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PCR Detection of Herpes Simplex -2 Virus in Human Placenta in Patients with Spontaneous Abortion

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Abstract : Background: HSV-2 is thought to account the majority of cases of neonatal herpes, which may cause severe complications in infected newborns.
Objective: This study was carried out to investigate the rate of HSV-2 infection in placental tissue of women with spontaneous abortion by conventional polymerase chain reaction technique (PCR).
Materials and methods: Placental tissue samples were collected from 100 pregnant women with spontaneous abortion. Twenty five gram of the placental tissue was homogenized, the homogenate centrifuged for about 15 min. at 5000 rpm and (2-8) °C. The supernatant used for DNA extraction using DNA isolation kit ((DNA-sorb-B (Sacace)/ Italy) Kit).
Results: The results showed that, this gene was present in 19 (19%) out of 100 placental tissue PCR product of this gene was 120 bp, the highest rate was observed in age group (30-39) years. The highest fetal losses in studied group were occurred in the first trimester, there was a statistical significant ($P<0.006$) association between number of pregnancy compared to infection with HSV-2 and highly significant differences ($P<0.001$) with pregnancy outcome.
Conclusions: Herpes Simplex -2 Virus may play a significant role in pregnancy loss and can be delivery outcome-related factor. Its detection by sensitive molecular techniques would allow prompt therapeutic intervention in order to increase the possibility of a successful future pregnancy.
Keywords : Herpes Simplex type 2, placental tissue, spontaneous abortion, first trimester, gpG gene.

Introduction:

Herpes simplex virus (HSV), a member of Alphaherpesvirinae family, belongs to the subfamily of the Herpesviridae is the most ubiquitous virus in the adult population. The main characteristic of Herpesviridae is lifetime latency after primary infection and ability to reactivation at any time (1). In the last years the infection with Herpes simplex virus (HSV) steadily rising and became one of most serious causes for spontaneous abortion as well as congenital abnormalities likewise prenatal HSV infection which increases in women within the reproductive age (2)

The risks of transmission of HSV-2 from the infected mothers to the babies during the duration of pregnancy, with probably devastating results to the fetus and neonate, have attracted more interest, particularly, primary or recurrent herpes simplex virus (HSV) infection in pregnancy and its severe consequences for the

fetus (3).

Transmission of HSV-2 to the new child can arise through transplacental hematogenous spread, in the course of delivery or inside the postnatal duration. This transmission can cause ocular and cutaneous lesions, meningoencephalitis, fetal malformations, both primary and recurrent maternal infections may cause congenital disease (4).

The aim of this study was to investigate the presence of HSV-2 infection in placental tissue of women with spontaneous abortion by conventional polymerase chain reaction technique (PCR).

Subjects and Methods:

Placental tissue samples were collected from 100 pregnant women with spontaneous abortion attending the Gynecology outpatient clinics, wards and emergency unit in Al-Emamain Al-Khadhmyain City Hospital, and Baghdad Teaching Hospital during the period from December 2015 to May 2016. None of the women or their husbands reported any clinically confirmed genital herpes in their medical history. Informed consent was obtained from all the women who participated in this study. This study approved by Research Ethical Committee (REC) in the College of Medicine /AL-Nahrain University, and the study was conducted in the Microbiology Department at the College of Medicine-Al-Nahrain University.

Preparation of tissue homogenate:

Twenty five grams of the placental tissue was homogenized with 10 ml of PBS by using tissue homogenizer (5) for about 1 min. at 4°C. The resulting suspension was subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenate centrifuged for about 15 min. at 5000 rpm and (2-8) °C. The supernatant then collected carefully and stored at (-80°C) till DNA extraction.

DNA extraction:

DNA was extracted from placental tissue using DNA isolation kit ((DNA-sorb-B (Sacace)/ Italy) Kit) according to the manufacturer's instruction. The concentration and purity of the purified DNA was quantified by the use of Nanodrop instrument following the instruction .

