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Human *Papillomavirus* in Spontaneous Abortion

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First trimester spontaneously aborted products of conception were obtained from 100 Egyptian women. Placental tissue specimens from 100 women of full term normal vaginal deliveries were used as a control group. Their age ranged from 17-42 years (mean = 29.31). The age at marriage lies between 16-36 years. All samples were tested for HPV-DNA by polymerase chain reaction (PCR) amplification using one pair of general primers which allowed for the amplification of HPV types 16, 18, 31, 33, 52 & 58. These primers were designed to amplify E6/E7 gene junction sequences. Twenty nine out of 100 (29%) samples of aborted products of conception were positive for HPV E6/E7 sequences. In comparison, only one of the placental tissue specimens was positive. All the specimens were positive for β-globin DNA. Positive PCR products were confirmed and subtyped by restriction endonuclease enzymes digestion. Seventeen were type 16, eight were type 18 and four were non-typeable. The highest rate of HPV-DNA positivity was found in patients with age less than 25 years, and also in patients with young age at marriage (≤ 20 years). High parity and use of hormonal contraceptives were found to be associated with high frequency of HPV positivity in aborted patients. On conclusion, human *Papillomavirus* types 16 and 18 might have a role in the currence of the first trimester spontaneous abortion among Egyptian women.

INTRODUCTION

Human Papillomavirus (HPV) infection is the most common sexually transmitted disease in the world. More than 100 types of HPV exist and are classified as low-, intermediate-, or high-risk (Sanclemente and Gill, 2002; Munoz *et al.*, 2003). More than 30 HPV types infect the genital tract. The association between certain oncogenic (high-risk) types of HPV and cervical cancer is well established and accounts for an estimated 12% of the global cancer incidence in women. HPV-16 is the most prevalent type detected in cervical cancer. In addition to cervical cancer, HPVs are also associated with the malignant transformation of other mucosal and skin cancers (Burd, 2003; Xi *et al.*, 2003). Thus, combination of the malignant potential of HPV and its high prevalence confers to it an importance of generalized clinical and virological significance (Sanclemente and Gill, 2002; Burd, 2003; Xi *et al.*, 2003).

Pregnancy represents a risk factor for infection with HPV or lead to an increased replication of persisting virus either by hormonal factors or by immunosuppression (Armbruster-Moraes, 1994; Fife *et al.*, 1996; Szepietowska *et al.*, 2002).

The most common complication of pregnancy is spontaneous abortion, which is estimated to occur in 10-15% of pregnancies (Beucher *et al.*, 2003). Viral infections are likely the second most common cause of spontaneous abortion after genetic cause (Kutteh, 1999). Viral infections associated with pregnancy loss include: herpes simplex (Sifakis *et al.*, 1998); *Mumps*; *Rubella*; *Cytomegalovirus* (Cruz *et al.*; 2002); *Epstein bar virus*; *Enteroviruses* and *Parvovirus* (Nunoue *et al.*, 2002).

The aim of this work is to study the possible role of human *Papillomavirus* (HPV) in spontaneous abortion by assaying for HPV-DNA in first trimester spontaneously aborted products of conception.

MATERIALS AND METHODS

Study subjects

This study included 200 women who attended the obstetric and gynecology department of Ain-Shams university hospital, their age ranged from 18-40 years (mean = 29.31 years). They were divided into two groups:

1. Aborted group consisted of 100 women with unexplained spontaneous first trimester abortion. Products of conception from them were collected in sterile plastic tubes.
2. Control group consisted of 100 women with full term pregnancy in labour ended by normal vaginal deliveries. Placental tissue specimens from them also were collected in sterile plastic tubes.

Extraction of DNA

DNA was extracted from abortus and placental tissue specimens using high pure PCR template preparation kit (Roche Molecular Biochemicals) as recommended by the manufacturer.

