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Research Paper

Co-infection Of *Ureaplasma urealyticum* And Human Papilloma Virus In Asymptomatic Sexually Active Individuals

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Abstract

This study aimed to determine the role of asymptomatic bacterial sexually transmitted infections (STIs), such as *Chlamydia trachomatis* (*Ct*), *Mycoplasma genitalium* (*Mg*), *Mycoplasma hominis (Mh)*, and *Ureaplasma urealyticum* (*Uu*) in human papillomavirus (HPV) infection.

In total, 264 asymptomatic outpatients aged between 21 and 80 years were prospectively enrolled in this study during routine gynecological screening tests. Specimens collected with a Cervex Brush were routinely analyzed with the Hybrid Capture 2 assay for HPV. Simultaneously, a specimen obtained with an endocervical swab was used to detect *Ct* and *Mg* with a monoplex real-time polymerase chain reaction (PCR) and to confirm *Mh* and *Uu* with a Mycoplasma IST 2 kit.

The detection rates (%) of HPV, *Ct*, *Mg*, *Mh*, and *Uu* were 82/264 (31.1), 6/264 (2.3), 5/264 (1.9), 16/264 (6.1), and 95/264 (36.0), respectively. Of 95 *Uu*, 32 (33.7%) showed high density colonization (HDC, $\geq 10^4$ color-changing units/mL). HDC-*Uu* was significantly associated with HPV infection (p=0.014, chi-square test). *Mg* infection and *Mh* infection were not associated with HPV infection (p=0.981 and p=0.931, chi-square test). Age was not associated with HPV infection or bacterial infection.

Our data suggested that asymptomatic HDC-*Uu* was closely associated with HPV infection. Therefore, simultaneous evaluation for *Uu* and HPV should be performed during gynecological screening, even in asymptomatic individuals.

Key words: Mycoplasma; *Ureaplasma urealyticum;* human papillomavirus

Introduction

Human papillomavirus (HPV) has been identified the etiologic agent of warts, cervical intraepithelial neoplasia (CIN), and cervical cancer. Among sexually transmitted infections (STIs), only HPV infection is known to be a major cause of cervical cancer.[1] However, cervical carcinogenesis is not facilitated by HPV infection alone, but it also associated with environmental factors, such as multiple sex partners and sexually transmitted diseases.[2]

A Recent study investigated the relationships between HPV and vaginal bacteria, including *Mycoplasma* spp. and *Chlamydia trachomatis* (*Ct*), and found that HPV was associated with *Ct* and *Ureaplasma urealyticum* (*Uu*).[3] Therefore, detection of *Mycoplasma* or *Chlamydia* infection may play a role in decreasing the prevalence of cervical cancer. Althouogh, the majority of individuals infected with *Chlamydia* and *Mycoplasma* were asymptomatic,[4,5] they could be carriers of *Chlamydia* and *Mycoplasma* infections in the community. A recent study

conducted in Korea showed an incidence (%) of 5.6, 0.3, 22.1 and 11.6, respectively, for *Ct*, *M. genitalium* (*Mg*), *Uu,* and *M. hominis (Mh)* in asymptomatic individuals.[6] However, to our knowledge, no epidemiologic study has evaluated the association between asymptomatic bacterial STIs and HPV infection in sexually active individuals.

The aim of this study was to evaluate the relationship between bacterial STI and HPV infection among asymptomatic sexually active women.

