

# Human papillomavirus infection prevalence in female university students in Novi Sad, Serbia

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## SUMMARY

**Introduction** Cervical cancer, attributed to human papillomavirus (HPV) infection, represents the fourth most common and lethal cancer in Serbian women, and the second most common cancer in women aged 15–44.

**Objective** The aim of the study was to determine the presence of high-risk and low-risk HPV types in population of unvaccinated female university students in Novi Sad, Serbia, and to evaluate possible risk factors for HPV infection.

**Methods** Sample consisted of 250 young women (19–26 years of age) attending outpatient clinics for screening gynecological examination. All participants in the study completed a specially designed anonymous questionnaire. For the detection of HPV DNA, two commercial kits – High Risk HPV Real-TM and Low Risk HPV 6/11 Real-TM (Sacace Biotechnologies, Como, Italy) were used. Thirty positive samples were retested by GenoFlow HPV Array Test (DiagCor Bioscience Incorporation Limited, Hong Kong, China).

**Results** The overall prevalence rate of HPV was 61.6%. The most common HPV types in the present study were as follows: HPV 16, 31, 51, 52, and 18. Female students with only one sexual partner had significantly lower chance of having HPV infection. Other variables describing lifestyle did not show statistical significance.

**Conclusion** The present paper provides data on the prevalence of high- and low-risk HPV genotypes among university students in Novi Sad. Obtained results indicate the need for educational activities on sexually transmitted infections, including HPV, together with promotion of healthy lifestyles. According to our results, bivalent and quadrivalent prophylactic vaccines have the potential to prevent over 50% of infections. Percentage of protection with a second-generation prophylactic nonavalent vaccine would be more than 80%.

**Keywords:** human papillomavirus; real-time PCR; students

## INTRODUCTION

Human papillomavirus (HPV) is one of the most prevalent sexually transmitted infections in the world. The important consequence of this infection is cervical cancer (CC), the fourth most common cancer among women worldwide (15%) and the second most common in developing countries [1]. According to the latest data from GLOBOCAN (2012) study, the incidence of cervical cancer in Serbia is 23.8 per 100,000 inhabitants, ranking Serbia fourth among European countries – after Romania, Lithuania, and Bulgaria. Currently, with 1,501 new cases and approximately 609 deaths per year, cervical cancer is the fourth most common and lethal cancer in Serbian women, while in the group of women aged 15–44 it is in second place [2].

HPV infection is common in young sexually active population. Large number of newly acquired HPV infections are transient, but certain percentage of infections persist, and the persistent infections are involved in the development of cervical cancer and its immediate precursor lesions [3]. However, it is also well known that the development of cervical cancer involves complex interactions among various factors. Although viral infection is necessary for neoplastic transformation, evidence sug-

gests that host and environmental co-factors also play a significant role [4].

Primary prevention of HPV infection, and thus prevention of its sequelae, is important for public health [5]. Early detection of HPV infection in the population of young, sexually most active women, together with the introduction of prophylactic vaccines, represent effective prevention of cervical cancer.

## OBJECTIVE

The main objective of this study was to determine the presence of most common high-risk (HR) and low-risk (LR) HPV types in the population of unvaccinated female university students from Novi Sad, Serbia. The second objective was to gain insight into lifestyle habits proven to be risk factors for acquiring HPV infection.

## METHODS

### Samples

Sample consisted of 250 young, unvaccinated women (age 19–26 years; mean age 22.5 years) who gave consent for participation in the study.

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Subjects attended gynecological outpatient clinics for gynecological examination as part of a required health evaluation for students, at the Department of Gynecology, Institute for Health Protection of Students, Novi Sad.

All potential participants in the study were informed of the aims of the project by trained interviewers before gynecological evaluation. Informed consent was obtained from all individual participants included in the study. They were told that their participation is voluntary and that they have a full right to withdraw from the study at any point. Only after all the information was provided, subjects completed a specially designed anonymous questionnaire.

The questionnaire contained 24 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 habits (age at first intercourse, number of sexual partners). Additional questions were asked on history of sexually transmitted infections, knowledge about HPV infections, frequency of visits to a gynecologist and family history of cancer.