Polymerase chain reaction technique:

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of HSV-2 gene (6) as shown in table (1). Human Beta-globin forward and reverse primers were used as an experimental control during protocol of PCR (7), and as a positive control for confirming the acceptability of the extracted DNA to template, those genes synthesized in Alpha DNA® (Canada) shown in table (1). To produce a DNA fragment of 120 base pair, the primers (HSV-2, and β-globin genes) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 2µl from forward and reverse primers were added. Five µl of DNA template was added for each reaction tube. Twelve and a half µl of GoTaq® Green Master Mix was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease–Free distal water and tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components.

PCR mixture without DNA template (non-template negative control) was used as a negative control. The tubes were placed on the PCR machine (Cleaver Scientific Thermal Cycler TC32/80) and the PCR program, with the right cycling conditions pre-installed, Amplification was as follows: 94°C for 5 min followed by 40 cycles of 94°C for 20 sec, 65°C for 20 sec, and 72°C for 20 sec, terminating in 72°C for 5 min.

Gel electrophoresis:

10 µl of each PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 µg /ml; Sigma). Five µl of the 100 bp DNA ladder (KAPA BIOSYSTEMS) (100, 150, 200, 300,

400, 500, 600, 800, 1000. 1200, 1600, 2000, 3000, 4000, 5000, 6000, 8000, 10000) were mixed with one µl of blue/orange 6X loading dye and subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis amplicon visualization was performed using an UV light transilluminator and then photographed using digital camera (Sony-Japan).

Table (1): Forward and reverse oligonucleotide sequences for HSV-2 and *β-globin* gene.

Gene target	5' position		Primer sequence (5' 3')	Reference	Product Size(bp)
HSV-2	**gpG**	F	TACGCTCTCGTAAATGCTTC	Yun Ji *et al*.,2014 (6)	120
	gpG	R	GCCCACCTCTACCCACAA		
β-globin	**GH20**	F	GAAGAGCCAAGGACAGGTAC	Saiki *et al,* 1988 (7)	408
	GH21	R	GGAAAATAGACCAATAGGCAG		

Statistical Analysis:

Statistical Analysis system (SAS) software was used for all statistical analysis continuous variables were expressed in mean ± standard deviation (SD). The Pearson's Chi-square test or Fisher exact test was used for comparing the categorical variable. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference.

Results:

Demographic data of pregnant women:

The age of the study population are shown in table (2).

Table (2): Age distribution of pregnant women.

Variable	No.	%
Age mean±SD (27±6.6 years)		
<20 years	19	19
20-29 years	40	40
30-39 years	30	30
≥40 years	11	11
Total	100	100.0

Molecular detection:

The results of the amplification of gpG gene by conventional PCR with using of specific set of primers sequences showed that gpG gene was present in 19 (19%) out of 100 placental tissue, and the PCR product of this gene was 120 bp. Figure (1).