Materials and methods

Study population

From a group of asymptomatic women who visited a gynecological outpatient clinic for routine screening between January 2012 and August 2017, 264 volunteers were enrolled in this study. Informed consent was obtained from all women participating in the study. The institutional review board of The Catholic University of Korea approved this study (No. VC15RISI0094). Cervical specimens for high-risk HPV detection and identification of other microorganisms were taken from the asymptomatic volunteers during a routine pelvic examination. Cytology was graded according to the Bethesda system, using standard forms, and was classified as negative, low-grade (atypical squamous cells of undetermined significance, atypical glandular cells of undetermined significance, low-grade squamous intraepithelial lesion), or high-grade (atypical squamous cells - cannot exclude high- grade squamous intraepithelial lesion).[7]

All women aged between 21 and 80 years who engaged in sexual activity at least once a month were eligible. Exclusion criteria were: 1) any urogenital symptoms, such as vaginal and/or urethral discharge, itching at the external genitalia, dyspareunia, dysuria, vaginal spotting, or hematuria; 2) antimicrobial treatment within 1 month prior to the present study; 3) recently diagnosed cervical disease within 6 months prior to the present study; 4) current pregnancy.

All participants were interviewed regarding obstetric history and the number of sexual partners in the prior 2 years.

HPV detection

Specimens were collected with a Cervex Brush (Rovers Medical Devices B.V., The Netherlands) and rinsed with PreservCyt fixative solution (Cytyc Corp., Roxborough, MA, USA). Specimens were placed in denatured alcohol at 65 ± 2°C for 45 min, and mixed with a 1:25 diluted HPV probe (for high-oncogenic risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) in a 96-well plate with the help of a rotary shaker at 1,100 rpm. The mixture was incubated at 65 ± 2°C for 60 min. The hybridized mixture was moved a Capture micro-plate (Digene Corp., Gaithersburg, MD, USA), with shaking at 1,100 rpm at 20-25°C for 60 min. Finally, the conjugation process was performed by adding the detection reagent (CDP-Star®, Tropix, Inc., MA, USA) to the Capture micro-plate. After the washing process, the test result was reported in relative light units using a DML 2000 Luminometer (Qiagen, Crawley, UK).

Detection of other microorganisms

The swab specimen in the collection tube was equilibrated to room temperature and mixed by vortexing. After centrifugation at 13,000 rpm for 10 min, the supernatant was discarded and the pellet was re-suspended in 1 ml of 1× phosphate-buffered saline by vortexing thoroughly to re-dissolve and disperse the sample. The mixed specimen was transferred to 1.5 ml Eppendorf tubes. DNA was then extracted from the pretreated specimens using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. *Ct* and *Mg* were detected with a monoplex polymerase chain reaction (PCR) using Seegene DPO™ technology.[8] PCR amplification was performed with the Seeplex® *C. trachomatis* Detection kit and Seeplex® *M. genitalium* Detection kit (Seegene, Seoul, Korea) according to the manufacturer's instructions.

Both *Mh* and *Uu* were detected using the commercially available Mycoplasma IST 2 kit (bioMérieux, Marcy-l'Etoile, France). Using this kit, we detected *Mh* and/or *Uu* at a cut-off value of 10^4 color-changing units (CCU)/mL. A positive test was indicated by a change in broth color from yellow to red (urea for *Uu* and arginine for *Mh*). An endocervical swab specimen was inoculated into the R1 solution (transport medium) according to the manufacturer's instructions, then combined with a vial of R2 (lyophilized growth medium) and vortexed. A 55-µL aliquot of the solution was dispensed into each of 22 test wells on the strip. Two drops of mineral oil were added to each well. The strips were incubated at 37.8°C for 24-48 h. Antimicrobial susceptibility against doxycycline, josamycin, ofloxacin, erythromycin, tetracycline, ciprofloxacin, azithromycin, clarithromycin and pristinamycin was also tested using this commercial kit.

However, *U. parvum* (biovar 1) and *Uu* (biovar 2) could not be discriminated with this kit. Therefore, we used *Uu* for both biovars as in previous research.[9]

Statistical analysis

SPSS® software (SPSS 15.0, SPSS, Chicago, IL, USA) was used for the statistical analysis. Student's

t-test, the chi-squared test, and Fisher's exact test, were used for statistical evaluations of the associations between groups using nominal data. Logistic regression was used for multivariate analysis and $p < 0.05$ was considered statistically significant.