Speculum examination was performed by gynecologists. In addition to smear taken for the conventional cytology, a smear for HPV testing was taken and placed in a container containing preservation solution (Copan Diagnostics Inc., Murrieta, CA, USA). After the collection and transport of samples for HPV DNA assay, the samples were examined for the presence of individual HPV genotypes.

Ethical Committee of the Institute of Public Health of Vojvodina approved the study protocol.

### Nucleic acid isolation

Cervical swab samples were preserved in specimen transport medium (Copan Diagnostics Inc.). HPV DNA isolation protocol was carried out according to manufacturer's instructions (Sorb-A DNA extraction kit, Sacace Biotechnologies, Como, Italy). DNAs were extracted from an aliquot of 100 µL specimen. The extracted DNA was eluted in 100 µL elution buffer and stored at -20°C.

### Nucleic acid amplification

HPV DNA detection and genotyping was performed by real-time polymerase chain reaction (PCR) at the Center of Virology, Institute of Public Health of Vojvodina. Twelve high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were determined in the endocervical swabs with HPV High-Risk Typing Real-TM kit, (Sacace Biotechnologies). Regarding the validity of the test used, the authors relied on the certificate provided by the manufacturer. The certificate ensured satisfactory clinical validity of the used test in relation to hybrid capture method in terms of sensitivity and specificity, according to the international rules. The comparison was performed by the manufacturer.

Viral E7 gene was amplified using multiplex primers and four TaqMan probes. To prove the HR HPV, multiplex

reaction was performed in a volume of 13 µL. The process of amplification was performed in 45 cycles under the following conditions: 15 minutes at 95°C, 20 seconds at 95 °C, and 60 seconds at 60°C.

Two LR HPV types (6, 11) were determined in the endocervical swabs with HPV 6/11 Real-TM kit (Sacace Biotechnologies). Viral L1 gene was amplified using the primers and TaqMan probes. Reproduction of target DNA for the detection of LR HPV was performed in a volume of 25 µL. The process of amplification is performed in 45 cycles under the following conditions: 15 minutes at 95°C, 20 seconds at 95°C, and 60 seconds at 60°C.

### GenoFlow test

The GenoFlow Array Test kit (DiagCor Bioscience Incorporation Limited, Hong Kong, China) uses biotin-labeled primers and specific probes to detect 33 common HPV types (18 HR HPV types and 15 LR types). The extraction DNA (Sorb-A DNA extraction kit, Sacace Biotechnologies) was mixed with PCR reagent mix and DNA *Taq* polymerase provided with the GenoFlow test kit, and PCR-amplified using the thermocycling condition stated in the manual. The amplicons were genotyped using flow-through hybridization according to manufacturer's instructions. Retesting was done on 30 samples previously genotyped by HPV High Risk Typing Real-TM kit, (Sacace Biotechnologies) with GenoFlow HPV Array Test kit (DiagCor Bioscience Incorporation Limited).

### Statistical analysis

Data were statistically processed by two-way analysis of variance (ANOVA), for each variable. Analysis of variance and LSD-tests were performed on data transformed by arcsine transformation in order to meet normal distribution of frequencies. The results were considered significant if the probability of test was less than 0.05. For statistical analysis of data, STATISTICA 12 (StatSoft Inc., Tulsa, OK, USA) statistical package was used [6].

## RESULTS

### Demographic characteristics of the study population

A total of 250 non-vaccinated, young women (mean age of 22.5 years, age range 19–26 years) were enrolled in the study from September 2012 to December 2014. The group of University of Novi Sad students mostly consisted of students from Vojvodina (90%), and only a small part (10%) was from other parts of Serbia. Almost all of young women were single 240 (96%), nine (3.6%) were married, and one (0.4%) was divorced. Seven of them had at least one full-term pregnancy. Nine (3.6%) of the participants had previous abortion.

## HPV Type Distribution

Overall prevalence of HPV DNA in a sample of young females was 61.6% (154/250). Among positive samples, oncogenic HPV types were detected in 96.1% (148/154), and LR HPV types in 28.6% (44/154).

Infections with a single HPV type was detected in 49.4% (76/154) participants, while most co-infections (23.4%; 36/154) involved two HPV genotypes; multiple infections with three HPV types were found in 17.5% (27/154) of the participants; four or more HPV genotypes were found in 9.7% (15/154) of the cases (Table 1).

