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Keywords:	HPV genotyping, HPV prevalence, PCR



**PREVALENCE OF DIFFERENT HPV TYPES AND ESTIMATION OF
PROGNOSTIC RISK FACTORS BASED ON THE LINEAR ARRAY HPV
GENOTYPING TEST**

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Abstract

The aim of the study was to evaluate the prevalence and risk factors of HPV in a gynecological population attending outpatient clinics using two new molecular tests.

The Amplicor HPV test and the Linear Array HPV Genotyping test (LA) were used for the detection of HPV DNA in 320 women. Multiple logistic regression was used to identify independent prognostic factors of HPV positivity.

The agreement between the two methods in terms of their qualitative results was 89.3% (kappa: 0.63). Based on the LA results, the overall prevalence of HPV DNA was 49.1% (95% CI: 43.5%, 54.7%). The prevalence of high-risk HPV types was 30.3%. The predominant types were HPV-6 (24.8%) and HPV-16 (20.4%). Among women with normal cytology, the prevalence of HPV was much higher in those presenting other findings, such as inflammation, than those without other abnormal findings (49.5% vs. 31.5%). On the basis of multivariate analysis, the risk of HPV infection was higher among women with multiple sexual partners (>3 vs. 1: OR=3.1, 95% CI: (1.5, 7.2)), Pap smear findings (low/high-grade lesions vs. negative: OR=2.8, 95% CI: (1.2, 6.5)), the presence of warts (yes vs. no: OR=3.0, 95% CI: (1.5, 6.3)) and no history of child birth (no vs. yes: OR=2.6, 95% CI: (1.0, 6.7)). Younger age was an additional risk factor for HPV infection with carcinogenic genotypes (OR for 1-year increase =0.93, 95% CI: (0.89, 0.98)).

Key words: HPV prevalence, HPV genotyping, PCR

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Introduction

Cervical cancer is the second most frequent female cancer worldwide resulting in about 270,000 deaths annually [Ferlay et al., 2005; Munoz., 2000; Parkin et al., 1990].The association of HPV infection with the development of cervical cancer and its precursor lesions has been well established [Munoz., 2000; Bosch et al., 2002; Clifford et al., 2003; Wallboomers et al., 1999; Wallin et al., 1999]. Recent studies have shown that HPV DNA can be found in 99.7% of all cervical cancers, with HPV types 16,18,45,31 being the most frequent [Wallboomers et al., 1999; Koutsky et al., 2002]. HPVs are non-enveloped double-stranded DNA viruses which belong to the Papillomaviridae family, which consist of more than 100 different HPV genotypes, 40 of which infect the genital tract [Chan et al., 1995; de Villiers et al., 2004; zur Hausen., 1991] and cause mainly transient infections [Evader et al., 1995; Rosenfeld et al., 1992; Hinchilfe et al., 1995]. Based on the etiological role in the progression to cervical cancer, 15 HPV genotypes have been classified as high risk, 3 genotypes (26,53,66) have been classified as probable high risk, 12 genotypes (such as 6,11 which are detected frequently in benign lesions such as condylomata accuminata) have been classified as low risk and 3 genotypes (34,57,83) are classified as of undetermined risk [Munoz et al., 2003].

Genotyping is, among others, important for epidemiological studies for the evaluation of HPV infections worldwide [van Hamont et al., 2006; Ferenczy et al., 2002; Meier et al., 2006]. The aim of the study was to evaluate the prevalence and risk factors of HPV among women who visited the outpatient clinics of four hospitals of Athens, for gynecological examination and Pap-test, using a linear assay HPV DNA test assessing the 37 most common anogenital HPV genotypes.

METHODS

Patients

320 cervical scrapes were collected from November, 2005 until March, 2008 from 320 apparently healthy women 16-71 years of age who attended the gynecological or the infectious diseases outpatient clinic of Aretaieion, A.Syggros, "P&A Kyriakou" Childrens' and Laiko hospitals of Athens, Greece. Many of the women had genital warts, abnormal Pap smears or other vaginal infections. The study was approved by the Institutional Review Board of Athens University Medical School and an informed consent from the patients was obtained.

