# HUMAN PAPILLOMAVIRUS-SPECIFIC ANTIBODY STATUS AMONG UNVACCINATED SUBJECTS IN THE REGION OF VOJVODINA, SERBIA

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**SUMMARY**

*Objectives:* The aim of the study was to evaluate the immune status of young people from the Vojvodina province, Serbia, through the detection of IgG antibodies specific for the L1 protein of HPV types 6, 11, 16, and 18 contained in quadrivalent vaccine.

*Methods:* The study enrolled 514 healthy persons of both genders, aged between 18 and 30 years. All potential participants were informed about the project's aims by trained interviewers before venous blood collection. Also, participants completed a specially designed anonymous questionnaire to identify socio-demographic characteristics and individual behaviours associated with HPV seroprevalence. VPL HPV L1-specific IgG antibodies were measured using a semi-quantitative HPV IgG ELISA kit (Dia.Pro, Italy).

*Results:* A total of 472 (91.8%) young subjects had no detectable antibodies against high- and low-risk HPV types covered by the quadrivalent vaccine. A slightly higher number of seropositive individuals were detected in the age group of 26–30 years compared to younger than 25. Multivariate analysis showed that the number of lifetime sexual partners was the most powerful predictor of HPV seropositivity (OR = 3.483, 95% CI: 1.294–9.379).

*Conclusions:* Obtained data point out low levels of naturally induced HPV-specific serum antibodies among the target population in the Vojvodina province. The present work highlights the significance and potential benefits of HPV vaccination. Routine HPV vaccination should be the public health priority in our country and should be included in the national immunization programme as soon as possible.

*Key words:* anti-HPV IgG antibodies, ELISA, human papillomavirus, L1 antigen

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*https://doi.org/10.21101/cejph.a7257*

## INTRODUCTION

During past decades, human papillomaviruses (HPV) have been intensively studied due to their oncogenic activity. The vast majority of HPV infections cause no symptoms and resolve spontaneously within eight months to two years, indicating that the immune system can usually eliminate these infections (1). Persistent infection with certain high-risk strains represents one of the most hazardous factors for developing precancerous lesions and carcinoma (2). The contribution of oncogenic HPV types in carcinogenesis has been most intensively studied and described in the example of cervical carcinoma. Besides cervical carcinoma, these types are causally related to several human cancers, including vulvar, vaginal, penile, anal, and a subset of head and neck carcinomas. About 63,000 new cases of HPV-related cancer are registered annually, accounting for 4.5% of all cancers worldwide (3).

Today it is well known that HPV infections are exclusively local and intraepithelial. From the onset of infection to the appearance of the lesion, the virus stays in the host for an extended period, efficiently avoiding the host's immune mechanisms (4). HPV infection frequently induces a humoral immune response mediated by specific antibodies, primarily specific to viral capsid antigens (4, 5). However, humoral immunity, mediated by HPV L1-specific IgG antibodies, is usually slow, weak, and varies considerably among the population (5–7). It is important to note that seroconversion occurs months or even years after infection. In some infected individuals, antibodies are never detected, as only 50–70% of women eventually became seroconverted (5). Furthermore, it is unclear whether produced antibodies protect against subsequent infection and whether natural immunity is maintained throughout life (6). Information on immune responses against HPV can have important implications for possible vaccination strategies.

The discovery of virus-like particles (VLPs) enabled a better understanding of the immune response during HPV infection (7). Expression and self-assembly of the HPV major capsid protein (L1) in eukaryotic cells result in the spontaneous formation of structures that resemble intact virions. These particles display conformational, type-specific epitopes recognized by the humoral

immune system as pathogen-associated molecular patterns (4, 5). Due to the structural and antigenic similarity to authentic virions, HPV type-specific VLPs are used in various applications in immunization, therapeutics, and diagnostics. HPV VLP-based enzyme-linked immunosorbent assay (ELISA) can measure type-specific HPV antibodies.

Several serological studies have been conducted worldwide and reported different levels of naturally induced HPV-specific serum antibodies within the general population (8–11). However, none of the serological studies was conducted in Serbia, where the HPV DNA prevalence is very high (12, 13). This study aimed to assess the young population's immune status concerning antigens of the HPV quadrivalent vaccine and to investigate their susceptibility to the virus. The study included unvaccinated subjects of reproductive age (age range 18–30 years). Additionally, identification of the demographic variables associated with HPV seropositivity was made.