Our analysis involved determination of 12 highly oncogenic HPV types, type 16 being the most frequent with 48.1% (74/154), followed by types 31, 51, and 18. Two low-oncogenic types were detected – HPV type 6 was the predominant one, with 27.3% (42/154), while HPV 11 was considerably rare, with 3.9% (6/154) (Table 2).

Vaccinal HPV types 16/18 were present in 54.5% (84/154) of the cases; types 16/18/6/11 in 64.2% (99/154), and HPV types 6/11/16/18/31/33/45/52/58 included in the new nonavalent prophylactic vaccine were present in 87% (134/154) of the cases, both as single infections and in combination with other HPV types (Table 3).

## Comparison between HPV High Risk Typing Real-TM kit (Sacace Biotechnologies) and GenoFlow HPV Array Test kit (DiagCor Bioscience Incorporation Limited)

In the total of 30 samples, the comparison between the kits showed complete agreement in 89.3%, partial agreement in 7.1%, and disagreement in 3.6% of the samples. By applying the GenoFlow test, additional HR HPV types were detected: HPV 73 (5/30 cases); HPV 66/68 (4/30), HPV 81 (4/30); HPV 53 (2/30); HPV 71, 82, and 83 (1/30). From LR HPV types, the following additional types were detected: HPV 26/84 (5/30 cases), HPV 40/61 (5/30); HPV 43/44 (4/30); HPV 54/55 (2/30); HPV 42 (1/30).

## Overall HPV DNA prevalence and factors associated with HPV infection

In our study, about 22.8% of the young women had a history of smoking, approximately for 4.9 years and 12.2 cigarettes per day. Out of 250 young women, 35.2% reported condom use, 26.4% use of oral contraceptives, 12.8% reported a history of sexually transmitted infections, and 20.9% of the participants had condylomata. Approximate age of first sexual intercourse was 17.7 years. Average number of sexual partners in the previous two years was two. Significant differences were found among HPV DNA positive and negative women only for variables associated with number of sexual partners in the previous two years ( $p = 0.009$ ). Women who had one sexual partner in the previous two years showed lower rates of HPV infection compared to women who had two or more sexual partners.

**Table 1.** Distribution of single, double, and multiple HPV infections (n = 154)

HPV infection	n (%)
Single HPV infection	76 (49.4)
Co-infection with 2 HPV types	36 (23.4)
Co-infection with 3 HPV types	27 (17.5)
Co-infection with 4 HPV types	15 (9.7)

HPV – human papillomavirus

**Table 2.** Frequency of 12 high-risk HPV and two low risk genotypes in university students in Novi Sad (n = 154)

HPV genotypes	n (%)	
High-risk	16	74 (48.1)
	31	28 (18.8)
	51	28 (18.8)
	52	24 (15.6)
	18	19 (12.3)
	56	15 (9.7)
	59	13 (8.4)
	33	12 (7.8)
	58	11 (7.1)
	45	8 (5.2)
	39	7 (4.5)
	35	5 (3.2)
Low-risk	6	42 (27.3)
	11	6 (3.9)

**Table 3.** Frequency of vaccinal HPV types (n = 154)

Vaccinal HPV types	N (%)
16/18	84 (54.5)
6/11/16/18	99 (64.2)
6/11/16/18/31/33/45/52/58	134 (87.0)

Other variables, as tobacco use ( $p = 0.955$ ), oral contraception method used ( $p = 0.806$ ), condom use ( $p = 0.293$ ), and history of sexually transmitted infections were not statistically significant ( $p = 0.825$ ) (Table 4).

A summary of variables which describe individual health care are provided in Table 5. Our research shows that 60% of the young women had knowledge about HPV infection and sources of information were internet sites and primary care physicians. Eighteen percent of the interviewed women had undergone an HPV test in the recent past. Of all the participants, 94% had regular gynecological check-ups, while 6% of the women went to a gynecologist irregularly.

## DISCUSSION

Among 250 non-vaccinated university students in Novi Sad, Serbia (19–26 years of age, with mean age of 22.5 years), the overall HPV prevalence was 61.6%.

