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ORIGINAL ARTICLE

Overall human papilloma virus and types 16/18 prevalence in women with normal cervical cytology in Serbia: is it time for human papillomavirus testing and/or vaccination?

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Summary

Purpose: *Infection with high-risk human papilloma viruses (HR-HPV), especially types 16/18, is the main factor in cervical carcinogenesis. Although the incidence of cervical cancer in Serbia is among the highest ones in Europe, data about HPV infection are insufficient. The aim of this study was to investigate the presence of overall and HPV16/18 infections in women with healthy appearance and cytologically (Pap) normal cervix.*

Methods*: The study was performed on women who participated in this cervical cancer screening pilot study. Cervical HPV infection was detected by GP5+/6+ PCR. HPV16/18 were detected by amplification of E7/E1 viral gene, respectively.*

Results*: In 350 women we got the following results: cytological abnormalities (10.3%); visible cervical changes (20.3%); previous precancerous lesion (2.3%); normal Pap and speculum finding without history of precancerous lesion (67.1%).*

In the last group overall HPV prevalence was 41.3%, with 10.5% HPV16 and 23.7% HPV18. The rate of multiple HPV16 plus HPV18 infections was 2.6%. HR-HPV16/18 comprised 31.6% of the total HPV positive participants.

Conclusion*: Owing to the high prevalence of overall and HPV16/18 infections in women with healthy appearance and cytologically normal cervix, we postulate that testing/prophylaxis for these HR-HPV types could be introduced in cervical cancer screening and preventive programmes in Serbia.*

Key words*: human papilomavirus, normal cervical cytology, screening, type 16/18, vaccination*

Introduction

Cancer of the uterine cervix is the third most frequent malignancy in women worldwide. More than 85% of the cases occur in developing countries [1], mainly because of the lack of routine screening, which would allow early detection of preneoplastic cervical lesions. Cervical cancer is a serious health problem in women in Serbia. Whereas cervical cancer screening programmes dramatically reduced the incidence rates of this malignancy in many developed European countries, Serbia named as "country in transition" has one of the highest incidence rates of cervical cancer in Europe [2]. Beside the absence of organized screening programmes, socio-economic status and inadequate education of women contribute to such high incidence.

Cervical cancer arises from precursor dysplastic lesions (cervical intraepithelial neoplasia- CIN) that are classified on the basis of cell abnormalities. Genital infection with HPVs is a major factor in the development of CIN and cervical cancer. It is one of the most common sexually transmitted infections, and the lifetime risk of genital HPV infection for sexually active women is more than 80%. Most of these infections clear spontaneously within two years. In 10–20% of

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women, infection remains persistent, triggering the risk of development to CIN and invasive cervical cancer in the period of a few to 10–20 years. In humans, at least 40 of 120 characterized HPV genotypes infect the genital tract. Based on their malignant potential, genital HPVs can be divided into low-risk or non-oncogenic HPV (LR-HPVs) and high-risk or oncogenic HR-HPVs. Genital LR-HPVs cause benign anogenital warts, while HR-HPVs cause anogenital intraepithelial neoplasia and cancer of the cervix, vagina, vulva, penis and anus [3].

HPV infection is present in 90% of cervical cancer cases and in more than 70% of CIN [4]. HR-HPVs cause malignant transformation of cervical epithelial cells by integration of viral DNA in the DNA of host cells. This integration results in uncontrolled and higher expression of E6 and E7 viral oncoproteins, which inhibit the function of P53 and RB tumor suppressors and other cellular proteins and lead to cell proliferation, disruption of DNA repair, differentiation and apoptosis [3]. HPV genotypes 16 and 18 are the most common and most aggressive among HR-HPVs [5], contributing for at least 70% of all cervical cancers [6].

In women with cytologically normal cervical epithelium, HPV DNA is present in approximately 12%, with higher prevalence in less-developed regions of Sub-Saharan Africa, Latin America and Caribbean, Eastern Europe and Southeastern Asia. Remarkable differences were perceived among countries [7] that arise from socio-economical, cultural, lifestyle and biological factors. Thus, it is of great importance to know the prevalence rate of HPV in each country in order to define its strategies for cervical cancer prevention.

