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CERVICAL HUMAN PAPILLOMAVIRUS INFECTION IN SERBIA: RISK FACTORS, PREVALENCE AND GENOTYPE DISTRIBUTION IN WOMEN WITH NORMAL CERVICAL CYTOLOGY

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Abstract - Sexual behavioral and other risk factors and the prevalence of genital *human papillomavirus* (HPV) infection are very important for the monitoring of HPV infection and cervical cancer prevention strategies. The purpose of this study was to determine the risk factors, prevalence of cervical HPV infection and genotype distribution among asymptomatic young women with normal cytology in Serbia. A total of 204 consenting young women were enrolled in this study and interviewed about risk factors. The presence of HPV DNA was assessed using the PCR method. HPV genotypes were identified by direct sequencing. Cervical HPV infection was detected in 19.1% of women. Out of nine identified HPV genotypes, types 16 and 52 were the most frequent. A significant association was found only between the number of sexual partners and HPV positivity ($p<0.05$). The obtained results showed the high prevalence of high-risk HPV types among young women in Serbia.

Key words: Cervical HPV infection, risk factors, HPV genotypes

INTRODUCTION

Cervical cancer is the leading cause of cancer mortality among women in Serbia, where the incidence of cervical cancer is among the highest in Europe. According to the data of the Cancer Registry of Serbia, from 1973-1982 the incidence was 14.7 to 18.2 per 100.000, while in the year 2002 the age-standardized incidence rate of cervical cancer was 27.2 per 100.000. Recent data from 2003-2009 show incidence rates from 21.6 to 27.1 per 100.000 (Kesic et al., 2007; Cancer Registry of Serbia, 2011).

Throughout the last decades, a strong and causal association between HPV and cervical cancer has been demonstrated and established (Bosch et al., 2008). To date, 120 HPV genotypes have been identified and according to the oncogenic potential, they are classified into 2 groups. High-risk genotypes are associated with cervical cancer while low risk types are associated with genital warts. All genotypes can cause an abnormal Pap test (Bernard et al., 2010; Clifford et al., 2006).

Genital HPV infection is one of the most frequent sexually transmitted infections. Usually it is without symptoms and in most cases the immune system and in particular cellular immunity, clears the infection, while in some cases persistent infection is established. The establishment of persistent infection

is a key point in the development of cervical carcinoma. The peak incidence of HPV infection occurs in adolescents and young women, while cervical cancer typically follows 20-30 years later (Baseman and Koutsky, 2005; Trottier and Franco, 2006).

Risk factors for genital HPV infection can be classified as factors for HPV acquisition and cofactors for the progression of infection and development of cervical cancer. The most important factors for HPV acquisition are younger age and sexual behavioral factors such as an earlier age of initiating sexual intercourse, the lifetime number of sexual partners and the sexual practices of male partners. The most important cofactors for the progression are immunosuppression, other infectious agents (especially sexually transmitted infectious agents like *Herpes simplex virus* type 2, *Chlamydia trachomatis*, etc.), smoking, parity, oral contraceptives, dietary factors and genetics (Trottier and Franco, 2006; Bosch et al., 2008). Therefore, the determination of sexual behavioral patterns and other risk factors, as well as the prevalence and HPV genotype distribution, is very important for the defining and monitoring of HPV infection and cancer prevention strategies (Bosch et al., 2008).

Based on the epidemiological data that the peak incidence of genital HPV infection is in young women, the aim of this study was to determine the risk factors, prevalence of cervical HPV infection and genotype distribution among asymptomatic young women with normal cytology in Serbia.

MATERIALS AND METHODS

Study population and specimen collection

This study was reviewed and approved by the Ethical Committee, Faculty of Medicine, University of Belgrade. A total of 204 consenting women aged 19 to 31 years (mean age 23.08±2.46) attending Gynecology Department, University Policlinics in Belgrade, were enrolled in this study. Inclusion criteria for the women recruited for this study were normal cervical cytology and the absence of genital clinical manifestations. Participants were interviewed using a questionnaire about sexual behavior and lifestyle.

Cervical cell samples, used for HPV testing, were collected with an endocervical brush and placed in 5 ml of sterile phosphate-buffered saline, refrigerated and transported to the laboratory.

HPV DNA amplification and detection

Samples were centrifuged (3000 g for 10 min) and the supernatant was removed. The pellet was used for DNA extraction with a QIAamp DNA Mini Kit (QIAGEN Inc., CA, USA) according to the manufacturer's instructions.