## MATERIALS AND METHODS

### Study Design

A cross-sectional study was conducted to investigate the prevalence of anti-HPV antibodies among healthy persons of both genders (aged 18–30 years) who are residents of the Vojvodina province, Serbia. In total, 514 subjects were included in the study from November 2013 to December 2016. The data were collected using systematic random sampling based on the order of arrival in two health institutions: the Institute of Public Health of Vojvodina and the Institute for Health Protection of Students, Novi Sad.

All potential participants in the study were informed about the project's aims by trained interviewers before venous blood collection. Informed consent was obtained from all individual participants included in the study. They were told their participation was voluntary, with the right to withdraw from the study at any point. After full information was provided, subjects completed a specially designed anonymous questionnaire. The questionnaire contained multiple-choice questions regarding the demographic and educational characteristics, lifetime habits (tobacco smoking, oral contraceptive use, condom use), and other potential risk factors related to sexual practices (age of first intercourse, number of sexual partners). Participants did not have an obligation to declare their sexuality. Additional questions were about the history of sexually transmitted infections, knowledge about HPV infections, frequency of visits to gynaecologists, and family history of cancer. To calculate the minimal sample size (n) needed to estimate the seroprevalence of HPV IgG in examine cohorts (18–30 years of age), we used the following formula: $n = [(z^2)P(1-P)]/d^2$ (14), where z represents the statistic for the chosen 95% level of confidence, P is the expected proportion of affected individuals in the population, approximately 50% (5), and d is the allowable error and represents the maximum risk acceptable in sample size estimate, and here it was set at conventional level of ± 5%. We implemented these findings in our sample estimated calculations.

This study protocol was approved by the Ethical Committee of the Institute of Public Health of Vojvodina (No. 01-1692/2 of 8th October 2013).

### Serologic Assay

Venous blood (3 mL) was collected in EDTA-free tubes following standard operational procedure. After centrifugation, serum was transferred into a clean polypropylene tube and stored at 4–8°C until analysis, for periods longer than 7 days, serum samples were frozen at −20°C. The HPV VLP IgG ELISA (Dia.Pro Diagnostic Bioprobes S.r.l., Milan, Italy) based on the anti-HPV L1 IgG capture was used in the study. All the steps of the ELISA protocol were carried out at room temperature. Standards and samples were added to the appropriate microtitre plate wells coated with recombinant VLPs derived from HPVs 6, 11, 16, and 18 and incubated for 60 minutes. The microtitre plates were washed before adding biotin-conjugated polyclonal antibodies specific to human IgG. After the incubation for 60 minutes and washing, TMB substrate solution was added to each well, and after 20 minutes, the reaction was stopped with the sulfuric acid solution. Only wells that contained HPV IgG, biotin-conjugated antibody and enzyme-conjugated avidin exhibited colour change. The formula for calculation of cut-off value optical density (OD) 450nm (OD of negative control plus 0.25) was used to set the threshold for determining the positive (reactive) and negative (not reactive) results. For statistical analysis purposes, serological results were interpreted qualitatively, as positive and negative. The manufacturer reported that sensitivity amounted to >1.0 (S/Co) with the internal panel of positive sample, while sensitivity amounted to >1.1 (S/Co) with the international standard WHO NIBSC.

### Data Analysis

Descriptive statistics were used for categorical data, presented as absolute frequencies and percentages. The Chi-squared or Fisher's exact tests (when only a few observations for individual cells were reported) were used for categorical variables. Invariable and corrected multivariable logistic regression analyses were used to determine potential predictors of HPV serological status. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All statistical analyses were performed using the software package Stata v.16, and the statistical significance was set at $p < 0.05$.

