

PREVALENCE AND GENOTYPING OF THE HUMAN PAPILLOMAVIRUS IN THE CERVICAL SPECIMENS AMONG WOMEN OF SOUTHERN CROATIA (DALMATIA COUNTY)

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SUMMARY

Aim: The aim of this study was to evaluate the prevalence of HPV types in the female population with either normal or abnormal Pap smears in Southern Croatia (Dalmatia County) and to evaluate the distribution of HPV types and their association with different cytological findings.

Methods: During one year period, we routinely screened 1,160 women for HR HPV at the Educational Public Health Institute of Split and Dalmatia County, by using Hybrid Capture 2 HPV DNA test. Only HR HPV positive specimens determined by HC2 were further genotyped by polymerase chain reaction (PCR) based assay at the Department of Pathology and Forensic Medicine, Split.

Results: Out of the total number of tested women, 406 (35%) of them were positive to HR HPV. The following frequency was observed: HPV 16 in 10.8%, HPV 18 in 7.8%, HPV 31 in 2.3%, HPV 33 in 1.1%, HPV 52 in 0.9%, HPV 45 in 0.6%, and HPV 59 in 0.4% specimens.

Conclusion: The data obtained from our study indicate that HPV 16 is the most common HPV type found in the cervical specimens among women of Southern Croatia, as in most studies worldwide, followed by HPV 18 and HPV 31. The positivity rates in our study slightly differed for HPV types 18 and 31 from those found in the other studies conducted in Northern Croatia. These suggesting geographic and environmental factors are related to the prevalence of HPV types. We consider it very important to gather information on regional variations in the HPV type spectrum.

Key words: HPV, high-risk genotypes, Dalmatia County, Croatia

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INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide. There are about 490,000 newly registered cases every year. There are about 270,000 cervical cancer deaths in the world annually, 85% of which take place in developing countries because of absent or poor screening programmes. Infection with Human Papillomavirus (HPV) is the main cause of cervical cancer. HPV is also one of the most frequent causal agents of sexually transmitted diseases. It can cause various clinical conditions, from asymptomatic infections to benign and malignant diseases of the genital region. Luckily most HPV infections are spontaneously cleared by host immune response. At present, there are more than 200 known HPV types. More than 40 types attack the epithelium of the genital tract, and are transmitted mainly through sexual intercourse. The types that are most commonly related to the development of cervical cancer, so called oncogenic or high-risk types (HR HPV), are: 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70 (1–3).

Genital HPV infection is rarely reported (there is no legal obligation). While its worldwide prevalence is assumed to be higher than 20 million, the incidence of HPV infection in the United States ranges from 1 million to 5.5 million per year (2).

The prevalence of HPV DNA in cervical cancer cases is 99.7% (3), compared to 5% to 20% of cervical specimens in women without cervical cancer (4–6). HPV 16 and HPV 18 are associated with 70% of cervical cancer worldwide (1, 7). The prevalence and distribution of the different HPV types in cervical samples with normal cytology and low grade cervical lesions vary around the world (2).

Croatia has a lower cancer incidence than most Central and Eastern European countries. There are about 355 new cases (incidence rate 13.3/100,000), and about 100 deaths each year (mortality rate 5.0/100,000 women) (8).

Very little is known about prevalence and distribution of HPV types in Croatia. There are a few studies from Northern Croatia about HPV prevalence related only to cervical specimens of women with abnormal Pap smear (9–11). There is only one small scale study for Southern Croatia (Split and Dalmatia County) conducted by Kaliterna et al. related to cervical samples of women with either normal or abnormal Pap smear (12).

We decided to expand our research on this topic in a larger and more detailed study. The aim was to evaluate the prevalence of HPV types in the female population with either normal or abnormal Pap smears in Southern Croatia (Dalmatia County). To go further with this study we wanted to evaluate the distribution

of HPV types and their association with different cytological findings (Pap smears).