Amplification of HPV DNA was carried out with the consensus primer pair MY09/MY11 for the HPV L1 gene (Gravitt et al., 2000). PCRs were performed in a 25 μL volume reaction mix containing Qiagen Taq PCR Master Mix-250U (QIAGEN Inc., Valencia, CA, USA), 1 μmol of each primer and 5 μl of extracted DNA. The PCR conditions were as follows: initial denaturation at 95˚C for 5 min was followed by 40 cycles: 30 s at 94˚C, 30 s at 58˚C and 1 min at 72˚C, and a final extension of 20 min at 72˚C. The specific 450 bp band for HPV DNA was detected by agarose gel electrophoresis (Gravitt et al., 2000).

HPV genotyping

HPV genotypes were identified by the direct sequencing method. PCR products were purified with a QIAGEN MinElute PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturer's instructions. The purified PCR products were subsequently sequenced with Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using PCR primers as sequencing primers. Sequencing reactions were analyzed on the ABI Prism 310 Genetic Analyzer.

The obtained nucleotide sequences were aligned and compared with documented virus sequences available in the GenBank database using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). The

Table 1. Features of 204 patients included in this study group

Risk factors	Mean (Range)	Std. Deviation	No.	%
Age	23,08 (19-31)	2,459	-	-
Menstrual cycle				
Irregular	-	-	14	6.9
Regular	-	-	190	93.1
Age of the first intercourse [a]	18,88 (14-27)	2,019	-	-
No. of lifetime partners [b]	3,00 (1-15)	2,338	-	-
Age difference of male partner [b]	4,52 (0-20)	3,519	-	-
Use of condoms[c]				
Always	-	-	42	21.2
Some use	-	-	119	60.1
Never	-	-	37	18.7
Frequency of gynecol. examinations[d]				
Once or more than once a year	-	-	171	87.7
Less than once a year	-	-	24	12.3
No. of pregnancies[e]				
0	-	-	176	89.8
1-4	-	-	20	10.2
No. of abortions[f]				
0	-	-	180	91.4
1-3	-	-	17	8.6
Smokers [b]	-	-	64	32.0

[a] Reported for 199 patients
[b] Reported for 200 patients
[c] Reported for 198 patients
[d] Reported for 195 patients
[e] Reported for 196 patients
[f] Reported for 197 patients

nucleotide sequence was assigned to an HPV type if it corresponded in more than 95% in 350-400bp with a known HPV genotype (Remmerbach et al., 2004; Lee et al., 2007).

Statistical analysis

Statistical analysis was performed with SPSS version 17.0. using the Chi-square, t test and Man-Whitney U test as appropriate. Differences with a *p*-value of <0.05 were considered to be significant.

RESULTS

At the time of enrollment, the female study participants were on average 23 years old (Table 1). Most of them had regular menstrual cycle (93.1%). The mean age at first intercourse was 18.8 (range 14-27), the average number of partners was 3 (range 1-15) and the average age difference of male partners was 4.5 (range 0-20). Only 21.2% of women enrolled in this study always used condoms. Two thirds of the interviewed women visited gynecologists once or

Table 2. The association between risk factors and HPV positivity

Risk factors	HPV positive No. of patients	HPV negative No. of patients	Statistical analysis
Age at the first intercourse[a]	38	161	t= -1.250; df=47.76 $p>0.05$
No. of lifetime partners[b]	38	162	Z=-2.76 **$p<0.01$***
Age difference of male partners[b]	38	162	Z=-1.662 $p>0.05$
Use of condoms[c]			
Always	5	37	
Some use	27	92	X^2=2.588; df=2 $p>0.05$
Never	6	31	
No. of pregnancies[e]			
0	35	142	X^2=0.000; df=1 $p>0.05$
1-4	3	16	
No. of abortions[f]			
0	35	145	X^2=0.000; df=1 $p>0.05$
1-3	3	14	
Smokers [b]			
Yes	17	47	X^2=3.498; df=1 $p>0.05$
No	21	115	

[a] Reported for 199 patients
[b] Reported for 200 patients
[c] Reported for 198 patients
[e] Reported for 196 patients
[f] Reported for 197 patients

more than once a year. There were 32% of smokers in our study group (Table 1).