Polymerase chain reaction (PCR)

Simultaneous amplification of HPV E6/E7 and β-globin DNA. The Co-amplification of human β-globin was used as an internal control for the integrity of the chromosomal DNA and the presence of PCR inhibitors in each sample.

PCR was done using one pair of general primers for detection of HPV types 16, 18, 31, 33, 52, and 58 amplifying E6/E7 sequences. The amplicon varied in lengths from 231 to 268 base pairs (bp) depending on the HPV type as the following: HPV-16 (238) bp., HPV-18 (268) bp., HPV-31(233) bp., HPV-33 (244) bp., HPV-52 (231) bp., HPV-58 (244) bp. (Fujinaga *et al.*, 1991; Zhang *et al.*, 1995). The sequence of these primers from 5' to 3' was: primer 1 (PU–1M) 5'-TGTCAAAA ACCG TTGTGTCC - 3'; primer 2 (PU – 2R) 5'-GAGCTGTCGCTTAATTGCTC - 3'. One pair of primers amplifying a 268 bp sequence of the human β-globin gene was used in separate reaction. The sequences of these primers from 5'to3'were:-Primer1 (GH2O) 5'-GAA GAGCCAAGGACAGGTAC-3'; Primer 2 (PCO4) 5' - CAACTTCATCCA CGT TCACC - 3'.

PCR was done in 50 µl contained 5 µl Taq DNA polymerase 10X buffer, 3 µl Mg Cl_2 (2.5 mM), 0.5 µl dNTPs (100 mM), 50 pmol of each Primer (1 µl), 2.5 U (0.5µl) Taq DNA polymerase and 300 ng (5 µl) and sterile nuclease free water to complete the reaction volume.

Using the thermal cycler Biometra model 2000, the following program was performed:- initial denaturation at 94°C for 5 minutes, then 94°C for 30 seconds, 52°C for 1 minute, and 72°C for 1 minute repeated for 40 cycles; finally, 72°C for 10 minutes as a chain elongation step.

The PCR products were analyzed by 2 % agarose gel electrophoresis and examined by U.V. transilluminator. The negative control was examined to exclude any source of contamination. The positive control was examined for the presence of sharp band at 238 or 268 bp. Samples were compared with controls. Photographs were taken by Polaroid camera (Sambrook and Russell, 2001).

Purification of amplified PCR products

After PCR amplification was complete, the total volume of each

PCR tube was adjusted to 100 µl and purified using PCR product purification kit (Roche Molecular Biochemicals) as recommended by the manufacturer.

Restriction endonuclease analysis

Pure PCR products positive for HPV DNA were undergone restriction endonuclease digestion for typing. Each restriction enzyme digestion of amplified fragments yields a distinctive fragment pattern for each HPV types. Three enzymes are used Ava II, Rsa I& Alu I for typing according to the manufacturer. The specific digestion pattern for each HPV type was detected by using 3% agarose gel for electrophoresis analysis and visualized by U. V. transilluminator and photographs were taken with Polaroid camera and gel documentation system

Statistical analysis

The results were tabulated and statistically analyzed using Chi-square test, regression and Pearson correlation at different probability values using computer program SPSS 11 (2001). Graphical representation of data was made through the Microsoft Excel computer program, 2000. The mean, standard deviation and standard error of the mean were obtained for numerical variables. For non-numerical variables, the frequency distribution and percentage were calculated.

RESULTS

Table (1) represents the frequency of detection of HPV DNA and β-globin DNA by PCR in aborted products and placental tissue specimens. HPV DNA was detected in 29% (29/100) in aborted products of conception and placental tissue specimens, in comparison to only 1% (1/100) of the placental tissue specimens. The difference was highly significant, $p < 0.001$. All tested samples were positive for β-globin DNA band at 268 bp. figure (1).