MATERIALS AND METHODS

The study involved a total of 1,160 women who approached the Educational Public Health Institute of Split and Dalmatia County, during one year period, with a request for HPV testing. The age of the female participants ranged from 17 to 58 years (the average age being 30 years). The study population included all women referred by their gynecologists for HPV DNA testing to our laboratory, on the occasion of the general population survey (with either normal or abnormal cytological diagnosis). All women underwent examination performed by a gynecologist, and two cervical scrapings were collected, for cytology and detection of HPV DNA. Informed consent was obtained from all participants. The cytology was reported using Bethesda system, as atypical squamous cell of undetermined significance (ASCUS), low grade squamous intraepithelial lesion (LSIL), and high grade squamous intraepithelial lesion (HSIL). The information from the Pap smear results was used to examine the relationship between HPV infections and histologically diagnosed cervical lesions.

The study had been approved by a suitably constituted Ethics Committee of the institution within which the work was undertaken and it conforms to the provisions of the Declaration of Helsinki.

HR HPV was detected in cervical exfoliated cells, by using a Hybrid Capture 2 (HC2) DNA test (Qiagene Corporation, Gaithersburg, MD USA). The HC2 test was used in screening specimens for high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). This test had been approved by the FDA for detection of HPV DNA in cervical specimens for diagnostic purpose, but it can not differentiate individual HPV types within the group (2).

Only HR HPV positive specimens detected by the Hybrid capture test were sent for further genotyping at the Department of Pathology and Forensic Medicine, Split University Hospital and School of Medicine. DNA was extracted from cervical cells, by using reagents Nucleo Spin Tissue (Machery-Nagel, Düren, Germany). Genotypes were distinguished with polymerase chain reaction (PCR) based assays using primer sequences for HR HPV types: 16, 18, 31, 33, 45, 52, and 59. Positive and negative controls were included in each reaction. PCR reactions were performed according to the manufacturer instruction on PCR system (Applied Biosystems, Foster City, USA). PCR products were detected using agarose gel electrophoresis (3% agarose gel). Clinical samples were checked for the integrity of the target DNA by standard amplification of the human β -globin, as described elsewhere. The specific primers for amplification of the sequence containing the E6 region of HPV types: 16, 18 and 33 were used (TaKaRa, Bio. Inc., Japan) (13). Amplification products yield DNA fragment of 140 bp for HPV types: 16, 18 and 141 bp for HPV type 33. The presence of HPV types: 31, 45, 52 and 59 was detected by using primers for amplification of the sequence containing the E7 region, described by Walboomers et al. (3), yielding a PCR product of 100 bp. Undetermined types were referred to as other types HPV.

Statistical analysis was carried out using Chi-square test (χ^2 test). The package SPSS 17.0 was used (SPSS Inc., Chicago, Ill., USA). Only $p < 0.05$ was considered statistically significant.

RESULTS

During routine HPV testing in Split and Dalmatia County, a total of 1,160 women with either normal or abnormal cytology were tested and 406 (35%) of them were positive to HR HPV, when samples were assessed by the HC2 test. Among HPV positive women, 79 (19.5%) were under 24 years of age, 197 (48.5%) were 25–34 years old, 82 (20.2%) were 35–44 years old, and 48 (11.8%) were ≥ 45 years old. Of these 406 HPV positive women, 24 (5.9%) had no SIL, 186 (45.8%) had ASCUS/LSIL, 148 (36.5%) HSIL and 48 (11.8%) had unknown cytology (Table 1).

Only HR HPV positive specimens by HC2 were further genotyped by PCR based assay. In this study a spectrum of seven HPV types were detected. Out of 1,160 tested women in our analysis, these are the frequencies of different genotypes: HPV 16 in 10.8%, HPV 18 in 7.8%, HPV 31 in 2.3%, HPV 33 in 1.1%, HPV 52 in 0.9%, HPV 45 in 0.6%, HPV 59 in 0.4%, while 12.2% of all tested specimens remained with unresolved genotype (Table 2). Among 406 HPV positive samples, HPV 16 was the most frequent type in 30.8% cases, followed by HPV 18 (22.2%), HPV 31 (6.7%), HPV 33 (3.2%), HPV 52 (2.5%), HPV 45 (1.7%), and HPV 59 (1.2%). While the other HPV types occurred at a frequency of 34.7% of positive specimens (Table 2). Multiple HPV infections were found in 12 (3%) cases out of 406 positive specimens.