Methods

The samples were collected in PreservCyt solution (Cytoc Corporation®, Marlborough, USA) with a Cervex-brush® (Rovers Medical Devices B.V., The Netherlands) and stored at 2-8°C according to the manufacturer's instructions. The cervical scrapes were examined by using two assays available commercially: the Amplicor® HPV test, and the Linear Array HPV Genotyping test (LA) (both from Roche Molecular Systems, Inc., Branchburg, NJ USA). The Amplicor HPV test is a qualitative in vitro test for the detection of 13 high risk HPV genotypes (16,18,31,33,35,39,45,51,52,56,58,59,68) while the LA is a qualitative in vitro test for the detection of 37 anogenital HPV genotypes (including high risk HPVs detected by the Amplicor HPV test) in cervical cells. Both tests are based on four major processes: specimen preparation, PCR amplification of the target DNA, hybridization of the amplified products to oligonucleotide probes and detection of the

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probe-bound amplified products by colorimetric determination [Gravitt et al., 2000; Davies et al., 2001].

HPV DNA was extracted from the samples using the Amplilute Liquid Media, (Roche Molecular Systems, Inc., Branchburg, NJ USA) according to the manufacturer's instructions. The Amplicor HPV test uses a pool of biotinylated primers which amplify an approximately 165bp sequence within the polymorphic L1 region of the genome of 13 high risk HPV genotypes.

The LA Genotyping test uses a pool of biotinylated primers designed to amplify an approximately 450 bp sequence within the polymorphic L1 region of the genome of 37 HPV genotypes. An additional 268 primer pair which targets the human β -globin gene is included in both assays to provide a control for cell adequacy, extraction and amplification. After completion of the PCR, amplified DNA is denatured and is added either into wells of a microwell plate which contain either HPV or β -globin probes or on strips which are covered with HPV and β -globin oligonucleotide probes.

Data analysis and epidemiological associations were based on Linear Array HPV Genotyping test. The prevalence of HPV was estimated as the proportion of women with HPV DNA positive test and the 95% confidence interval (95% CI) was also calculated. The chi-squared test or the t-test was used to examine the presence of association between HPV DNA status and the characteristics of the patient. Multiple logistic regression was used to identify independent prognostic factors for HPV positivity.

RESULTS

Study participants

The characteristics of the women participating in the study are shown in Table 1. Their age ranged from 16-71 years with a mean (SD) of 28.4 (8.7) years. Approximately one third of the women (31 %) had warts and 50.5% reported more than three lifetime sexual partners. The age of first sexual contact ranged from 13-34 years with a mean (SD) of 18.0 (2.6) years. Only 17.3% of the enrolled women had at least one child and 15.1% reported at least one abortion (Table 1).

Comparison of the Amplicor HPV test and the Linear Assay

The agreement between the two methods in terms of their qualitative results was 89.3% (kappa: 0.63). In one sample it was not possible to obtain a result by Amplicor HPV test. All Amplicor HPV negative samples ,except one, were either negative (75.2%) with the LA test or low risk genotypes (24.3%) were found (Table 5).

Prevalence of HPV

Taking into account all 37 genotypes, 157 women (49.1%) were found to be positive for any HPV type (95% Confidence Interval (CI): (43.6%, 54.6%)). Approximately one third of the infections (36.9%) involved only one HPV type while the prevalence of infections with two and three or more types was 33.8% and 29.3%, respectively.

The prevalence (95% CI) of high-risk HPV infections was 30.3% (25.3%, 35.7%).