Figure (2) represents the ethidium bromide stained 2% agarose gel electrophoresis profile of positive PCR products for different HPV-types from products of conception.HPV-52 (lane1), HPV-16 (lanes 2, 4, 6&8), HPV-18 (lanes 3, 7 &9) HPV-58 (lane 5) approximately.

Table (2) represents the frequency HPV types as detected by restriction endonuclease digestion. HPV-16 was detected in 17/29 (58.62%) and HPV-18 in 8/29 (27.59%). The non-typaeble HPVs were 4/29 (13.79%).

Figure (3) represents the ethidium bromide stained 3% agarose gel electrophoresis profile of HPV-DNA type 16 PCR products before and after digestion with restriction enzymes. Lanes 1, 3&5 show positive bands at 238 bp before cleavage. Lanes 2, 4 &6 show positive bands for HPV-16 after cleavage into two bands (157 & 81 bp).

Figure (4) shows the agarose gel electrophoresis profile of HPV-DNA type 18 PCR products before and after digestion with restriction enzymes. Lanes 2, 4&6 show positive bands at 268 bp before cleavage. Lanes 1, 3&5 show positive bands for HPV-18 after cleavage into two bands (172 & 96 bp).

Table (3) represents the status in aborted products of conception in relation to different variables including:- Maternal age, aborted women below 25 years show the highest rate(41.37%) of HPV-DNA positivity, then decreased with the age increase which was highly significant, $p < 0.001$. Age at marriage, there was significant correlation ($p < 0.001$) between HPV-DNA positivity and

young age at marriage. Parity, HPV detection rate was significantly increased ($p < 0.001$) with number of pregnancies. Type of contraceptive, hormonal contraceptive users show statistically significant ($p < 0.001$) rate of HPV-DNA positivity.

Table (1): Detection of HPV-DNA and β-globin by PCR in aborted products of conception and placental tissue specimens.

Study group	No.	+ve PCR		P value
		HPV-DNA	β-globin	
Aborted	100	29 (29%)	100	<0.001*
Control	100	1 (1%)	100	

* Highly significant

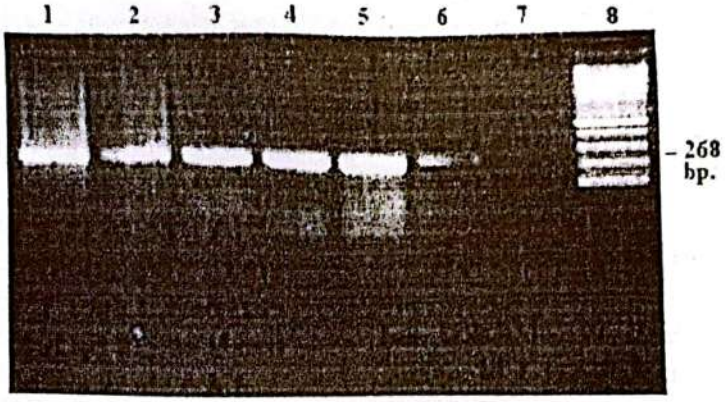

Fig. (1): Ethidium bromide stained 2% agarose showing the electrophoresis profile of β-globin DNA PCR products from placental tissue specimens. band at 268 bp. Lanes 1-5 show positive samples. Lane 6 shows positive control. Lane 7 shows negative control. Lane 8 shows molecular size marker 100 bp.