## RESULTS

### Characteristics of the Participants

A total of 514 unvaccinated young persons (mean age 24.7 years, age range 18–30 years) were enrolled in the study. A summary of variables that describe the socio-demographic characteristics, lifestyle factors, and sexual practice of participants is provided in Table 1. In the study population, 56.2% were females, and 54.1% of participants were younger than 25. Most of them had intermediate level of education (68.3%) and were not married (80.4%). The majority of participants (92.8%) self-assessed their economic status as middle. The habit of tobacco use was relatively frequent among young persons in our study (27.6%). According to our questionnaire, 45.8% of study population had the first sexual intercourse before the age of 18. The proportion of participants reporting more than three sexual partners was 68.9%, and only 7.8% reported ever having had a sexually transmitted infection. About half of the participants (52.7%) had any knowledge of what HPV was and

what effect it had on the human health, while the main sources of information were internet sites and primary care doctors. The age of the first intercourse among females was 18.1 years and 17.4 among men. The average number of sexual partners over a lifetime for males was 5.8 and 3.6 for females (data not shown).

## Serological Test Results

Results of HPV VLP ELISA showed that 91.8% (472/514) of study participants had no detectable HPV L1 IgG antibodies. High level of seronegativity was seen in both populations, males (91.1%) and females (92.4%). A slightly higher number of seropositive individuals were detected in the population aged 26–30 years compared to younger ones (Fig. 1).

## Influence of Risk Cofactors on HPV Seropositivity

The association between HPV seropositivity and sociodemographic and behavioural characteristics of participants as risk cofactors for acquiring an HPV infection was assessed by calculating odds ratios (OR) and 95% CI. Summarized results are presented in Table 2.

HPV seropositivity status did not significantly differ between genders or age groups of participants. Multivariable logistic regression analyses showed that the lifetime number of sexual partners was the only independent risk factor for HPV seropositivity (adjusted OR = 3.483, 95% CI: 1.294–9.379 for three or more partners vs. three partners). Other variables that describe behavioural risk factors did not show a statistically significant association with HPV serological status for anti-HPV 6, 11, 16 and 18 IgG antibodies.

## DISCUSSION

This paper provides data on the seroprevalence of four HPVs types (HPV 6, 11, 16, and 18) present in the quadrivalent vaccine. As far as we know, this is the first study evaluating HPV seroprevalence in Serbia among unvaccinated persons. In the period when current study was conducted, HPV vaccine coverage was less than 2% (15). VLP-based ELISA is easy to perform and does not require expensive equipment and additional laboratory space. However, it must be noted that although type-specific epitopes on the surface of VLP are structurally similar to authentic virions, their distortion may sometimes interfere with the serological type-specific detection (16). In this study, 514 sera from unvaccinated, sexually active, apparently healthy subjects of both genders were tested. The seropositivity rate of IgG antibodies to HPV vaccine types was low and amounted to 8.2% (7.6% in the female and 8.9% in the male population). It is difficult to compare the results of our research with the results of other authors because