The genotype which was identified most frequently among infected women was HPV6 (24.8%) followed by HPV16 (20.4%), HPV42 (17.8%), HPV51 (14.7%), HPVCP6108 and HPV31 (12.7%), HPV52 and HPV62 (12.1%), HPV66 (10.2%), HPV53 (8.9%), HPV84 (7.6%), HPV59 and HPV18 (7%), HPV56 and HPV61 (6.4%). (Table 2)

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Prognostic factors of HPV infection: Univariate analysis

The prevalence of HPV in women <25, 25-29, 30-34, 35-39 and over 40 years of age was 52.1%, 55.4%, 46.8%, 41.4%, and 29% respectively (p=0.104) (Fig.1). The prevalence of HPV increased with increasing number of sexual partners. More specifically, among women with one, two, three and more than three lifetime partners, the prevalence of HPV was 27.4%, 36.2%, 51.1% and 61.1%, respectively (p<0.001) (Table 3). According to the Pap test result, the prevalence of HPV was 100% for the high-grade smears (3/3), 62.9% for low-grade (44/70), 33.3% for the Atypical Squamous Cells of Undetermined Significance (ASCUS) smears (10/30) and 31.5% for the negative smears without any other findings (17/54) (p=0.001). In negative smears with other findings such as inflammation, trachilitis, fungi and condylomata the prevalence of HPV was 49.5% (51/103) (p=0.001) (Table 3).

The prevalence of HPV in women who had external genital warts was higher compared to those without warts (62% and 42.7% respectively, p=0.001). Among women who had given at least one birth, the prevalence of HPV was 29.1% (16/55) whereas in those with no births the corresponding rate was 53.6% (141/263), (p=0.001). HPV was detected at similar rates in smokers and non-smokers (52.8% and 46.7% respectively, p=0.282) (Table 3).

Considering only the high risk HPV genotypes, the detection rates in women <25, 25-29, 30-34, 35-39 and over 40 years of age were 34.7%, 32.6%, 31.9%, 20.7% and 12.9% respectively (Table 3). The prevalence of high risk types in women with one, two, three and more than three lifetime partners was 14.5%, 19.2%, 37.8%, and 37.6% respectively (p=0.002). The detection rates of one or more high risk genotypes in high grade, low grade, Atypical Squamous Cells of Undetermined Significance

(ASCUS), and negative Pap-smears without any other finding were 100% (3/3), 47.1% (33/70), 13.3% (4/30) and 14.8% (8/54) respectively. In negative smears with some other findings such as inflammation, trachilitis, fungi and condylomata the detection rate was 30% (31/103) ($p < 0.001$). In women who had external genital warts, high risk types were detected in 33% (33/100) of women ($p = 0.409$). High risk types were detected in 18.2% (10/55) of those who had given at least one birth whereas in those without a history of births the detection rate was 33% (87/263), ($p = 0.029$). Among smokers, high risk types were detected in 34.2% (55/161) of women and in non-smokers the respective rate was 27% (41/152) (Table 3).

Prognostic factors of HPV infection: Multivariate analysis

In multiple logistic regression, detection of any HPV was associated with presence of genital warts (OR=3.0, 95%CI: 1.5-6.3, $p = 0.003$), multiple sexual partners (>3 partners vs. 1: OR=3.2, 95%CI: 1.5-7.0 $p = 0.004$) and presence of low/high-grade lesions (OR=2.8, 95%CI: 1.2-6.5, $p = 0.014$) (Table 4). There was a marginal association between the absence of births and risk for HPV, i.e. women reporting no births were at higher risk of being HPV positive relative to those with children (OR=2.56, 95%CI: 0.99-6.66, $p = 0.054$) (Table 4).

Multiple logistic regression was also used to identify prognostic factors of HPV infection with high risk types. Older women had a reduced risk of being positive for a high risk HPV type (OR=0.9, 95%CI: 0.9-0.98, $p = 0.008$), while multiple sexual partners (3 partners vs.1) increased the risk by four times (OR=4.0, 95%CI: 1.4-12.0, $p = 0.012$). In women with low/high-grade lesions the risk of infection with a high risk type was increased compared to women with a normal Pap test (OR=5.6, 95% CI: 2.1-14.5, $p < 0.001$) (Table 4).