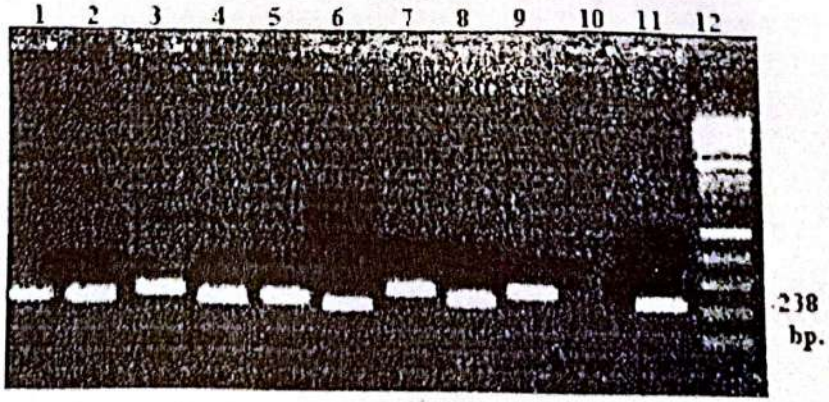

Fig. (2): Ethidium bromide stained 2% agarose gel electrophoresis profile of HPV-DNA positive PCR products from aborted products of conception. Lanes 1-9 show positive samples. HPV-52 (lane 1), HPV-16 (lanes 2, 4, 6, & 8), HPV-18 (lanes 3, 7, & 9) HPV 58 (lane 5) approximately. Lane 10 shows negative control. Lane 11 shows positive control. Lane 12 shows molecular size marker 100 bp.

Table (2): Detection of HPV-types by restriction endonuclease digestion

Study group	No. of +ve	No.(%)1 of HPV-type			P value
		HPV-16	HPV-18	Non- typeable	
Aborted	29	17 (58.62)	8 (27.59)	4 (13.79)	< 0.001*
Control	1	-	-	1(100)	

/ Calculated in relation to the total (29) positive cases.
* Highly significant

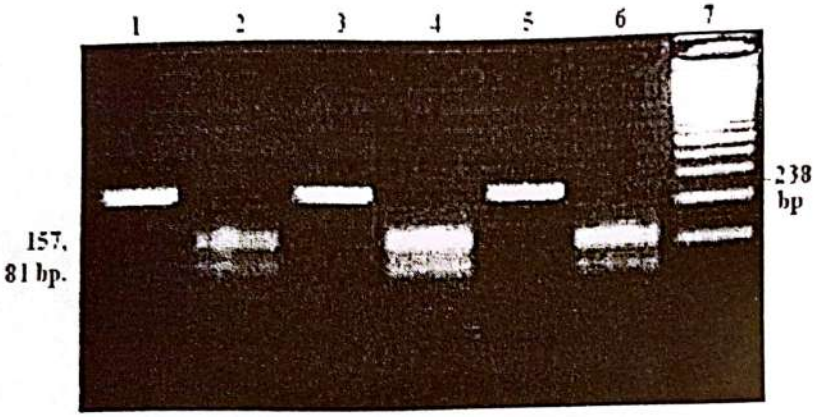

Fig. (3): Ethidium bromide stained 3% agarose gel electrophoresis profile of HPV-DNA type 16 PCR products before and after digestion with restriction endonucleases . Lanes 1, 3, 5 show positive bands for HPV-16 before cleavage at 238 bp. Lanes 2, 4, 6 show positive bands for HPV-16 after cleavage into two bands (157 & 81bp.). Lane 7 shows molecular size marker 100 bp.