Among HPV positive women, HPV 16 was found to be the most prevalent type within each histological category (37.5% among no SIL, 29.0% ASCUS/LSIL, and 34.5% HSIL), followed by HPV 18 (29.2% among no SIL, 19.9% ASCUS/LSIL, and 22.3% HSIL), HPV 31 (8.3% among no SIL, 6.5% ASCUS/LSIL, and 6.8% HSIL). The frequency of unresolved HPV types (other HPV types) was approximately the same in cervical specimens of women with ASCUS/LSIL and those with HSIL, 38.2% and 29.7%, respectively. In our study there were no significant differences in the prevalence of each HPV type regarding different histological finding ($p = 0.58$) (Table 3).

As we expected, most of the HPV positive women have had abnormal cytological findings (45.8% ASCUS/LSIL and 36.5%

Table 1. Age distributions and cytological findings among HPV positive women ($n = 406$)

Years	n (%)
≤ 24	79 (19.5)
25–34	197 (48.5)
35–44	82 (20.2)
≥ 45	48 (11.8)
Total	406 (100.0)
Cytology	n (%)
No SIL	24 (5.9)
ASCUS/LSIL	186 (45.8)
HSIL	148 (36.5)
Unknown cytology	48 (11.8)
Total	406 (100.0)

No SIL – without squamous intraepithelial lesion
 ASCUS – atypical squamous cell of undetermined significance
 LSIL – low grade squamous intraepithelial lesion
 HSIL – high grade squamous intraepithelial lesion

Table 2. HPV type specific prevalence among women in Southern Croatia (Dalmatia)

HPV type	No. HPV positive N = 406*	Percentage distribution of HPV types	
		Whole population N = 1,160	HPV positives N = 406
16	125	10.8	30.8
18	90	7.8	22.2
31	27	2.3	6.7
33	13	1.1	3.2
52	10	0.9	2.5
45	7	0.6	1.7
59	5	0.4	1.2
Other types	141	12.2	34.7

*Some women counted more than once because of multiple infections, which were found in 12 cases.

Table 3. HPV type specific prevalence in the group of patients with different cytological findings (PAP smear)

HPV type	n (%) of no SIL cases N = 24	n (%) of ASCUS/ LSIL N = 186	n (%) of HSIL N = 148	n (%) of unknown cytology cases N = 48
16	9 (37.5)	54 (29.0)	51 (34.5)	11 (22.9)
18	7 (29.2)	37 (19.9)	33 (22.3)	13 (27.1)
31	2 (8.3)	12 (6.5)	10 (6.8)	3 (6.3)
33	2 (8.3)	5 (2.7)	5 (3.4)	1 (2.1)
52		7 (3.8)	3 (2.0)	
45		5 (2.7)	2 (1.4)	
59		2 (1.1)	3 (2.0)	
Other types	4 (16.7)	71 (38.2)	44 (29.7)	22 (45.8)

($\chi^2=19.03$; ss=21; p=0.58)

No SIL – without squamous intraepithelial lesion

ASCUS – atypical squamous cell of undetermined significance

LSIL – low grade squamous intraepithelial lesion

HSIL – high grade squamous intraepithelial lesion

HSIL) (Table 1), but no significant differences were found with regard to the frequency of abnormal cytology in relation to any individual HPV type. Out of the total of 125 HPV 16 positive specimens 7.2% had no SIL, 43.2% had ASCUS/LSIL, and 40.8% HSIL. Among 90 HPV 18 positive specimens 7.8% had no SIL, 41.0% had ASCUS/LSIL, and 36.7% HSIL. Out of 27 HPV 31 positive specimens 7.4% had no SIL, 44.5% had ASCUS/LSIL, and 37.0% HSIL (Table 4).

DISCUSSION

HPV DNA has been identified in almost all cases of cervical cancer worldwide, with the most frequent types 16 and 18 in 70% of cases (14). In Europe, HPV prevalence in invasive cervical cancer is 85.9%, in exfoliated cells of women with low grade lesions in Pap smears is 67.8%, while in cervical specimens of women with normal cytology the HPV prevalence is 8.1% (7, 15, 16).