Out of the 204 women, HPV DNA was detected in 39 (19.1%). The prevalence rates of type-specific infections are presented in Fig. 1. Out of 9 identified HPV genotypes, types 16 and 52 were the most frequent (23% and 15.4%, respectively) and together accounted for 38.4% of all isolates. This group was followed by HPV types 33, 42 and 58, which all appear with the frequency of 7.7%. All other types were detected at frequencies ≤5%. According to the cervical oncogenicity, high-risk HPV types were detected in 64.1% of HPV-positive women. In 25.6% of the HPV-positive women, genotypes were not identified due to the appearance of numerous ambiguous or overlapping peaks in the DNA sequencing tracings, indicating the possibility of the presence of more than one HPV genotype.

The evaluation of sexual behavioral risk factors showed that the presence of HPV infection was not associated with the age at first intercourse or the age of the partner ($p>0.05$) (Table 2). However, significant association was found between the number of sexual partners and HPV positivity ($p<0.05$).

The analysis of other risk factors revealed no significant association with HPV positivity ($p>0.05$) (Table 2).

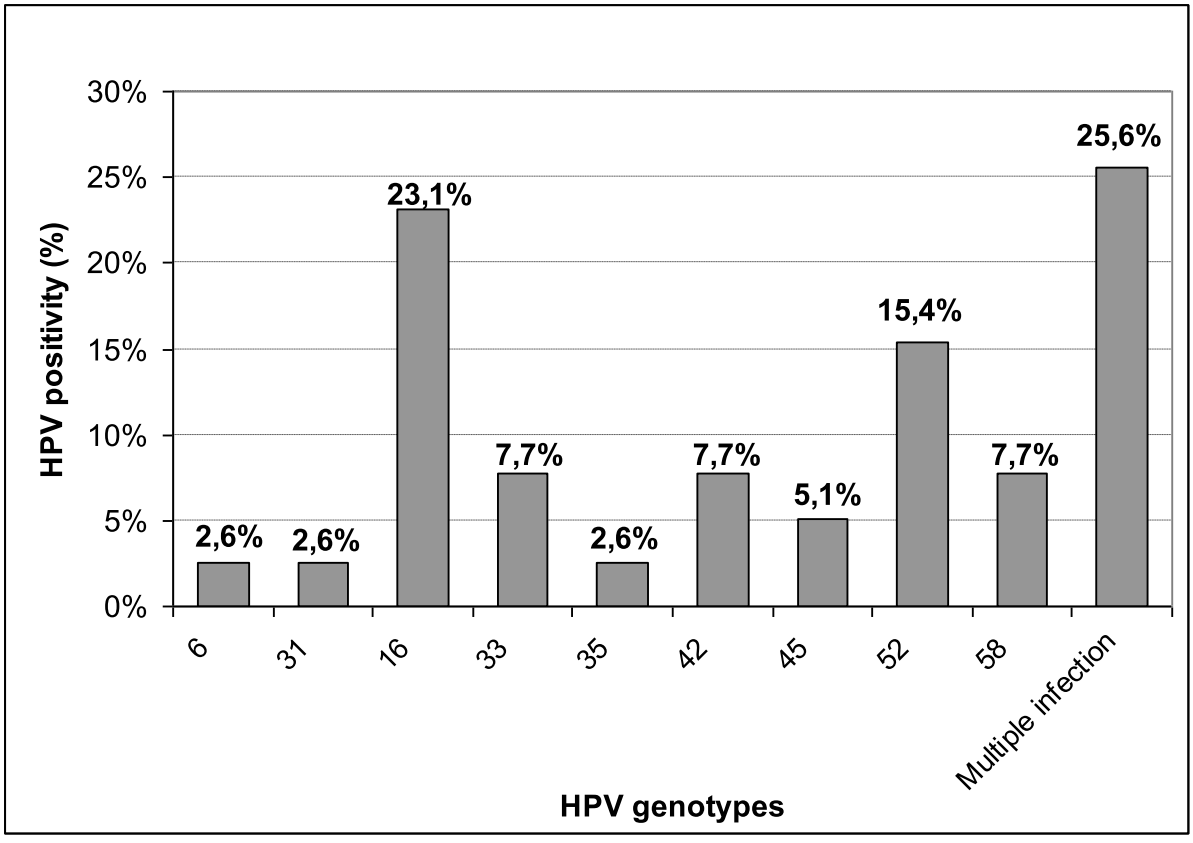

Fig. 1. Distribution of HPV genotypes

DISCUSSION

The recognition that HPV infection is essential for the development of cervical carcinoma has led to the introduction of HPV testing in cervical cancer prevention programs. It has been suggested that within cervical screening programs, HPV testing could be suitable for primary screening in the absence of, or in conjunction with cytology, management and triage of women with low-grade disease and test of cure of treatment of high-grade disease (Cuschieri and Cubie, 2005; Cuzick et al., 2008).