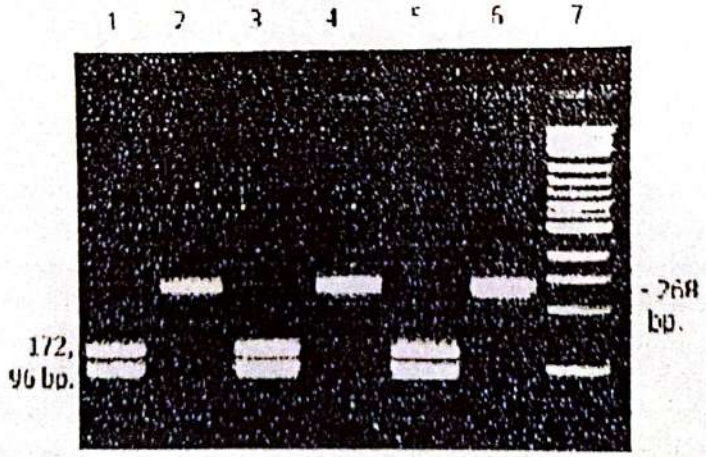

Fig. (4): Ethidium bromide stained 3% agarose gel electrophoresis profile of HPV-DNA type 18 PCR products before and after digestion with restriction endonucleases. Lanes 2, 4, 6 show positive bands for HPV-18 before cleavage at 268 bp. Lanes 1, 3, 5 show positive bands for HPV-18 after cleavage into two bands (172 & 96 bp.). Lane 7 shows molecular size 100 bp. Marker.

Table (4): The frequency of HPV-types in aborted products of conception in relation to maternal age, age at marriage, parity and method of contraceptive

Variable	No.	HPV status		P value
		No. of +ve (%)	No. of -ve (%)	
-Maternal age in years				
<25	26	12 (46.15)	14(53.85)	<0.001*
25-29	25	7 (28)	18(72)	
30-34	26	6 (23.07)	19(76.93)	
≥ 35	23	4 (17.39)	19(82.61)	
-Age at marriage in years				
<20	17	13 (76.47)	4(23.53)	<0.001*
20-24	42	11 (26.19)	31(73.81)	
25-29	32	4 (12.50)	28(87.50)	
≥30	9	1 (11.11)	8(88.89)	
-Parity				
0	17	2 (11.76)	15(88.24)	<0.001*
1	26	5 (18.52)	21(81.48)	
2	27	8 (30.77)		
>3	30	14 (46.67)		
-Contraceptives:				
Hormonal	43	19 (44.19)	24(55.81)	<0.001*
IUD	25	5 (20)	20(80)	
Condom	5	0	5(100)	
Non-users	27	5 (18.52)	22(81.48)	

* Highly significant.

DISCUSSION

Among known pregnancies, the rate of spontaneous abortion is approximately 10-20% and usually occurs between the 7th and 12th weeks of pregnancy (Beucher *et al.*, 2003).

Few studies in the last years reported that the human papillomavirus (HPV) might linked with spontaneous abortion (Hermonat *et al.*, 1997; Rabreau and Saurel 1997; Hermonat *et al.*, 1998; Matovina *et al.*,2004).

Many studies showed that the presence of cervical HPV infection is more frequent in pregnant women than in non pregnant ones with a prevalence of some distinct types especially 16, 18 and 33 (Armbruster-Moraes *et al.*,2000; de Villiers 2003; Moodley *et al.*, 2003).,

The pregnancy represents a risk factor for infection with HPV, or help replication of persisting virus either by hormonal factors or by immunosuppression (Armbruster-Moraes, 2000; Morales –Peza *et al.*,2000 ; Szepietowska *et al.*, 2002).

In the present study the role of HPV in the etiology of spontaneous abortion among the Egyptian women was evaluated by PCR and typed by restriction endonuclease digestion. We selected primers homologous to the E6/E7 ORF, and amplify HPV-DNA types 16, 18, 31, 33, 52, & 58 according to Fujinaga *et al.*, (1991) and Zhang *et al.*, (1995); which are useful for predicting the presence of multiple HPV infection in the first screening. HPV-DNA was detected by PCR in 29 out of 100 (29%) of the first trimester spontaneously aborted products of conception. While only one sample out of 100 (1%) placental tissue specimens gave positive result for HPV-DNA amplification by PCR.

Unfortunately, few studies targeting this issue were found and their results support our findings. Hermonat and his colleagues (1997) have used E6-E7 junction primer set to identify HPV-DNA by polymerase chain reaction in the aborted products of conception from first trimester spontaneously aborted cases in comparison to cases of elective abortion. They found that HPV-DNA was positive in 60% (15/25) of spontaneously aborted cases but 20% (3/15) of cases of elective abortion were positive for HPV-DNA. Rabreau and Saurel (1997) found HPV in the deciduous membranes of early abortion products in 13 out of 20 (65%) cases by in situ hybridization technique

But in these studies the frequency of HPV detection in aborted materials was much higher than those obtained in our study. This is probably due to the association of HPV infection with other risk factors including promiscuous sexual behavior, multiple partners, cigarette smoking and alcohol consumption; which are prominent in Europeans and Americans in contrast to Egyptians where the religious ethics control the behavior of the people.