DISCUSSION

This is the first study on HPV prevalence conducted in Greece which is based on an up-to-date laboratory method. The main question was to estimate both the prevalence and the associated risk factors of HPV in a gynecological population attending outpatient clinics using two nucleic acid amplification-based tests.

Our sample allowed the estimation of the prevalence with a precision of 5.5%. To date, data on this topic in the Greek population are very limited. Patients' participation in the study was rather slow and a prolonged period for sample collection would be needed to attain the sample size goal which was 500 patients. The population studied does not represent the general population as it includes women who were attending the clinic for medical advice and treatment for vaginal infections. Of course there were women who underwent cytology testing as a routine examination. It should be noted that in Greece there is no organised screening programme so many women choose to have a Pap test while others had never been tested cytologically until symptoms developed.

The prevalence of the different HPV types was based on the Linear Array test. In addition all the samples were also examined by the Amplicor HPV test in order to evaluate the performance of the two tests. Both tests are based on nucleic acid amplification. The use of the LA results allowed an estimation of the prevalence of any HPV and of high-risk types as well as the associated risk factors.

The agreement between the two methods in terms of their qualitative results was 89.3% (kappa: 0.63). In one sample it was not possible to obtain a result by the Amplicor HPV test (Table 5). All Amplicor HPV negative samples ,except one, were either negative (75.2%) by the LA test or identified low risk genotypes (24.3%). This

indicates that the Amplicor test could be used as a screening test for high risk HPV types followed by the LA test which provides information not only on the involved genotype but also on the persistence with the same genotype, which is a major risk factor for progression to cervical cancer.

In the study by Mo et al (2008) the Amplicor test was compared with the Hybrid Capture II and since both assays did not differ in terms of sensitivity and specificity they could be used for the detection of high risk HPV DNA with similar efficacy in specimens collected in the PreservCyt solution. On the other hand, Sandri et al (2006) found that the Amplicor test provided a larger number of positive results in women with normal smears and this difference may be due to the fact that the Hybrid Capture II is characterized by a signal amplification step.

In this study, the prevalence of the HPV types among women attending outpatient gynecological clinics was found to be 49.1%. When only women with negative Pap test and the absence of other findings were considered, the corresponding prevalence was 31.5%. From the limited epidemiological data in Greece, Kroupis et al [2007] mentioned that the prevalence of HPV in an almost similar study group was 60% with types 16, 53 and 6 being the most frequent. In women with normal cytology the respective rate was 23.6%. Panotopoulou et al [2007] found a prevalence of 53.6% of HPV, with types 11 and 18 being the most frequent. It should be noted that the above study group differed from the present study as it involved a 10% of women with Squamous Cell Carcinoma. Nevertheless, in the women with normal cytology of that study the prevalence of HPV was 26.4%, whereas in another study conducted by Agorastos et al [1995] for asymptomatic women with normal cytology in Northern Greece, the respective prevalence was 36.3%. The higher detection rates of HPV observed in younger women, when compared to older women, falls with increasing

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age and this finding is in agreement with many other published studies [Jacobs et al., 2000; De Roda et al., 1995; Cuzick et al., 1999]. This can be a result of either reduced sexual activity and less high risk behavior factors such as unprotected sex or acquired immunity to HPV from past exposure [Burk et al., 1996a;]. Furthermore, older women were also found to have a reduced risk of infection with a carcinogenic HPV type.

It is known that there is a positive association between the number of sexual partners and the infection with HPV [Burk et al., 1996b; Tarkowski et al., 2004]. In the present study, multiparity increased the risk of harboring HPV by 3-4 times when compared with the women who declared a single partner.

The presence of carcinogenic HPV types is greater in abnormal smears and many studies have demonstrated that the prevalence of HPV increases in parallel with the severity of cervical lesions [Jacobs et al., 2000; Grce et al., 2001; Evans et al., 2006]. In terms of finding of HPV in normal smears, the results of the current study suggest that in women with negative smears but with inflammation, trachilitis, fungi and genital warts, the prevalence of high risk HPV types was greater than in those with negative smears without any other findings. In previous studies [Singh et al., 1995; Parashari et al., 1995] negative smears with inflammation were suspicious for HPV infection, whereas in the study of Arora et al., [2005] a 10% of women who had a negative Pap smear with inflammation had high-risk types HPV16 and 18.

