API 32C.

HSV1 and HSV2 detection

Cervical samples collected in Phosphate Saline Buffer (PBS) were centrifuged at low speed and the cell pellets underwent DNA extraction using QIAampTissue kit (Qiagen, Italy).

For HSV1 and HSV2 detection, a multiplex PCR was performed. This molecular analysis is a nested PCR (Nanogen Advanced Diagnostics, s.r.l.) that allows the amplification of a region of the HSV-1 glycoprotein D gene (*gpD*) and a region of the HSV-2 glycoprotein G gene (*gpG*). The assay was performed using two sets of primers that amplify the US6 region of HSV-1 glycoprotein D gene and the US4 region of HSV-2 glycoprotein G gene obtaining a 160-bp long fragment for HSV-1 *gpD* and a 81-bp long fragment for HSV-2 *gpG*. PCR amplifications were run in a reaction volume of 50

μl containing 5 μl of the DNA sample. Thermal cycling of the first amplification was initiated with a preliminary denaturation step of 1 min at 95°C, followed by 35 cycles of 1 min at 55°C for primers annealing and 1 min at 72°C for DNA extension and by a final cycle of 5 min at 72°C. Taq Polymerase was activated by heating the mix at 95°C for 4 min. Thermal cycling of the second amplification was initiated with a preliminary denaturation step of 1 min at 95°C, followed by 30 cycles of 1 min at 55°C for primers annealing and 1 min at 72°C for DNA extension and by a final cycle of 5 min at 72°C. Taq Polymerase was activated by heating the mix at 95°C for 2 min. All assays include positive (plasmid containing target sequences of HSV-1 and HSV-2) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results. This assay was performed in a GeneAmp PCR System 9700 (AB Applied Biosystems). The PCR products were visualized by electrophoresis on 2% agarose gel by ethidium bromide staining.

Multiplex PCR is able to detect about 1000 molecules of target sequences in 5 μl of extracted DNA used in the reaction.

Statistical analysis

The χ^2 test was used in the statistical analysis, and the Odd Ratio (OR) was used to measure the strength of the association with HSV infection. Statistical tests were considered significant if the p value was 0.05 or less. Logistic regression analysis was used to assess the simultaneous effect of more than one variable on the risk of HSV1 and/or HSV2 infection and to identify possible confounding factors. The results obtained were used to estimate the sample size necessary for the proposed main study.

RESULTS

The demographic and behavioural characteristics of the study population are presented in Table I. The women were aged between 20 and 44 years (mean 32.11 years). The variables analyzed in univariate analysis for association with HSV1 and/or HSV2 DNA detection were: patient's age, age at first intercourse, number of lifetime sexual partners, parity, condom use and smoking habits. The Odds Ratio (OR) test found no significant association between HSV1 and/or HSV2 infections and these variables (Table I).

Of the 109 enrolled outpatients, Table I shows that 33/109 (30%) were HSV1 and/or HSV2 positive, of whom 10 out of 33 (30%) were infected with both HSV1 and HSV2, 6 out of 33 (18%) were infected with HSV1 only and 17 out of 33 (52%) with HSV2 only (Fig. 1A). There is a strong association between HSV1 and HSV2 infection ($p = 0.0002$); the prevalence of HSV2 infection in women infected with HSV1 is 63% compared with only 18.3% among women who are HSV1 negative (Fig. 1 B, C).

Of 102 pregnant women analysed for *Candida albicans*, 21.6% were positive; of 106 patients tested for *Mycoplasma spp*, 16.0% were positive.

Wet mount examination was carried out on only 67 women and 62.0% were normal (*Doderlein bacillus* positive), 10.5% and 22.4% had aerobic bacterial vaginitis and vaginal mycosis respectively, bacterial vaginosis was observed in only one patient; finally *Trichomonas vaginalis* was found in only two women.

The examination for *Streptococcus agalactiae* was performed on all 109 patients and was positive in 7.3%, whereas bacterial vaginitis caused by aerobic common germs (cultural assay) was positive in 11.1%.

All samples were negative for *Chlamydia trachomatis*.

The prevalence of HSV1/2 varied in presence of other vaginal microorganisms. Of 22 patients positive for *Candida albicans*, only 9 (41%) were co-infected with HSV1/2; of 17 patients positive for *Mycoplasma spp*, only 1 (6%) was co-infected with HSV1/2; of 7 patients with bacterial vaginitis (microscopic fresh evaluation), only 3 (43%) were co-infected with HSV1/2; of 15 patients with vaginal mycosis, only 5 (33%) were co-infected with HSV1/2; of 8 patients with *Streptococcus agalactiae*, only 1 (13%) was co-infected with HSV1/2; of 12 patients with bacterial vaginitis by aerobic common germs, only 3 (25%) were HSV1/2 co-infected; finally, of the three patients with bacterial vaginosis and *Trichomonas vaginalis* infection, no patients were co-infected with HSV1/2. These data are shown in Table II.

U. urealyticum is a common commensal of the female lower genital tract and seems to be an important opportunistic pathogen during pregnancy and in the presence of other genital diseases such as cervicitis (16); its pathogenic potential seems to be related to a density >10,000 CCU/ml. Of the 106 women tested for *Mycoplasma spp*, all 17 who

resulted positive were infected with *U. urealyticum*, and none was positive for *M. hominis*. Of these 17 women, 8/17 (47%) had a density >10,000 CCU/ml, and the only woman co-infected with HSV and *U. urealyticum* had a density < 10,000 CCU/ml (data not shown).

The number of the specimens in which *T. vaginalis* was detected was low in HSV negative women (4.4%), compared to women co-infected.

Statistical analyses did not reveal any significant association between HSV presence and the microorganisms listed above, and the logistic regression analyses did not substantially change these results (Table II).

DISCUSSION

Herpes simplex virus infection is one of the most common sexually transmitted viral diseases worldwide. Herpes simplex virus type 2 causes most genital herpes and frequently is sexually transmitted. Herpes simplex virus type 1 is usually transmitted via non-sexual contacts.

The aim of this cross-sectional study is to evaluate the prevalence of HSV cervical infection and HSV co-infection with other genital microorganisms associated with poor neonatal outcome.

We focused our attention on pregnant women attending the "Cervico-vaginal Pathology Unit" of the Gynaecology and Obstetrics Department of Policlinic between January 2007 and December 2008.

Our results show that of the 109 enrolled outpatients, 30% was HSV1 and/or HSV2 positive, of which 30% was infected with both HSV1 and HSV2, 18% was infected with HSV1 only and 52% with HSV2 only. A significant association between HSV1 and HSV2 infection was found and the prevalence of HSV2 infection in women infected with HSV1 was 62% compared with only 18.3% among women who are HSV1 negative.

The prevalence of HSV1/2 varied in the presence of other vaginal microorganisms but statistical analyses did not reveal any significant association between HSV presence and other microorganisms and the logistic regression analyses did not substantially change these results.

Nevertheless, the size of the population studied (109 patients) is probably too small to have sufficient power to obtain statistically significant results. Using these results we have calculated the number of patients we will need to enrol to detect an increase of 50% in HSV infection due to the presence of other genital infection, socio-demographic characteristics, sexual behaviour, reproductive history or smoking habits. We calculated that, with a statistical power of 95% and significance 5%, about 530 patients with comparable features should be enrolled. Further larger studies are required to shed more light on HSV infection epidemiology and to optimize management strategies in pregnancy.

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