Manavi and coworkers (1992) reported that HPV DNA type 16 or 18 was detected in aborted material in 30.8% by dot blot hybridization. This low percentage may be due to the lower sensitivity of the technique in comparison to PCR (Birner *et al.*, 2001). In addition, geographical differences and the limited number of examined cases in the other studies may be responsible for this variation.

However, Genest and his colleagues (1999) have reported an absence of association between spontaneous abortion and HPV detection as they assessed 30 cases of first trimester spontaneous abortion for HPV-DNA by in situ PCR using general primers derived from the L1 region, and restriction fragment polymorphism analysis. This difference in the results may be related to the accuracy of sampling, where they detected the human β-globin in only70% (21/30) of the studied cases. Also, they used different primer set and in situ PCR technique.

Sikstrom and his colleagues (1995) found that women with past history of spontaneous abortion showed a significant correlation with cervical HPV infection. Cervical tissue specimens from our patients were not available as the cervices were dilated and taken, also blood contaminations were difficult to be avoided and difficulty of follow up of these women.

In the present study HPV-DNA positive PCR products were confirmed and typed by restriction enzymes digestion. HPV-16 was detected in 17 out of 29 (58.62%) HPV positive

samples, while 8 (27.59%) HPV positive samples were HPV-18. The remaining 4 (13.79%) samples were non typaeble by the used restriction endonucleases. They are most probably HPV-type 52, or 58 (according to the specificity of the used primers) or may be new types. So the high risk HPV types especially type-16 followed by type-18 are the most types that might lead to spontaneous abortion. Findings of other studies can support this result.

Hermonat and his colleagues (1997) typed the positive PCR products for HPV-DNA from first trimester spontaneous abortion material by dot blot hybridization using probe of HPV-16 sequence. They found that all positive samples were HPV-16.

In the present study, HPV detected in the aborted material might indicate infection of embryonic tissues rather than contamination from the genital tract of the mothers. Findings of other studies can support this argument:- HPV has been detected by PCR amplification in 60% of amniotic fluid samples of pregnant women with cervical lesions indicating that HPV has the capacity to cross the placental barrier when already present in the cervix (Ambruster-Moraes *et al.*, 1994).

Pao and others (1995) used PCR technique with primers targeted the E6 and E7 genes of HPV types 16 and 18. Positive samples were typed by restriction endonuclease digestion and DNA sequencing. They found that HPV-18 was present in 2/11 (18%) hydatiform moles and 4/8 (50%) of choriocarcinomas (malignant trophoblasts) while 9 normal placentas were negative for HPV-DNA.

Hermonat and coworkers (1998) identified that the placental syncytiotrophoblasts were the predominant cell type being targeted by the HPV in cases of spontaneous abortion by in situ PCR.

Liu and others (2001) demonstrated that the HPV-16 genome replicates in 3A trophoblasts cell line in vitro with a peak at days 15-27. They also found that HPV-16 was fully active and carrying out its complete life cycle in trophoblasts.

These findings also will support the hypothesis of a possible link between HPV and spontaneous abortion.The role of HPV in promoting spontaneous abortion could be due to HPV DNA integration into the host genome, causing genomic alterations as a result of gene interruption and loss of chromosome heterozygosity (Gallego and Lazo, 1995).

Epple and his colleagues (2000) found significant association between low risk HPV infection of the pregnant women and elevated risk of chromosome aberration and abnormal fetal karyotyping.