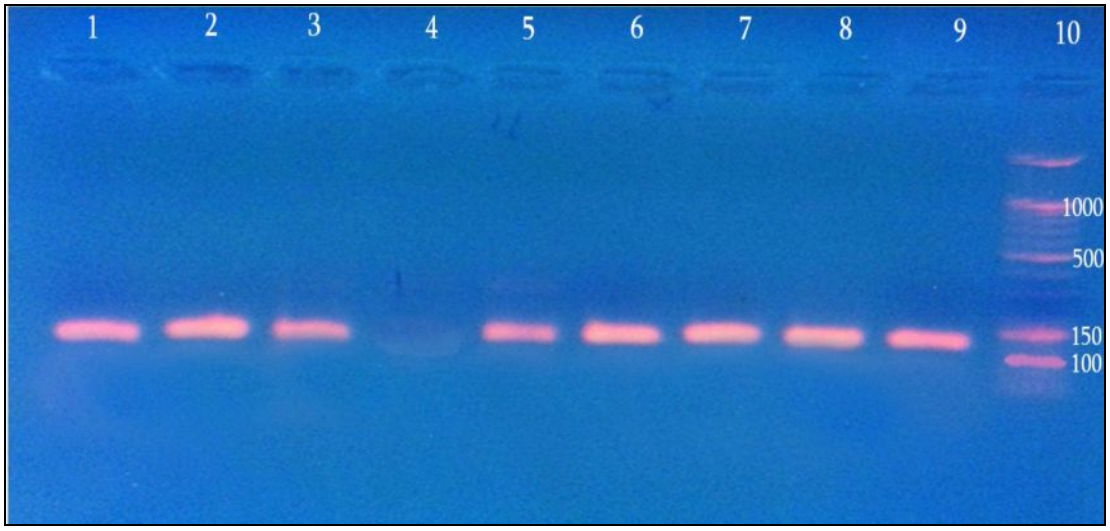

Figure (1): Gel electrophoresis (2% agarose, 7v/cm, 1.5hrs) of the PCR products, lane10: 100bp DNA ladder, lane (1, 2, 3, 5-9): Positive sample for herpes simplex viruses type 2 (120 bp); lane 7: Negative control.

The rate of HSV-2 among the age groups is presented in Table (3). In the current study 19 patients proved as HSV-2 positive out of 100, the highest rate was observed in age group (30-39) years in 7(18.9 %). The association between age and HSV-2 positive pregnant women was highly significant (P<0.001).

Table (3): The prevalence of HSV-2 among age groups.

Variable	Yes		No		χ^2	P
	No.	**%**	**No.**	**%**		
Age					29.8	**<0.001 Highly Significant**
<20 years	1	6.25	15	93.7		
20-29 years	5	9	50	90.9		
30-39 years	7	18.9	30	81.0		
≥40 years	6	54.5	5	45.4		

Analysis of patients that were subsequently proved to be positive for HSV-2 in association with adverse pregnancy outcome was studied and the results showed a highly significant association as shown in table (4) with P<0.05 (P=0.0001).

Table (4): The relation between adverse Pregnancy outcome and Positive HSV-2.

Type of adverse pregnancy outcome	**Positive**	
	No.	**%**
Abortion	8	42.1
Still birth	6	31.5
Congenital abnormalities	3	15.7
Total	19	100%
Chi-square	9.0294 **	
P-value	0.00216	

The association between gestational age at the time of miscarriage and the infection with HSV-2 in studied groups is presented in table (5). The highest fetal losses in studied group were occurred in the first trimester in 75 (75%) out of 100 patients. Regarding aborted cases in positive samples results demonstrated that

6 (31.5%) out of 19 gave abortion in first trimester and only 2 (36.9%) gave abortion in second trimester, Table (6).

Table (5): Distribution of studied groups in relation to gestational age and abortion.

Variable	No.	%
Abortion in first trimester		
Positive	75	75.0
Negative	25	25.5
Total	100	100.0
Abortion in second trimester		
Positive	7	7.0
Negative	93	93.0
Total	100	100.0

Table (6) Distribution of positive cases with gestational age and abortion.

Variable	Positive		Negative		χ^2	P value
	No.	%	No	%		
Abortion at 1st trimester	**6**	**31.5**	**13**	**68.4**	**7.79**	**0.005 Highly Significant**
Abortion at 2nd trimester	**2**	**10.5**	**17**	**89.4**		

The relation between HSV-2 positive cases (n=19) compared to the number of pregnancy and delivery outcome were presented in table (6), there was a statistical significant (P<0.006) association between number of pregnancy compared to infection with HSV-2 and highly significant differences (P<0.001) with pregnancy outcome.

Table (6): Positive cases for HSV-2 compared to pregnancy number and pregnancy outcome.

Variable	Yes		NO		χ^2	P value
	No.	%	No.	%		
Number of pregnancy					10.3	**0.006 Significant**
1-2	3	8.3	33	91.6		
3-4	5	10	45	90.0		
>4	11	33.3	22	66.6		
Pregnancy outcome					12.5	**<0.001 Highly Significant**
Normal	0	0.0	75	100.0		
Abnormal	19	15.2	106	84.8		

Discussion:

According to our knowledge, this is the first study of HSV infection detection in placental tissue using conventional PCR technique in Iraq.