Results

The mean age of participants was 50.76 ± 11.04 years and the mean body mass index (kg/m^2) was 23.04 ± 4.19. Baseline participant characteristics are presented in Table 1. Among epidemiologic factors, 2 or more sexual partners in the prior 2 years showed a greater association with current HPV infection than 1 partner (p=0.001, chi-square test). Obstetric history was not significantly associated with current HPV infection.

The incidences (%) of HPV, *Ct*, *Mg*, *Uu* (<10^4 CCU/mL), high-density colonization (HDC)-*Uu* (>10^4 CCU/mL), *Mh* (<10^4 CCU/mL), and HDC-*Mh* (>10^4 CCU/mL) in the present study was 31.1, 2.3, 1.9, 23.9, 12.1, 4.9, and 1.1, respectively. Among the 264 asymptomatic volunteers, HPV tested positive in 82 (31.1%) and 69 (26.1%) had abnormal cytology. HPV infection and both low- and high-grade cytologic abnormalities, were not associated with patient age. Bacterial infections were also not related to patient age. The distribution of HPV infection, cytologic abnormalities, and bacterial infections according to age is shown in Table 2.

Two *Ct* infections (2/6) were found in the HPV-infected group (p=0.903, chi-square test). HDC-*Uu* (>10^4 CCU/mL) was significantly associated with HPV infection (p=0.014, chi-square test). Even after adjusting for confounding epidemiologic factors, such as multiple sexual partners (≥3), HDC-*Uu* was still significantly associated with HPV infection (p=0.045, logistic regression analysis). *Uu* (<10^4 CCU/mL) was not associated with HPV infection (p=0.284, chi-square test). *Mg* and *Mh* (both <10^4 CCU/mL) were also not associated with HPV infection (p=0.158 and p=0.981, respectively, chi-square test). The distribution of each microorganism is shown according to the presence of HPV infection in Table 3.

Antimicrobial susceptibility testing of *Uu* showed that the microorganisms were resistant to fluoroquinolones, such as ofloxacin and ciprofloxacin. Meanwhile, *Mh* tended to be resistant to macrolides such as erythromycin, azithromycin and clarithromycin (Table 4).

Table 1. Baseline characteristics of study population (N=264)

	HPV negative (n=182)	HPV positive (n=82)	Total (n=264)	OR	*p*-value, 95% CI
Age (mean)	51.5±8.06	49.11±12.55	50.76±11.04		0.376‡, -2.234 ~ 5.865
Number of partners					
1 partner	155 (85.2%)	39 (47.6%)	194 (73.5%)	1.00	
2 partners	14 (7.7%)	20 (24.4%)	34 (12.9%)	5.678*	0.001§, 2.634 ~ 12.24
3 or more partners	13 (7.1%)	23 (28.0%)	36 (13.6%)	7.032*	0.001§, 3.270 ~ 15.12
Parity					
Nulliparous	43 (23.6%)	22 (26.8%)	65 (24.6%)	1.00	
Multiparous	139 (76.4%)	60 (73.2%)	199 (75.4%)	1.54*	0.330§, 0.641 ~ 3.715
Abortion	25 (13.7%)	23 (28.0%)	48 (18.2%)	2.29†	0.198§, 0.752 ~ 6.948

Each mean data was presented with standard deviation
OR* is the odds ratio compared with the first category, and OR† is the odds ratio between HPV negative and positive group.
p-value‡ and *p*-value§ were measured by t-student test and Chi-square test, respectively.