Numerous studies focused on the prevalence of HPV in women show that the highest percentage of infection is present in youngest women, i.e. < 25 years. HPV infection in women has been consistently found to have a peak just after the onset of sexual activity, usually starting from 15 years of age [7], reaching prevalence of up to 80% in some populations [8]. Results obtained in this study regarding the frequency of HPV infection in women 19–26 years

**Table 4.** Characteristics of life style and sexual habits in relation to the presence of HPV

Variables	% of HPV-positive women (n = 154)	% of HPV-negative women (n = 96)	p-value (F-test)
Tobacco users	23.1 <sup>a</sup>	22.6 <sup>a</sup>	0.955
Oral contraceptive users	25.4 <sup>a</sup>	26.8 <sup>a</sup>	0.806
Condom users	31.6 <sup>a</sup>	38.8 <sup>a</sup>	0.293
First sexual intercourse before 17 years of age	49.9 <sup>a</sup>	55.2 <sup>a</sup>	0.412
One sexual partner in previous two years	57.3 <sup>b</sup>	31.8 <sup>a</sup>	0.009*
≥ 2 sexual partners in previous two years	42.7 <sup>b</sup>	68.2 <sup>a</sup>	
Previously reported STDs	12.1 <sup>a</sup>	12.9 <sup>a</sup>	0.825

\* statistically significant difference

<sup>a, b</sup> Labels of homogenous groups: values with the same letter do not differ significantly at level  $\alpha = 0.05$ **Table 5.** Personal health habits and knowledge about HPV in relation to the presence of HPV

Variables	% of HPV-positive women (n = 154)	% of HPV-negative women (n = 96)	p-value (F-test)
Regular visit to gynecologist	95.5 <sup>a</sup>	93.5 <sup>a</sup>	0.652
Papa test	87.9 <sup>a</sup>	81.3 <sup>a</sup>	0.125
Family history of cancer	28.9 <sup>a</sup>	37.4 <sup>a</sup>	0.255
Knowledge about HPV	61.1 <sup>a</sup>	58.7 <sup>a</sup>	0.707
Recent HPV analysis	18.6 <sup>a</sup>	16.4 <sup>a</sup>	0.586

<sup>a</sup> Labels of homogenous groups: values with the same letter do not differ significantly at level  $\alpha = 0.05$ 

of age are in accordance with data found in neighboring countries. Grozdanov et al. [9] tested a group of 95 18- to 29-year-old women in Bulgaria and detected HPV DNA in 63% of samples, while in Romania, Moga et al. [10] reported the highest HPV prevalence in women of age < 25, being 54.93%. The authors further state that prevalence decreases with age. A study on a group of 37 young women (age < 29) in Montenegro detected HPV prevalence of 53% [11]. Differences in prevalence depend of geographic areas, diversity of population samples, type of the test used for DNA HPV detection, and other factors [12]. Most common HPV types in the present study were HPV 16, 31, 51, 52, and 18, which is in accordance with the data of Bruni et al. [13] stated in the report provided by the Information Centre on HPV and Cancer. A significant feature of the present study is high prevalence of HPV 16, meaning that nearly one half (48.1%) of HPV DNA-positive young women had infection with HPV 16. This result is similar to the findings of other authors from the region [14, 15, 16]. HPV 18 was detected less frequently and ranked fifth, similarly as in other countries in women of the same age group [14, 17]. Also, considerably high percentage of multiple infections was noticed (49.4%), which is in accordance with the results from neighboring countries [9, 10, 15]. Multiple infections may lead to the increased risk of viral persistence and neoplastic progression [18].

Detection and monitoring of HPV infection is an important step in prevention of cervical cancer development, and incorporation of a broader HPV DNA genotype range into screening programs would be desirable. Our pilot study on 30 samples retested by GenoFlow HPV Array Test method showed high agreement (89.3%) with the real-time PCR test (Sacace Biotechnologies) routinely used in our laboratory. GenoFlow HPV Array Test offered a broader range of genotypes determination (33 vs. 14 genotypes), and indicated a relatively high frequency of some genotypes not covered by a conventional real-time PCR tests. Some

of the detected genotypes have been detected for the first time in Serbia. By applying the GenoFlow test, we observed that additional HR HPV types (53, 66, 68, 71, 73, 81, 82) are present in population of young women in our region. As for the LR HPV, besides genotypes 6 and 11, we detected the presence of HPV 26, 40, 42, 43, 44, 54, 55, 61, and 84. According to these preliminary results, even higher prevalence rate could be postulated, especially of LR HPV types.