Herpes simplex virus infection is one of the maximum not unusual sexually transmitted infections. Because the infection is not unusual in women at the reproductive age, it can be contracted and transmitted to the fetus during pregnancy and the newborn. Herpes simplex virus is an important cause of neonatal infection, which could result in death or long-term incapacity (8).

In the present study, the mean age of the patients who were suffered from spontaneous abortion and those from whom the specimens were collected was (27) years with range of (20-40) years, the results of

current study revealed that the infection with Herpes Simplex Virus raise steadily with age (9% among women aged 20-29 to 18.9 % among women aged 30-39 years**)**. There was a significant association (P<0.001) between infection with Herpes Simplex Virus and age. These findings are compatible to studies reported by Zainab Khalil. , *et al* and Biswas. *et al.,* (9,10).

Partially this may be related to the idea in our community of older women that they are old enough and had the experience to pass the pregnancy without the help provided by the antenatal care units, and also to the fact that older age mothers more porn to gets infections.

In the present study, the prevalence of HSV-2 in placental tissue was higher (19%) on comparison with other studies which have reported prevalence rates of 2.6–6.8%. **(**11-13**)**, and lower than prevalence in a study done by Nikiforos and Dimosthenis who reported the prevalence of HSV 2 in gestational tissue samples was 43.2% (14).

This discrepancy probably due to the methods of sample homogenization and the quantity or the physical status of viral DNA in the gestational tissue of pregnancy loss does not permit its easy detections likewise the differences in the size of samples in each study as well as selection bias can hardly explain this findings.

Our data showed significant association between Herpes Simplex infection and adverse pregnancy outcome range from stillbirth, abortion, congenital anomalies and neonatal death.

Which may reflect the possible role of Herpes Simplex-2 in adverse pregnancy outcome, our result comes with agreement with Iraqi studies reported by Zainab Khalil *et al.,* (9) and with abroad studies by Robb *et al* (15) they found that placental HSV positivity was significantly correlated with adverse pregnancy outcome (39% positive out of 200 cases)**.** Some previous studies showed no relation or low relation between HSV infection and adverse pregnancy outcome. Chow *et al.,* (16) examined 105 pregnant women using multiplex PCR to explore prevalence of HSV they did not detect any HSV infection, the result of their study may be due to low number of abortion cases. While in our study the selection group enrolled only women with bad obstetric history.

The present study reported the highest fetal losses in studied group (n=100) were occurred in the first trimester (75%). Regarding positive HSV 2 (n=19) results demonstrated that 12 (63.1%) out of (19) gave abortion in first trimester and only 7 (36.9%) gave abortion in second trimester. The methods by means of which HSV infection is concerned in being pregnant loss is a matter of speculation. It is able to be cause dysregulation of the Th1 to Th2 cytokine shift mechanism (17).

Reactivated endometrial HSV infection may be leads to a subsequent increase in natural killer cell activity which has been found to have great role in pregnancy loss (18). Our results come with agreement with Bujko M *et al*., (19); Smith and Robinson. (20).

Results obtained in this study showed a statistical significant (P<0.006) association between number of pregnancy compared to infection with HSV-2 and highly significant differences (P<0.001) with pregnancy outcome. The possible explanation for this results that multipara women more prone to different type of infection which may be lead to exaggerated immune responses such as elevated IL-17 responses which induce a lethal immune pathology during viral infection (18), this in accordance with the study of Bushra Al-rubaii., *et al.,* (21), Fabiana, *et al.,* (22).

In conclusion, HSV may play a significant role in pregnancy loss and can be delivery outcome-related factor. Its detection by sensitive molecular techniques would allow prompt therapeutic intervention in order to increase the possibility of a successful future pregnancy.

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