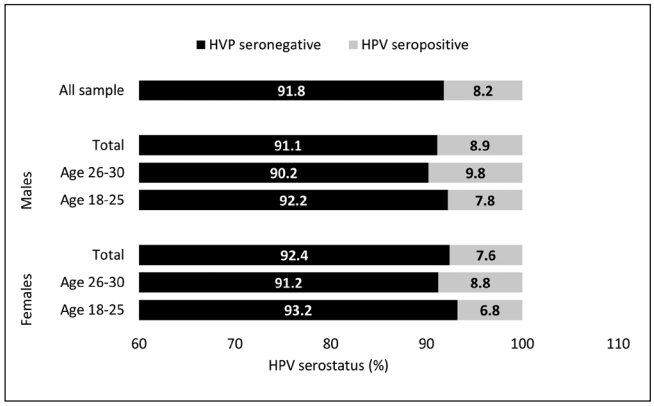


***Fig. 1.*** *HPV IgG serostatus stratified by gender and age.*

***Table 1.*** *Demographic and socioeconomic characteristics of the study group (N = 514)*

| Variables | Group size n (%) |
|---|---|
| Gender | |
| Male | 225 (43.8) |
| Female | 289 (56.2) |
| Age (years) | |
| ≤ 25 | 278 (54.1) |
| 26–30 | 236 (45.9) |
| Education level | |
| Primary | 2 (0.4) |
| Intermediate | 351 (68.3) |
| University | 161 (31.3) |
| Marital status | |
| Others | 413 (80.4) |
| Married | 101 (19.6) |
| Economic status | |
| High | 17 (3.3) |
| Middle | 477 (92.8) |
| Low | 20 (3.9) |
| Tobacco smoking | |
| Yes | 142 (27.6) |
| No | 372 (72.4) |
| Age of first intercourse (years) | |
| < 18 | 235 (45.8) |
| ≥ 18 | 279 (54.2) |
| Number of sexual partners | |
| < 3 | 160 (31.1) |
| ≥ 3 | 354 (68.9) |
| Condom use | |
| Yes | 176 (34.2) |
| No | 338 (65.8) |
| Previously reported STDs | |
| Yes | 40 (7.8) |
| No | 474 (92.2) |
| Family history of cancer | |
| Yes | 149 (29.0) |
| No | 365 (71.0) |
| Informed about HPV | |
| Yes | 271 (52.7) |
| No | 243 (47.3) |

STD – sexually transmitted disease; HPV – human papillomavirus

***Table 2.*** *Socio-demographic/behavioural characteristics and HPV serological status of subjects tested for anti-HPV 6, 11, 16, and 18 IgG antibodies (N = 514)*

| Variables | HPV seronegative n = 472 (91.8%) n (%) | HPV seropositive n = 42 (8.2%) n (%) | p-value[1] | Univariate odds ratio (95% CI) | p-value | Multivariate odds ratio[2] (95% CI) | p-value[2] |
|---|---|---|---|---|---|---|---|
| Gender | | | | | | | |
| Female | 267 (56.6) | 22 (52.4) | 0.600 | Reference | | | |
| Male | 205 (43.4) | 20 (47.6) | | 1.184 (0.629–2.228) | 0.601 | NA | NA |
| Age (years) | | | | | | | |
| 18–25 | 258 (54.7) | 20 (47.6) | 0.380 | Reference | | | |
| 26–30 | 214 (45.3) | 22 (52.4) | | 1.326 (0.705–2.495) | 0.381 | NA | NA |
| Education level | | | | | | | |
| University | 149 (31.6) | 12 (28.6) | 0.774 | Reference | | Reference | |
| Intermediate | 321 (68.0) | 30 (71.4) | | 1.160 (0.578–2.330) | 0.676 | 1.253 (0.613–2.558) | 0.536 |
| Primary | 2 (0.4) | 0 | | NA | NA | NA | NA |
| Marital status | | | | | | | |
| Married | 92 (19.5) | 9 (21.4) | 0.762 | Reference | | Reference | |
| Other | 380 (80.5) | 33 (78.6) | | 0.888 (0.410–1.920) | 0.762 | 1.127 (0.487–2.610) | 0.780 |
| Economic status | | | | | | | |
| Low | 15 (3.2) | 2 (4.8) | 0.251 | Reference | | Reference | |
| Middle | 440 (93.2) | 37 (88.1) | | 0.631 (0.139–2.864) | 0.550 | 0.605 (0.132–2.774) | 0.518 |
| High | 17 (3.6) | 3 (7.1) | | 1.324 (0.194–9.020) | 0.775 | 1.479 (0.213–10.263) | 0.692 |
| Age of first intercourse (years) | | | | | | | |
| < 18 | 216 (45.8) | 19 (45.2) | 0.948 | Reference | | Reference | |
| > 18 | 256 (54.2) | 23 (54.8) | | 1.021 (0.542–1.925) | 0.948 | 0.996 (0.520–1.908) | 0.991 |
| Lifetime sexual partners | | | | | | | |
| < 3 | 155 (32.8) | 5 (11.9) | 0.005* | Reference | | Reference | |
| > 3 | 317 (67.2) | 37 (88.1) | | 3.618 (1.395–9.388) | 0.008* | 3.483 (1.294–9.379) | 0.014* |
| Tobacco smoking | | | | | | | |
| No | 344 (72.9) | 28 (66.7) | 0.388 | Reference | | Reference | |
| Yes | 128 (27.1) | 14 (33.3) | | 1.344 (0.686–2.634) | 0.389 | 1.242 (0.626–2.464) | 0.535 |
| Condom use | | | | | | | |
| No | 306 (64.8) | 32 (76.2) | 0.137 | Reference | | Reference | |
| Yes | 166 (35.2) | 10 (23.8) | | 0.576 (0.276–1.201) | 0.141 | 0.601 (0.286–1.263) | 0.179 |
| Family history of cancer | | | | | | | |
| No | 334 (70.8) | 31 (73.8) | 0.677 | Reference | | Reference | |
| Yes | 138 (29.2) | 11 (26.2) | | 0.859 (0.420–1.757) | 0.677 | 0.888 (0.433–1.823) | 0.747 |
| Previously reported STDs | | | | | | | |
| No | 436 (92.4) | 38 (90.5) | 0.557 | Reference | | Reference | |
| Yes | 36 (7.6) | 4 (9.5) | | 1.275 (0.431–3.772) | 0.661 | 1.328 (0.447–3.948) | 0.610 |
| Informed about HPV | | | | | | | |
| No | 228 (48.3) | 15 (35.7) | 0.117 | Reference | | Reference | |
| Yes | 244 (51.7) | 27 (64.3) | | 1.682 (0.872–3.243) | 0.121 | 1.682 (0.857–3.304) | 0.131 |