From the public health perspective, in countries like Serbia, where resources for screening are scarce, population of university students could be a clever choice for gaining the epidemiological insight into HPV genotype prevalence. Taking into account great variety of circulating genotypes in this age group, even a limited number of samples could give a valuable data on the circulating genotypes for the entire population.

Routine HPV vaccination programs are implemented in 22 out of 31 EU/EEA countries [19]. In addition to the two vaccines against HPV infection approved previously by the Food and Drug Administration (FDA) – quadrivalent (HPV4) Gardasil (2006; Merck & Co., Inc., Kenilworth, NJ, USA) and bivalent (HPV2) Cervarix (2009; GlaxoSmithKline, London, UK) [20] – FDA recently approved a nonavalent vaccine Gardasil9 (HPV9) (December 2014, Merck & Co., Inc.) [21]. Cervarix (HPV2) and Gardasil (HPV4) both protect against HPV types 16 and 18. In addition, HPV4 protects against HPV types 6 and 11. HPV9 offers protection against five additional HPV types: 31, 33, 45, 52, and 58, which cause approximately 20% of cervical cancers and are not covered by previously FDA-approved HPV vaccines. The HPV9 vaccine has a potential to prevent approximately 90% of cervical, vulvar, vaginal, and anal cancers [22].

Coverage rates vary in different EU countries and range between 17% in Luxembourg and 84% in Portugal [19]. Although both HPV2 and HPV4 vaccines are registered in Serbia, vaccination programs have not



yet been implemented. It should be emphasized that in our study, vaccine-covered HPV types 16/18/6/11 were present in 64.2% (99/154) of the cases, and HPV types included in the new nonavalent prophylactic vaccine (6/11/16/18/31/33/45/52/58) were identified in 87% of the cases (134/154), both as single infections and in combination with other HPV types [23].

It has been shown that among healthy young women, most of the HR HPV infections tend to be cleared without treatment and become undetectable without clinical consequences. However, some infections persist and a subset of persistent infections may progress to cervical intraepithelial neoplasia or invasive cancer [24]. Our intention was to gain insight into the lifestyle, sexual habits, and knowledge of HPV infections, the factors of significance for the health of young women in our region which correlate with the frequency of acquiring HPV infections.

Habit of tobacco use is rather frequent in women from our region (22.8%). However, this factor was not associated with HPV DNA positivity in the present study. This is not surprising, since only the persistency was proven to be associated with heavy smoking [25, 26]. About 26.4% of the young women in our study used oral contraceptives, most of them reporting the period of one year. Research suggests that long-term use of oral contraceptives could increase the risk of cervical cancer by up to fourfold in women with HPV infection [27]. This variable is presently not associated with HPV DNA positivity, but similarly to tobacco use it may contribute to persistency of HPV infection and the increased risk for development of precancerous cervical lesions and cancer in the future.

The results of the statistical analysis demonstrated that HPV infection was associated with some of the parameters of sexual behavior, such as average age of the first intercourse and number of sexual partners in previous two years (Table 4). Nielsen et al. [28] found that lifetime number of sexual partners was the main risk factor for HR HPV infection. In our research, the average age of the first intercourse among young women was 17.7 years. Only women who had one partner in the previous two years had statistically lower chance of HPV infection. It is well known that monogamous lifestyle can reduce the risk of infection and consequently the occurrence of precancerous lesions. Other variables describing sexual habits including condom use and history of sexually transmitted infections were not statistically significant in the present study. The absence of any significant association between HPV and past sexually transmitted diseases might be accounted for by the fact that most women are reluctant to disclose the details of previously sexually transmitted diseases, as well as the number of sexual partners. Recent studies suggest that condom use provides only partial protection against the transmission of genital HPV types, the effectiveness being up to 70% due to the fact that HPVs can infect portions of skin not covered by condom [29]. Recommendations for the use of condoms are still given, in order to reduce the risk of transmission of HPV warts and spread to other parts of healthy skin and

mucous membranes, as well as to prevent transmission of other sexually transmitted diseases.