At present, there is limited data regarding the prevalence and distribution of HPV types in Croatia. There are a few studies from Northern Croatia (Zagreb County), about HPV prevalence related

only to cervical specimens of women with abnormal Pap smear. According to these studies, HPV prevalence in cervical specimens of women with abnormal Pap smears ranges from 58.8% to 64.0%, caused by the most common types: HPV 16 (11.4% to 15.9%), HPV 31 (5% to 8.7%), HPV 18 (2% to 3.8%), and HPV 33 (2.7% to 2.8%) (9–11). There is only one small scale study (570 women) for Southern Croatia (Split and Dalmatia County) related to cervical samples of women with either normal or abnormal Pap smear. Out of the total number of tested women, 200 (35%) of them were positive to HR HPV, with the most common types: HPV 16 in 10%, HPV 18 in 6.1%, HPV 31 in 2.6%, and HPV 33 in 1.9% specimens (12).

In the present study, our research of this topic expanded and was conducted in more detailed way. The aim was to evaluate the prevalence of HPV types in the female population with any cytological findings in Southern Croatia (Dalmatia County) and to evaluate the distribution of HPV types and their association with different cytological findings (Pap smears). Similar to the results of our previous study, out of the total number (1,160) of tested women, 35% (406) of them were HPV positive, with the most common types: HPV 16 in 10.8%, HPV 18 in 7.8%, HPV 31 in 2.3%, and HPV 33 in 1.1% specimens.

Table 4. Correlation between cytological findings and different HPV types

Cytology	n (%) of HPV 16 cases N = 125	n (%) of HPV 18 cases N = 90	n (%) of HPV 31 cases N = 27	n (%) of HPV 33 cases N = 13	n (%) of HPV 52 cases N = 10	n (%) of HPV 45 cases N = 7	n (%) of HPV 59 cases N = 5	n (%) of other types N = 141
No SIL	9 (7.2)	7 (7.8)	2 (7.4)	2 (15.3)				4 (2.8)
ASCUS/LSIL	54 (43.2)	37 (41.1)	12 (44.5)	5 (38.5)	7 (70.0)	5 (71.4)	2 (40.0)	71 (50.4)
HSIL	51 (40.8)	33 (36.7)	10 (37.0)	5 (38.5)	3 (30.0)	2 (28.6)	3 (60.0)	44 (31.2)
Unknown	11 (8.8)	13 (14.4)	3 (11.1)	1 (7.7)				22 (15.6)

($\chi^2=19.03$; $ss=21$; $p=0.58$)

No SIL – without squamous intraepithelial lesion

ASCUS – atypical squamous cell of undetermined significance

LSIL – low grade squamous intraepithelial lesion

HSIL – high grade squamous intraepithelial lesion

Our results of HR HPV genotyping are slightly different from those found in the studies conducted in Northern Croatia. The frequency of HPV types in these studies are similar for HPV 16 (10.8 versus 11.4–15.9%) and HPV 33 (1.1 versus 2.7–2.8%), but discordant for HPV 18 (7.8 versus 2.0–3.8%) and HPV 31 (2.3 versus 5.0–8.7%). The prevalence of HPV type 18 in LSIL in Europe is 5.1%, but ranges from 2.2% to 31.4% when compared by region (15). The reason for these discrepancies could be because (a) in previous studies regarding HPV prevalence in Northern Croatia, mainly women with abnormal Pap smears were tested for the presence of HPV. While in our study population included all women who were referred by their gynecologists for HPV DNA testing to our laboratory (with any cytological diagnosis), (b) the choice of type-specific primers for DNA amplification (different, but still clinically validated), and (c) different distribution of HR HPV types in both Counties. Split and Dalmatia County is an important tourist region of Croatia with a high number of tourists passing through. It is also a maritime centre with the port and significant number of seamen that are carriers of HPV types gained from all over the world and spread within this region.

In conclusion, the data obtained from our study indicate that HPV 16 is the most common HPV type found in the cervical specimens among women of Split and Dalmatia County as in most studies worldwide, followed by HPV 18 and HPV 31. The positivity rates by HPV type in our study slightly differed for HPV types 18 and 31 from those found in the other studies conducted in Northern Croatia, which suggests that geographic and environmental factors are related to the prevalence of HPV types.

Although it is important to have international updates on the prevalence and type distribution of HPV in the worldwide population, we consider it very important to gather information on regional variations in the HPV type spectrum, particularly in the context of HPV vaccine application. However, this data on the prevalence and distribution of HPV types in women with any cytology finding in Southern Croatia may contribute to increasing knowledge of the HPV epidemiology in Croatia.

Conflict of Interest

None declared

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