Table 2. Characteristics of human papillomavirus infection, cervical cytology and sexually transmitted bacterial infection by age (N=264)

Age distribution			21~50 (n=139)	51~80 (n=125)	OR	*p*-value	95% CI
HPV							
	HPV negative (n=182)		93/139 (66.9%)	89/125 (71.2%)	1.00		
	HPV positive (n=82)		46/139 (33.1%)	36/125 (28.8%)	1.223,	0.4516*	0.724 – 1.652
Cytology							
	Negative cytology (n=195)		101/139 (72.6%)	94/125 (75.2%)	1.00		
	Low grade abnormality+ (n=28)		14/139 (10.1%)	14/125 (11.2%)	0.888	0.766*	0.406 – 1.945
	High grade abnormality‡ (n=41)		24/139 (17.3%)	17/125(13.6%)	1.326	0.412*	0.675 – 2.603
Bacterial infection							
	Chlamydia trachomatis (n=6)		4/139 (2.9%)	2/125 (1.6%)	1.822	0.487*	0.328 – 10.13
	Mycoplasma genitalium (n=5)		4/139 (2.9%)	1/125 (0.8%)	3.674	0.216*	0.405 – 33.34
	Ureaplasma urealyticum (n=95)	<10^4CCU/ml (n=63)	32/139 (23.0%)	31/125 (24.8%)	0.907	0.735*	0.515 – 1.598
		>10^4CCU/ml (n=32)	18/139 (12.9%)	14/125 (11.2%)	1.179	0.664*	0.560 – 2.483
	Mycoplasma hominis (n=16)	<10^4CCU/ml (n=13)	4/139 (2.9%)	9/125 (7.2%)	0.382	0.105*	0.115 – 1.273
		>10^4CCU/ml (n=3)	1/139 (0.7%)	2/125 (1.6%)	0.446	0.500*	0.040 – 4.978

Data in bracket was presented as positive cases/all cases, percentage
* Chi-squared test; +Low grade: ASCUS, AGUS, LSIL; ‡High grade: ASC-H, HSIL

Table 3. Relationship between sexually transmitted bacterial infections and HPV infection (N=264)

Bacterial infection		HPV negative (n=182)	HPV positive (n=82)	*p*-value, OR, 95% CI
C. trachomatis† (n=6)		4 (2.2%)	2 (2.4%)	0.903*, 1.113 0.199-6.202
M. genitalium‡ (n=5)		2 (1.1%)	3 (3.7%)	0.158*, 3.418, 0.559-20.86
U. urealyticum§ (n=95)	<10^4 CCU/ml (n=63)	40 (22.0%)	23 (28.0%)	0.284*, 1.384, 0.762-2.512
	>10^4 CCU/ml (n=32)	16 (8.8%)	16 (19.5%)	0.014*, 2.515, 1.189-5.322
M. hominis‖ (n=16)	<10^4 CCU/ml (n=13)	9 (4.9%)	4 (4.9%)	0.981*, 0.985, 0.294-3.299
	>10^4 CCU/ml (n=3)	2 (1.1%)	1 (1.2%)	0.931*, 1.111, 0.099-12.44

Each value was presented as negative/positive.
*Chi-squared test
†*Chlamydia trachomatis*
‡*Mycoplasma genitalium*
§*Ureaplamsa urealyticum*
‖*Mycoplasma hominis*

Table 4. Antimicrobial susceptibility pattern of *Ureaplasma urealyticum* and *Mycoplasma hominis* in asymptomatic sexually active women (N=111*)

	Ureaplasma urealyticum (n=95)			*Mycoplasma hominis* (n=16)		
	S† (%)	I‡ (%)	R§ (%)	S† (%)	I‡ (%)	R§ (%)
Doxycycline	95(100.0)	0 (0.0)	0 (0.0)	16(100.0)	0 (0.0)	0 (0.0)
Josamycin	95(100.0)	0 (0.0)	0 (0.0)	16(100.0)	0 (0.0)	0 (0.0)
Ofloxacin	38 (40.0)	48 (50.5)	8 (8.4)	14 (87.5)	2 (12.5)	0 (0.0)
Erythromycin	83 (87.4)	10 (10.5)	2 (2.1)	0 (0.0)	0 (0.0)	16(100.0)
Tetracycline	95(100.0)	0 (0.0)	0 (0.0)	16(100.0)	0 (0.0)	0 (0.0)
Ciprofloxacin	10 (10.5)	39 (41.1)	46 (48.4)	11 (68.8)	2 (12.5)	2 (12.5)
Azithromycin	77 (81.0)	18 (18.9)	0 (0.0)	0 (0.0)	4 (25.0)	12 (75.0)
Clarithromycin	93 (97.9)	0 (0.0)	2 (2.1)	0 (0.0)	0 (0.0)	16(100.0)
Pristinamycin	95(100.0)	0 (0.0)	0 (0.0)	16(100.0)	0 (0.0)	0 (0.0)