The risk of detecting HPV in the cervix was three times higher when patients had genital warts. Other studies show that women with genital warts may be at an increased risk for harboring high risk HPV types [Mosciki et al., 2001] and have a Cervical Intraepithelial Neoplasia risk up to six times greater [Ward et al., 1994] than

women in the general population [Sellors et al., 2000] since external genital warts may be a marker of a relative deficiency in immune response [Shlay et al., 1998].

The absence of births in a woman doubles approximately the risk of infection with HPV. Several studies [Veress et al., 1996; Fife et al., 1996; Fife et al., 1999; Nobbenhuis et al., 2002] show that during pregnancy the rate of HPV detection increases due to hormonal changes or immunological factors which lead to activation of HPV. This activation may trigger the immune system to respond and thus clear the virus.

Smoking did not seem to influence the prevalence of HPV in this and other studies [Tarkowski et al., 2004; Deacon et al., 2000]. Other studies show that smoking is not only associated with an increased prevalence of HPV, but also that the prevalence of HPV increases with smoking [Vaccarella et al., 2008;] whereas Hildesheim et al [1994] and Ho et al [1998] found that smoking was a potentially protective factor against persistent HPV infection.

The availability of HPV vaccines may change the future epidemiology of HPV. It would be of interest to monitor this natural experiment by periodical studies on the prevalence of HPV types in cervical cells.

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Table 1: Characteristics of 320 healthy women with cervical scrapes.

AGE (years)	
<25	121 (37.8%)
25-29	92 (28.8%)
30-34	47 (14.7%)
35-39	29 (9.1%)
40+	31 (9.7%)
NUMBER OF LIFETIME SEXUAL PARTNERS, n(%)	
1	62 (19.9%)
2	47 (15.1%)
3	45 (14.5%)
>3	157 (50.5%)
NUMBER OF DELIVERIES, n(%)	
0	263 (82.7%)
1	25 (7.9%)
2	23 (7.2%)
3	7 (2.2%)
NUMBER OF MISCARRIAGES, n (%)	
0	304 (95.6%)
1	9 (2.8%)
2	2 (0.6%)
3	3 (0.9%)
NUMBER OF ABORTIONS, n (%)	
0	270 (84.9%)
1	32 (10.1%)
2	10 (3.1%)
3	4 (1.3%)
4	2 (0.6%)
PRESENCE OF WARTS, n (%)	
NO	218 (69.0%)
YES	100 (31.0%)
SMOKING, n (%)	
NO	152 (49.0%)
YES	161 (51.0%)
AGE OF FIRST SEXUAL CONTACT	
Mean (SD)	18 (2.6)
Range	13-34

Table 2. Prevalence of HPV types in single and any infections

HPV type	Single infections (N=58)	Any infection (N=157)
6	15.5%	24.8%
11	0.0%	0.6%
16	15.5%	20.4%
18	1.7%	7.0%
26	0.0%	0.0%
31	12.1%	12.7%
33	0.0%	2.6%
35	0.0%	1.9%
39	0.0%	2.6%
40	0.0%	0.0%
42	5.2%	17.8%
45	0.0%	0.6%
51	5.2%	14.7%
52	3.5%	12.1%
53	1.7%	8.9%
54	1.7%	3.2%
55	3.5%	3.2%
56	1.7%	6.4%
58	0.0%	4.5%
59	5.2%	7.0%
61	1.7%	6.4%
62	3.5%	12.1%
64	0.0%	1.3%
66	5.2%	10.2%
67	3.5%	5.7%
68	1.7%	3.8%
69	0.0%	0.6%
70	1.7%	0.6%
71	0.0%	0.6%
72	0.0%	0.6%
73	0.0%	4.5%
82	0.0%	0.0%
81	0.0%	3.8%
83	0.0%	3.2%
84	5.2%	7.6%
IS39	0.0%	0.6%
CP6108	5.2%	12.7%

Table 3: Univariate associations between potential risk factors and HPV or high risk prevalence of HPV.