According to our questionnaire, 94% of the participants had the habit of regular gynecological check-ups. This percentage is much higher than the one for general population and can be attributed to the fact that we surveyed female university students. The 2013 Ipsos survey (Ipsos Strategic Marketing, Belgrade, Serbia) showed that the percentage of women of fertile age in Serbia who had visited a gynecologist in the previous year was 47.5%. Differences in this respect exist by region, type of settlement, education, and financial situation of the household [30].

Literature data show different levels of knowledge about HPV infections among young population worldwide. While only 17.7% of students from Nigeria (368 female students, aged 16–29 years) have some knowledge on HPV infection, an international study showed that higher percentage of young adults in the USA (62%), the UK (44%), and Australia (40%) are informed about HPV infection [31]. Results from the present study indicate that 40% of the respondents have no knowledge on HPV, and that 82% of them have never submitted themselves to an HPV test.

## CONCLUSION

The present work provides data on the prevalence of HR and LR HPV genotypes and potential risk factors for HPV infection among university students in Novi Sad, Serbia. The results obtained in this study indicate the need for educational activities on sexually transmitted infections, including HPV. In countries such as Serbia, where HPV vaccination programs have not yet been implemented, and resources for HPV screening programs are scarce, university students could be a target population group for the HPV testing and awareness raising, together with the promotion of healthy lifestyles. Undertaken at this moment in their lives, the activities will most probably make a long lasting effect on individual, as well as reproductive health of the entire population.

Furthermore, our data suggest that currently licensed prophylactic vaccines, if applied, have the potential to prevent approximately 50% of infection, while percentage of protection with a second-generation prophylactic nonavalent vaccine would be more than 80%. However, a wider-scale HPV screening with a test for broader genotype range is needed as a starting point in prevention actions, as well as a better algorithm for cancer diagnosis and adequate treatment.

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## Учесталост инфекције хуманим папилома вирусом код студенткиња у Новом Саду у Србији

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### КРАТАК САДРЖАЈ

**Увод** Рак грлића материце, проузрокован ХПВ инфекцијом, представља четврти најчешћи летални карцином код жена у Србији и други најчешћи малигнитет у групи жена од 15 до 44 године.

**Циљ рада** Циљ рада је био да се одреди присуство *HR* и *LR* ХПВ типова у популацији невакцинисаних студенткиња Универзитета у Новом Саду и процене могући фактори ризика за ХПВ инфекцију.

**Методе рада** Узорак се састојао од 250 младих жена (старости 19–26 година) укључених у студију током систематских гинеколошких прегледа. Све учеснице су попуниле специјално дизајниран анонимни упитник. За детекцију ХПВ ДНК коришћена су два комерцијална кита: *High Risk ХПВ-real-TM* и *Low risk ХПВ 6/11 real-TM* (*Sacace Biotechnologies, Italy*). Тридесет позитивних узорак је ретестирано употребом *GenoFlow ХПВ Array Test* (*DiagCor, Bioscience*).

**Резултати** Укупна стопа учесталости ХПВ инфекције је износила 61,6%. Најчешћи типови ХПВ у овој студији били су: ХПВ 16, 31, 51, 52 и 18. Студенткиње са само једним сексуалним партнером су имале сигнификантно мању шансу за стицање ХПВ инфекције. Остале варијабле које описују начин живота нису показивале статистичку значајност.

**Закључак** Презентовани рад пружа податке о учесталости високо и нискоризичних генотипова ХПВ међу студенткињама Универзитета у Новом Саду. Добијени резултати указују на потребу едукације о сексуално преносивим инфекцијама, укључујући и ХПВ, заједно са промоцијом здравих стилова живота. Према нашим резултатима, бивалентна и четворовалентна профилактичка вакцина би могле да спрече преко 50% инфекција. Процент заштите применом деветовалентне вакцине износио би преко 80%.

**Кључне речи:** хумани папилома вирус; *real-time PCR*; студенти

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