STD – sexually transmitted disease; HPV – human papillomavirus;
[1]using the Chi-squared test or Fisher's exact test (when appropriate); [2]adjusted for age and sex of the participants;
*p < 0.05

the study design varies considerably across the different investigations. Low seroprevalence of 6.6% among 91 female subjects aged 16–40 years was detected using the Dia.Pro ELISA kit in a study performed by Adekunle et al. (11). In Nigerian study using the same kit, Okonko et al. (17) revealed high anti-HPV seronegativity of 95.1% among 182 women of childbearing age (age range 19–45 years), while Faneye et al. (18) determined a slightly higher percentage antibody response in about 28.6% and 30% of unvaccinated men and women, respectively, attending the STI clinics. Also, using VLP-based ELISA kit Lu et al. (19) reported the presence of anti-HPV IgG antibodies to HPV16/18 in 13.3% and 19.8% of males aged 18–25 and 26–35 years, respectively.

In contrast to VLP-based ELISA used in current research, several surveys reported higher seroprevalence of anti-HPV L1 IgG antibodies using a competitive ELISA technique. For instance, in one Australian study, which included 2,797 participants aged 0–69, seroprevalence rates were 23.8% and 17% in female and male subjects, respectively (8). In the investigation carried out in the United States among participants aged 14–59 years, the prevalence of antibodies against HPVs 6, 11, 16, and 18 was higher among females than males (32.5% vs. 12.2%) (9). Besides ELISA, there are several other approaches to evaluate the antibody responses to HPV infection, such as the neutralization assay with native HPV (NA-HPV) or pseudoviruses (NA-PsV) and multiplex immunoassay systems (20). However, recent data proved that different serological assays correlate well, particularly in specimens with high antibody levels (21).

The finding that only 8.2% of the participants had detectable anti-HPV L1 IgG antibodies against HPVs 6, 11, 16, and 18 may lead to the conclusion that the rest of the tested persons had not been exposed to the examined HPV genotypes. However, according to data from a few regional health centres, the HPV DNA prevalence is very high in Serbia, ranging from 19.1% to 36.8% (12, 13). Among sexually active adolescents and young women under 25 years of age, the virus prevalence is around 50% (13). A complex interplay between HPV immune evasion mechanisms and the host immune system may explain a discrepancy between the prevalence of HPV DNA and anti-HPV antibodies. HPV has multiple strategies for evading the host defence, including downregulation of innate sensing signalling pathways, inhibition of interferon synthesis and Th1-inflammatory and cytotoxic response, as well as impaired production of adhesion molecules for antigen-presenting cells and MHC class I and II components which are essential for activation of adaptive immune response and antibodies production (4). The non-lytic and non-viremic nature of the HPV infection and the fact that HPV L1 protein is expressed in the superficial layers of the squamous epithelium, where antigen-presenting cells have limited access, additionally help the virus in escaping from the immune recognition (4–6). Therefore, we speculate that some participants in our study who were naturally exposed to HPV types 6, 11, 16, and 18 did not seroconvert or had low, undetectable antibody responses.