Testing today for HR-HPVs, particularly HPV16 and 18, is part of cervical screening programmes in developed countries. Also, an effective tool for prevention of cervical cancer is prophylactic HPV vaccination. In Serbia, cervical cancer screening programmes as well as HPV vaccination are not implemented yet.

Because of the high incidence of cervical cancer in Serbian women as well as lack of data over HPV infection, the aim of this study was to determine the overall HPV and HPV16/18 cervical infection in women with healthy appearance and cytologically normal cervix.

Methods

Subjects

The study group consisted of 350 women who participated in the routine gynecological examination and cytological screening at the Institute for Oncology and Radiology of Serbia. The study was approved by the Ethics Committee of the Institute and all women gave written informed consent to participate in the study.

Speculum examination was performed by gynaecologists and cervical samples were obtained using a spatula. After spreading the cervical smear onto a glass slide, the tip of the spatula was placed in a container with 0.9% sodium chloride and then sent to the Laboratory for Molecular Genetics for HPV testing. The smears were processed for Papanicolaou (Pap) staining and reported by cytopathologists according to the 2001 Bethesda classification system.

The samples with visible cervical changes such as leukoplakia, erythroplakia, condyloma, polyp or any cytological evidence of atypical squamous cells / atypical glandular cells, squamous intraepithelial lesion, adenocarcinoma in situ or malignancy were excluded from the study group for HPV prevalence.

DNA extraction and detection of HPV infection

Cervical smear samples in 0.9% sodium chloride were vortexed to separate the cervical cells, centrifuged at 1700×g for 4 min and sediments of the cells were kept at -20 ° C until DNA isolation.

Genomic DNA was isolated by the salting-out method from exfoliated cervical cells [8]. The DNA was dissolved in sterile deionized water and stored at 20°C. DNA concentrations were measured by Biophotometer (Eppendorf, Germany).

Cervical HPV infection was detected by the presence of HPV DNA by polymerase chain reaction (PCR) amplification, using general primers GP5+ and GP6+ (Metabion, Germany). These primers have been designed to amplify the 150 bp conserved sequence of L1 viral gene of a broad spectrum of HPV genotypes (6, 11, 13, 16, 18, 30–35, 39, 40, 42, 45, 51–53, 56, 58, 61, 66, 68) [9]. DNA from HeLa cells, containing HPV18, was used as PCR positive control.

Human β globin amplification was used to test sample DNA quality. Primers' sequences and PCR conditions were described previously [10]. Presence of amplified L1 and β globin gene were checked by electrophoresis on a 2% agarose gel (ICN Biomedicals, USA).

HPV 16/18 genotyping

The HPV-positive samples were further analyzed for genotypes 16 and 18. Forward primer 5´- CAT GGA GAT ACA CCT ACA TTG C -3´ and reverse primer 5´CTG AGA ACA GAT GGG GCA CAC -3´ were used to amplify HPV16 DNA, and forward primer 5´- AAC AGT CCA TTA GGG GAG CGG CTG GA - 3´ and reverse primer 5´- TGC CGC CAT GTT CGC CAT TTG -3´ were used for HPV18 (Metabion, Germany). PCR reactions for HPV16 and HPV18 were carried out in a total volume of 25 µl containing 12.5 µl 2 X AmpliTaq Gold PCR Master Mix (Applied Biosystems, USA), 4 pmol of each