The route by which HPV is able to infect the trophoblasts of the placenta is uncertain. An increase in viral copy number should enable the dissemination of virus to more distant sites from the primary lesion, such as the placenta and amniotic fluid. Virus may reach the amniotic cavity through the transcervical route (Armbruster-Moraes *et al.*, 1994).

Tseng *et al* (1992) reported that HPV in the peripheral blood mononuclear cells may be involved in the possible transplacental transmission of HPV, which may occur during the intermittent low-level viremia released from these cells.

Another possibility might be infection of oocyte or zygot before or soon after implantation. The precursor cells of the trophoblasts would be most exposed to infection during this period (Hermonat *et al.*, 1998). Finally, initial infection of the oocyte by sperm carrying a latent infection is

yet one more possibility, because such HPV-carrying sperm have been demonstrated (Lai *et al* 1996).

The present study revealed that several factors may be associated with the increased HPV positivity and this association was statistically significant. These factors include: maternal age, age at marriage, parity and hormonal contraceptives.

The maternal age was one of the factors affecting the frequency of HPV infection, the highest rate of positivity (41.37%) was observed in the age group less than 25 years, declined with increasing age to reach the lowest rate (13.79%) in the age group equal to or more than 35 years. This finding was corresponding well with other reports of epidemiological studies which have shown that the key risk factors for HPV detection include age and sexual behavior, showing that women 20-25 years of age had the highest prevalence of HPV infection (Hellberg *et al.*, 1993; Ho *et al.*, 1998).

Also our finding is in agreement with Armbruster-Moraes and others (1994) that showed an association between the presence of HPV-DNA sequence amplification from amniotic fluid and maternal age, with the highest rate of HPV detection in the age group ranged from 20-24 years.

de Roda-Husman and coworkers (1995) studied the age related pattern of HPV prevalence in cytomorphological normal cervical scrapes of pregnant and non-pregnant women by PCR. They found that the highest prevalence of HPV was present in younger ages in both groups. Franco and coworkers (1999) found that the incidence rate of high HPV infection was 2 times higher in women < 35 years old than in older women.

In our study, young age at marriage is another factor affecting the rate of HPV-DNA positivity, where the highest rate (44.83%) of HPV infection was observed among women have married at age below 20 years while the lowest rate (3.45%) was found at age ≥ 30 years. This is in agreement with other studies. Kenney (1996) reported that women at highest risk for acquiring HPV infection had initiated sex before age of 15 years. Kahn and others (2002) reported that short interval between menarche and age of first sexual intercourse is associated with subsequent HPV infection.

In this study, high parity is also one of the factors affecting the rate of HPV detection with the highest rate (48.27%) observed in cases with ≥ 3 pregnancies while the lowest rate of detection (6.90%) in nonlliparous women, this finding is in agreement with other studies.

Gopalkrishna and his colleagues (1995) studied the rate of HPV infection in cervical scrapes from pregnant and non pregnant women and they reported that there was an increased incidence of HPV infection particularly HPV-16 during pregnancy, also there was a gradual but statistically significant increase in the frequency of HPV infection with the increasing number of pregnancies. The highest rate of HPV positivity (66.7%) was observed during the fifth pregnancy and the lowest (31.1%) was among primigravidae.

Bayo and coworkers (2002) studied the role of HPV in the etiology of cervical cancer, their data provided further evidence on the role of HPV in cervical cancer and show that high parity and poor genital hygiene conditions were the main co-factors for cervical cancer in this population with prevalent HPV infection.

In this study, the use of hormonal contraceptives was associated with the highest rate (65.52%) of HPV DNA when compared with the use of other contraceptives or with the non users.

This is in agreement with Sikstrom *et al* (1995) and Smith *et al*,.(2003).

From this study, it is concluded that human papillomavirus types 16 and 18 might have a role in the occurrence of the first trimester spontaneous abortion among Egyptian women.

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