A systematic review and meta-analysis conducted on more than 24,000 subjects from 18 countries showed that HPV antibodies acquired through natural infection provide modest and limited protection (22). The same study also highlights that the duration of innate immunity is still unknown. On the contrary, HPV vaccination generates 10- to 100-fold higher titres of anti-HPV L1 neutralizing antibodies than natural infection (23) and significantly reduces the risk of invasive cervical cancer, which is the ultimate intent of HPV vaccination programmes (24). Our results suggest the high susceptibility of the young population in our province to HPV infection. The burden of cervical cancer in our country is very high, and 1,205 new cervical cancer cases and 634 cervical cancer deaths are diagnosed annually (25). Although the Government of Serbia has pointed out on several occasions the obligation to reduce morbidity and mortality of HPV-related diseases, including cervical cancer, HPV vaccination at the state's expense was introduced only in 2022 (26). Vaccination against HPV infection is not part of the mandatory national immunization programme, and it is recommended for children aged over 9, before the first sexual intercourse. Also, teenagers of both genders aged 15–19 may receive the HPV vaccine free of charge (27).

Because individual behaviours and certain habits can influence the risk of HPV infection, we intended to estimate the correlation of HPV seropositivity with socio-demographic characteristics, lifestyle, and sexual habits. The only variable which showed a statistically significant association with HPV seropositivity was the lifetime number of sex partners. A number of lifetime sexual partners is considered to be a strong predictor of HPV infection (28, 29). Also, some studies have reported that HPV seroprevalence is associated with the age of sexual debut and tobacco use (28–30), but we could not confirm this association. Additionally, recent studies suggested that HPV seropositive women have greater antibody response than HPV seropositive men (18, 22, 29). However, we found a mild gender imbalance in favour of males. Furthermore, previous studies have shown that the anatomical site of exposure to HPV infection influences the probability of seroconversion and that heterosexual men have lower HPV seroprevalences compared to women (30). Our participants did not have an obligation to declare their sexuality. An explanation for why men are slightly more positive (8.9%) than women (7.6%) may be the fact that seropositive males included in our study have been older and had more sexual partners over their lifetime than females. In a current study, the average age of the first sexual intercourse among females was 18 years and 17 years among males. Considering that the time needed for HPV seroconversion is about 18 months (5), it is expected that the highest seroprevalence of HPV infections in our region should be up to 20 years. However, they recorded a slightly higher seroprevalence among subjects aged between 26 and 30 years compared to younger ones. This result potentially reflects the effect of multiple life-long sexual partners. Repeated antigenic stimulation through re-exposure to the virus stimulates the immune response and causes a detectable antibody response (5). Such observation was confirmed, especially in the case of HPV-16 (28, 29).

The current study has several limitations. First, we performed a cross-sectional study, and the results should be interpreted in the context of its limitations. The HPV DNA status of the participants before and at the moment of the sampling was unknown. The strength of our study was that all serological data were obtained using the same commercial kit, which enabled us to achieve a consistent and reliable interpretation of the results. However, some individuals seroconvert at deficient antibody levels, often below the detection limit of tests. It remains unknown whether the low HPV IgG seroprevalence we obtained is due to the serologic test of modest performance or to overvaluing the cut-off value of the serological test.

## CONCLUSION

We presented the results of the first study of HPV seroprevalence in the Province of Vojvodina, Serbia. The results highlight a low level of naturally induced HPV-specific serum antibodies and consequently the high risk of infection with prevalent high- (HPV 16, 18) and low-oncogenic (HPV 6, 11) types in unvaccinated young persons. Today, the carcinogenicity of HPV is mainly related to cervical cancer, but the increased incidence of other cancer types associated with HPV should also be noted. For these reasons, our data additionally stress the importance of the value, functions, and potentials of HPV vaccination. These data could be relevant starting point for national and regional health authorities in initiating additional research on HPV status after the HPV vaccine has begun to be applied on a more organized and mass basis.

**Acknowledgements**

This study was funded by the City of Novi Sad, City Administration for Health, in the period 2013–2016, Project No. XII-51-80-5-1/2013; XII-51-98-3/2014; XII-51-54-3/2015; XII-51-55-2/2016.

**Conflict of Interests**

None declared

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*Received December 27, 2021*
*Accepted in revised form March 4, 2023*