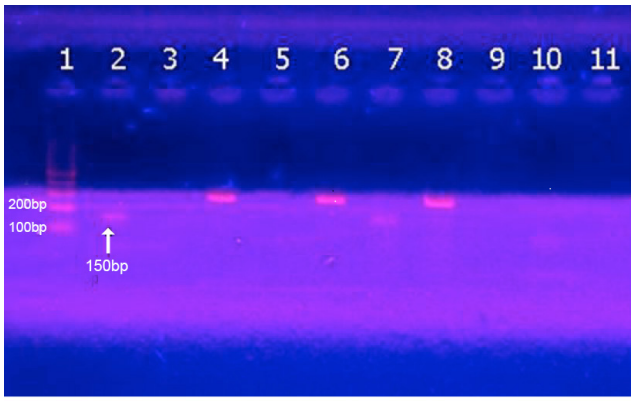

Figure 1. Line 1: molecular weight marker; line 2: PCR positive control (HeLa); lines 3 and 5: HPV-negative sample; line 7: HPV-positive sample; lines 4, 6 and 8: PCR product of β globin gene (268 bp); line 10: control without DNA; lines 9 and 11: empty wells.

primers and 200 ng of sample DNA. Thermocycler conditions for HPV16 were: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, with final extension at 72 °C for 10 min. Thermocycler conditions for HPV18 were: initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 70 °C for 1.5 min, and extension at 72 °C for 1 min, with final extension at 72 °C for 2 min. DNA from HPV16 positive sample, previously confirmed by direct sequencing, was used as PCR positive control for HPV16. DNA from HeLa cells was used as PCR positive control for HPV18.

The amplified fragments were visualized by electrophoresis on 2% agarose gels and stained with ethidium bromide.

Results

Based on speculum examination and cytological findings, the study group (N=350) was divided into four subgroups: the subgroup of women with cytological abnormalities (10.3%), the one with normal Pap test and visible cervical changes (20.3%), the one with normal Pap test and previous precancerous lesion (2.3%), and the one with normal Pap and speculum finding and no previous history of precancerous lesion (67.1%). The last subgroup, consisting of 235 women (aged 19-75 years, median 51), was target one for HPV testing.

DNA was successfully isolated in all of 235 cases by salting out procedure. The concentrations (measured by spectrophotometer) and quality of the DNA (checked by amplification of β globin gene) were adequate for HPV analyses. DNA was enough for further analysis in 225 cases.

HPV testing by PCR with GP5+/GP6+ primers (Figure 1) showed that 41.3% (93/225) samples

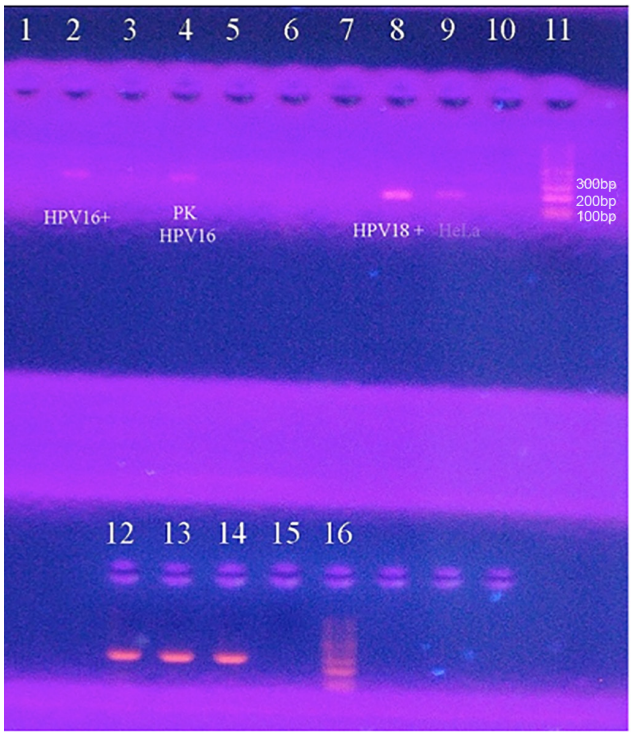

Figure 2. Line 1, 3: HPV16 negative sample; line 2: HPV16 positive sample; line 4: PCR positive control for HPV16 (285 bp); line 5: control without DNA; line 6, 7: HPV18 negative sample; line 8: HPV18 positive sample; line 9: PCR positive control (HeLa) (187 bp); line 10: control without DNA; line 11: O'GeneRuler™ 100bp DNA Ladder; line 12,13 and 14: PCR product of β globin; line 15: control without DNA; line 16: O'GeneRuler™ 100bp DNA Ladder.

were HPV-positive.