PROGNOSTIC FACTORS	N	HPV (+) (any type)		HPV (+) (high risk types)	
		n(%)	P	n (%)	P
AGE					
<25	121	63 (52.1)	0.104	42 (34.7)	0.253
25-29	92	51 (55.4)		30 (32.6)	
30-34	47	22 (46.8)		15 (31.9)	
35-39	29	11 (41.4)		6 (20.7)	
40+	31	9 (29.0)		4 (12.9)	
No. OF LIFETIME SEXUAL PARTNERS					
1	62	17 (27.4)	<0.001	9 (14.5)	0.002
2	47	17 (36.2)		9 (19.1)	
3	45	23 (51.1)		17 (37.8)	
>3	157	96 (61.1)		59 (37.6)	
PAP-TEST					
Negative	54	17 (31.5)	0.001	8 (14.8)	<0.001
Negative+other	103	51 (49.5)		31 (30.1)	
ASCUS [*]	30	10 (33.3)		4 (13.3)	
LGSIL [†]	70	44 (62.9)		33 (47.1)	
HGSIL [‡]	3	3 (100.0)		3 (100.0)	
WARTS					
NO	218	93 (42.7)	0.001	62 (28.4)	0.409
YES	100	62 (62.0)		33 (33.0)	
DELIVERIES					
NO	263	141 (53.6)	0.001	87 (33.1)	0.029
YES	55	16 (29.1)		10 (18.2)	
SMOKING					
NO	152	71 (46.7)	0.282	41 (26.1)	0.168
YES	161	85 (52.8)		55 (34.1)	

* ASCUS:Atypical Squamous Cells of Undetermined Significance

† LGSIL:Low Grade Squamous Intraepithelial Lesion

‡ HGSIL:High Grade Squamous Intraepithelial Lesion

Table 4: Multiple logistic regression model: Prognostic factors of HPV infection (any infection and high risk HPV)

	ANY HPV			High Risk HPV		
	OR	95% CI	P	OR	95% CI	P
Age(/year)	0.97	(0.93,1.02)	0.275	0.93	(0.89,0.98)	0.008
Number of lifetime sex. partners						
2 vs. 1	1.20	(0.46,3.14)	0.710	1.50	(0.49,4.55)	0.473
3 vs 1	2.27	(0.86,6.00)	0.098	4.04	(1.36,12.01)	0.012
>3 vs 1	3.19	(1.46,7.01)	0.004	2.92	(1.19,7.15)	0.019
Pap-test						
Ascus/Negative	0.70	(0.24,1.99)	0.503	0.86	(0.22,3.26)	0.819
Negative+other						
Finding/Negative	1.40	(0.64,3.10)	0.401	2.43	(0.98,5.98)	0.054
LGSIL,HGSIL/Negative	2.84	(1.24,6.52)	0.014	5.62	(2.17,14.52)	<0.001
Warts (Yes/No)	3.02	(1.46,6.27)	0.003			
Deliveries (No/Yes)	2.56	(0.99,6.66)	0.054			

ASCUS:Atypical Squamous Cells of Undetermined Significance

LGSIL:Low Grade Squamous Intraepithelial Lesion

HGSIL:High Grade Squamous Intraepithelial Lesion

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Table 5: Comparative performance between the Linear Array and Amplicor HPV test on 319 samples.

LINEAR ARRAY	AMPLICOR (+)	AMPLICOR (-)	TOTAL
High risk	96 (82.1%)	1 (0.5%)	97
Low risk	12 (10.3%)	49 (24.3%)	61
Negative	9 (7.6%)	152 (75.2%)	161
TOTAL	117	202	319

Figure 1. Prevalence of HPV according to age