The most sexually active women will be infected with one or more genital HPV types at some point during their lifetime. The most comprehensive data about HPV prevalence are in women with normal cytology because the majority of women with any cytological abnormality are known to be HPV positive (Baseman and Koutsky, 2005; Bosch et al., 2008).

The prevalence of cervical HPV infection varies greatly worldwide. Recent meta-analytical studies have shown that in Europe the overall prevalence of HPV infection in women is 9.7%, while in sub-Saharan Africa it is much higher (22.5%). Furthermore, it has been shown that the prevalence of HPV is age-dependent, with a peak in young sexually active women (<25 and 25-34 years) (Clifford et al., 2005; Bruni et al., 2010).

In this study, HPV prevalence was 19.1% in young women with normal cervical cytology. This result is very similar to HPV prevalence in other eastern and southern European countries in a similar age group and in women with normal cervical

cytology (WHO/ICO Information Centre on HPV and Cervical Cancer, 2010; Bruni et al., 2010).

In addition, comprehensive meta-analysis showed differences in the worldwide distribution of HPV genotypes, where the most common HPV type in either single or multiple infections was HPV16, followed by HPV42, HPV58, HPV31, HPV18, HPV 56, HPV81, HPV35, HPV33, HPV45 and HPV52. Type 16 was twice as frequent as any other high-risk type in all regions except in sub-Saharan Africa, where HPV35 was equally common. In Europe, HPV16 was present in 25.5%, followed by HPV31 (9%) (Clifford et al., 2006; Bruni et al., 2010).

The distribution of HPV genotypes identified in this study population, as well as the prevalence of high-risk genotypes (64.1%), is similar to the genotype distribution in other European countries, where the most common type is HPV16 (23%). However, the distribution of other high-risk HPV types (HPV52, HPV58 and HPV45) is higher in this study group compared to other European countries.

Although HPV 18 is reported to be among common high-risk infections in other countries, this type was not detected in our study population.

It has been suggested that infection with multiple HPV types is associated with an increased risk of disease progression. It is not clear if this observation is due to differences in host susceptibility, interaction between viruses or the independent probability of progression associated with each viral type. The prevalence of multiple infections is up to 35% of HPV-positive samples from patients with advanced cytological disorders, whereas multiple genotypes are less prevalent in carcinoma patients (Baseman and Koutsky, 2005; Molijn et al., 2005).

In our study population, cervical infections with more than one HPV type were found in 25.6% of HPV-positive women. This result is very similar to those of a recent meta-analytical study that showed that about 20% of HPV-positive women with normal cervical cytology have had infections with multiple HPV types (Bruni et al., 2010).

Numerous risk factors for HPV infections have been identified. The determination of sexual behavior patterns is fundamental for understanding HPV transmission dynamics (Burchell et al., 2006; Bosch et al., 2008).

Various epidemiological studies have shown that sexual debut usually occurs in the late teens between 15 and 24 years, usually around the age of 20 (Burchell et al., 2006; Vaccarella et al., 2006; Bosch et al., 2008). In our study, the average age of the first intercourse was 18.8, and most of the participants had had sexual debut between 17 and 20 years of age (74.4%), which is consistent with the data from these studies. However, there was no significant association between the age at first intercourse and HPV positivity.

The most consistent risk factor for HPV acquisition is an increased number of sexual partners as well as the age difference of the male partners (Burchell et al., 2006; Vaccarella et al., 2006; Bosch et al., 2008). This is supported in this study by the observed significant association between HPV positivity and the number of sexual partners, however, the presence of HPV infection was not associated with the age of the partner.

The significant association between HPV positivity and other risk factors (the use of condoms, number of pregnancies and abortions, smoking) are not shown in this study.

As a result of increased awareness of the rising incidence and mortality of cervical cancer in Serbia and the importance of early detection and treatment of this disease, the Ministry of Health of Serbia nominated a National Committee for the Prevention of Cervical Cancer to develop and implement a National Program for cervical cancer screening with the technical support of the World Health Organization (Nikula et al., 2009).

The obtained results of HPV prevalence, genotype distribution and risk factors for HPV acquisition, as well as the results of future studies with a larger cohort, may contribute to the development of a cervical carcinoma prevention program in Serbia.

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