For HPV16 and HPV18 testing, we had enough DNA in 76 of 93 HPV-positive cervical samples. We analyzed the presence of HPV16 and HPV18 in HPV-positive cervical smears by PCR with set of primers specific for these viral types (Figure 2). The prevalence of HPV16/18 in the group of women with cytologically normal and healthy-appearing cervix was 13.0%. Regarding HPV-positive samples, we obtained the following:

- the percentage of HPV16 infection in HPV-positive samples was 10.5%,
- the percentage of HPV18 infection in HPV-positive samples was 23.7%,
- the percentage of multiple infections (HPV16 plus HPV18) in HPV-positive samples was 2.6%,
- the percentage of HPV16 and/or HPV18 in HPV-positive samples was 31.6%.

The distribution of HPV infection by age groups <30, 30-39, 40-49, ≥50 years was: 58.3,

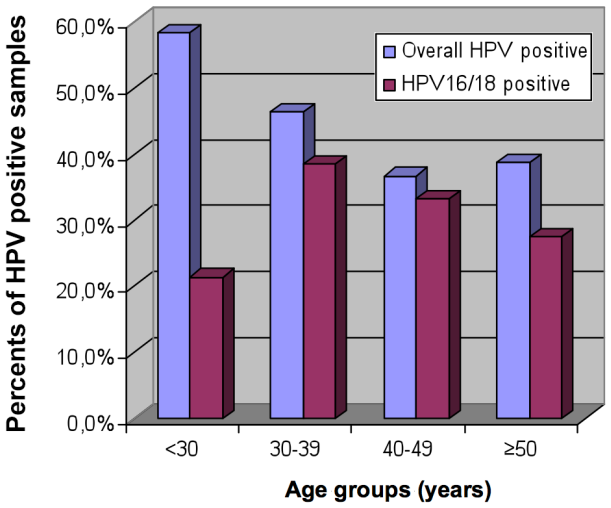

Figure 3. Overall HPV and HPV16/18 prevalence by age group in women with normal cervical cytology.

46.4, 36.7 and 38.7%, respectively. The distribution of HPV16/18 within the same age groups was 21.4, 38.5, 33.3 and 27.5%, respectively (Figure 3).

Discussion

Cervical cancer is preventable and curable disease if diagnosed at an early stage. The risk factors and causes of this malignancy are well-known, and the methods for early detection of the disease are developed. Despite all the above, cervical cancer is the third most common malignancy and the fourth leading cause of cancer-related death in females worldwide [1].

Every year, about 530 000 women are diagnosed with cervical cancer and 275 000 die of it. More than 85% of the cases and deaths occur in developing countries. The highest incidence rates are in Eastern and Western Africa, where the age-standardized incidence rates are over 30 per 100 000. Other high-risk regions with age-standardized incidence rates of over 20 per 100 000 include South Africa, South-Central Asia, South America, Melanesia, Middle Africa, Central America and the Caribbean. Rates are lowest in Western Asia, Australia and New Zealand, and North America, where the age-standardized incidence rates are less than 6 per 100 000 [1]. In Europe, the incidence rate is highest in Eastern and Central European countries. In most countries of Eastern Europe the incidence rates are more than 20 per 100 000. In some regions and some age groups these figures are reaching 40 per 100 000 (Romania, Serbia) [2].

Globally, the differences in prevalence and mortality of cervical cancer among high and low-recourse settings of the world and Europe as well arise from socio-economical, cultural, lifestyle and biological factors, and lack of cervical cancer prevention by screening and vaccination. Periodical screening (based on cytology and/or HPV testing) is very important and cervical cancer incidence can be reduced more than 80% by high quality screening [11]. In planning cervical cancer prevention programmes, it is significant to know HPV prevalence in a particular region.

HPV infection is a necessary event for cervical cancer development and it is present in about 90% of cervical cancer cases, with HPV16 and 18 in more than 70% of the cases [6]. Other co-factors in cervical cancer development include sexual behavior, hormonal factors, tobacco smoking, other sexually transmitted diseases and dietary factors [12]. HPVs exert tropism for epithelial cells of the skin and mucosa and are associated with a variety of clinical manifestations that range from benign lesions to cancer. Genital HPV infection is one of the most common sexually transmitted infections and the lifetime risk of genital HPV infection for sexually active women is more than 80% [3]. Although very few of these infections lead to cervical cancer, it is crucial to find pre-cancer cells before cancer occurs.

Cervical cancer is a serious health problem in women in Serbia. The incidence rate (age-standardized rate by world population- ASR-W) of cervical cancer in Serbia was 20.9/100 000 women in 2008, and it is among the highest in Europe along with Romania, FYROMacedonia and Bulgaria in the region of Southeastern Europe. The incidence was 2-fold higher than the European average (with ASR-W incidence of 10.6/100 000 women) and more than 3-fold higher than Western Europe countries (with ASR-W incidence of 6.9/100 000) for the same period. The ASR-W mortality of cervical cancer in Serbia in 2008 was twice as high than in Europe (9.2/100 000 and 4.5/100 000 women, respectively) [2].

The potential reasons for the high cervical cancer burden in Serbia may be sexual behavior (such as early onset of sexual life or number of sexual partners, sexual intercourse without using condom), other sexually transmitted infections (such as infection with hepatitis virus and Chlamydia), lifestyle factors (such as cigarette smoking), type of diet etc. A large epidemiological study is needed to estimate the impact of the mentioned factors to cervical cancer burden. Inadequate education of women about the disease (risk factors for cervical cancer development and

methods for prevention) and a national organized cervical cancer screening programme that is not implemented yet contribute to this situation. Additionally, we had no data of HPV infection in Serbian women, while preventive HPV vaccination is not recommended by Public Health authorities. Thus, in our study, we investigated HPV infection prevalence in women with normal cervical cytology in order to improve cervical cancer prevention strategies.

This is the first study to assess the prevalence of HPV infection in Serbian women. Since HPV infection is expected in cervical lesions (such as condylomas, leukoplakia, erythroplakia, polyps etc.) [13-16] as well as cytologically abnormal cervix cells, we tested HPV infection only in women with normal Pap and healthy appearance of the cervix and no previous history of precancerous lesion. Unfortunately, the study showed a high HPV-incidence of 41.3%. Additionally, nearly one third (31.6%) of HPV-positive women had HPV16/18 types, marked as the most aggressive HR-HPVs.

The estimated global HPV prevalence among women with normal cervical cytology, obtained from meta-analysis, was 11.7% [7]. African and Latin American regions showed higher average HPV prevalence than European, North American, and Asian regions [7]. HPV prevalence for Sub-Saharan Africa was 24.0%, while for Latin America and Caribbean was 16.1% [7]. In Asia and Europe, the highest prevalence was seen in Southeastern Asia (14.0%) and Eastern Europe (21.4%) [7].

Remarkable differences were observed among countries. Results similar to ours, of high overall HPVs and HR-HPVs infection burden, were reported in some studies from Africa, Latin America and Southeast Asia [17-20]. All studies were PCR-based.

On the contrary, European Union countries have low HR-HPVs incidence (ranged from 3% in Greece to more than 15% in Denmark, the United Kingdom, Ireland, France and Belgium) [21] and are partly dependent on the different age composition of the study populations. Studies were based on GP5+/6+ PCR and hybrid capture 2 (HC2) methodology. Studies using HC2 reported on average a higher prevalence of HR HPVs than studies using GP5+/6+ PCR. Age-standardised HR-HPVs prevalence in women from the general population in Europe (aged 30–64 years) ranged from 1.7% (Spain) to 12.5% (Belgium) [21]. In cervical cytological normal part of the general population of women from Serbia, only HPV16/18 type's prevalence was 13.0%. Consequently, we assume that overall HR-HPVs prevalence in the general population of women in our settings is even higher.

It is also worrying that high overall prevalence HPV infection in Serbian women appeared in women older than 30 years (from 36- 46%). Similarly, high percentage of HR-HPVs 16/18 infection was seen in groups older than 30. Namely, a peak in HPV infection is expected at younger ages (<25 years), declining to a lower prevalence plateau in middle age. This peak is related to the start of sexual intercourse and it is a mostly transient HPV infection that clears rapidly, while HPV prevalence is relatively low among middle-aged women in developed countries. In some regions, a second peak of HPV prevalence was observed in individuals aged >45 years, but it was of lower intensity [7]. In our study, HPV infection appeared in high percentage in women older than 30 years and could have persistent character. Similarly, some studies from low-resource settings of Africa and Asia reported high HPV prevalence in all as well as in older than 35 years age-groups [21-23].

Interesting data, obtained from our study, is that HPV18 type is more frequent than HPV16 type in HPV-positive cytologically normal cervical smears of Serbian women (10.5 vs 23.7%). The average proportion of HPV16 and 18 among HR-HPV-positive women from general population in Europe was 29.8 and 12.0%, respectively [21]. Meta-analyses showed that the most common worldwide HPV types in HPV-positive samples in women with normal cervical cytology are HPV16, followed by HPV18 in North America and Asia [7]. In Europe and Africa HPV16 is followed by HPV31 and 52, respectively [7]. The knowledge of specific HPV distribution and testing only for the subset of HR-HPV types in a particular geographical area improves the cost-effectiveness of screening. All these suggest that it is essential to screen the female population in Serbia for different HR-HPV types and determine the specific distribution of HR-HPV types in our country.

In developed countries, detection of HR-HPV DNA due to its high sensitivity and negative predictive value is now used in primary screening for cervical cancer (alone or in combination with conventional Pap-stained smear or liquid-based cytology) [24]. The HR-HPV testing is more sensitive than cytological test in primary screening for severe or moderate dysplasia (CIN 2/3) and cervical cancer (95 vs 55%) [25]. Also, HR-HPV test has a high negative predictive value (from 97% to more than 99%) [24] and allows for increased

length of screening interval [26]. The novel HPV assay allows for differentiation of HR-HPV16 and 18 from other HR-HPV types. It is based on the results of a study by Khan et al. [27] showing that HPV16 and 18 display a greater risk of CIN 3 than other HR-HPV types. This could permit less aggressive management of women with other HR-HPV infections [27]. Up until now, HR-HPV testing is not part of cervical cancer screening programme in Serbia. Before its potential implementation, it is necessary to conduct cost-effectiveness analysis of HPV testing/cotesting vs cytology in primary screening.

An effective tool for the prevention of cervical cancer is also prophylactic HPV vaccination. Currently, the Food and Drug Administration (FDA) has approved two preventive HPV vaccines: a quadrivalent vaccine (HPV4; Gardasil, produced by Merck) and a bivalent vaccine (HPV2; Cervarix, produced by GlaxoSmith-Kline). HPV2 is directed against two oncogenic types (HPV16 and 18). HPV4 is directed against two oncogenic types (HPV 16 and 18) and two non-oncogenic types (HPV 6 and 11) [28,29]. Both vaccines are highly effective against persistent infection and premalignant and malignant cervical lesions associated with HPV16 and 18 types [30,31]. HPV vaccination has become part of cervical cancer preventive programme in many developed countries, especially in Europe and North America. So far, there is no national recommendation for HPV vaccination in Serbia.

In conclusion, based on the obtained results of high prevalence of the overall HPV and 16/18 HPV infections in women with healthy appearance and cytologically normal cervix, we anticipate that testing/prophylaxis for these high-risk HPV types could be introduced in cervical cancer screening and preventive programmes. Studies on larger population could confirm this finding and may assist the Public Health Authorities in planning further strategies to prevent cervical cancer in Serbia.

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