*Among 264 patients, 95 patients were positive for *Ureaplasma urealyticum* and 16 patients were positive for *Mycoplasma hominis*. Antibiotics susceptibility was tested only for *Ureaplasma urealyticum* and/or *Mycoplasma hominis* positive patients.
†Susceptible
‡Intermediate
§Resistant

Discussion

The most important finding of this study was that HDC-*Uu* was associated with HPV infection in asymptomatic, sexually active woman at all age. Verteramo et al. suggested that *Ct* and *Uu* (>10^4 CCU/mL) may be risk factors for HPV infection in asymptomatic patients.[3] Lukic et al. emphasized that *Uu* (>10^4 CCU/mL) in asymptomatic woman could be a cofactor in HPV infection and may exert a negative influence on cervical cytology.[2]

This result was also similarly observed in symptomatic woman. Pisani et al. found parallel positive rates of *Uu* and HPV infection and suggested a synergistic effect between *Uu* and HPV infection in CIN and cervical cancer caused by HPV.[10]

Zhang et al. found that *Uu* was the most common infection among patients with HPV, which supports our result. However, in contrast to our results, there was no significant association between *Uu* and HPV infection.[11]

Other studies suggesting a relationship between HPV infection and *Uu* infection are described in table 5.[2,3,10,12-20] These studies strongly support an association between *Uu* and HPV infection. Coinfection of *Uu* with HPV contributes to development of CIN. Diagnosis and treatment can be delayed in asymptomatic women, but not in symptomatic women with *Uu* and HPV coinfection. Therefore, HPV testing is suggested in women with *Uu* infection, regardless of symptom status.

The number of sexual partners is a known risk factor for HPV infection, Kataja et al. reported that the number of sexual partners during prior 2 years is the strongest independent risk factor for HPV infections.[21] In our cohort, we tried to classify participants according to the number of sexual partners in the prior 2 years. Among 264 participants, 34 had 2 partners and 36 had 3 or more partners, which suggested that the cohort in our study consisted of sexually active individuals. Even after adjustment for the number of sexual partners (logistic regression analysis with cut-off at 1-2 versus 3 or more), HDC-*Uu* was significantly associated with HPV infection in the present study.

Kataja et al. reported that the risk for HPV infection varied with age, being highest in the 20-29 year old group, and thereafter declining in subsequent 10-year age groups.[21]

However, in our cohort, we found no relationship between age and HPV infection. Moreover, age was not associated with abnormal cytology or bacterial infection. Therefore, asymptomatic infection with HDC-*Uu* should be eradicated, regardless of age, for the prevention of HPV infection and subsequent CIN.

Table 5. Relationship between bacterial infection and HPV infection

Number	Parameter	Key findings	Authors
1	Bacterial infection, CIN	*Uu** detection in 40.5% of CIN+ patients	Szostek et al. [12]
2	Bacterial infection, HPV infection	Correlation between *Uu** and HPV infection	Pisani et al. [10]
3	Bacterial infection, HPV infection, Cytology	*Uu** (> 10,000 CCU/mL) as a cofactor of HPV infection Significant association between *Uu** infection and high grade CIN	Lukic et al. [2]
4	Bacterial infection, HPV infection	Significant association between HPV and *Ct*‡ Significant association between HPV and *Uu** (> 10,000 CCU/mL)	Verteramo et al. [3]
5	Bacterial infection, HPV infection	Significant association between HPV and *Ct*‡ Significant association between HPV and *Uu** (> 10,000 CCU/mL)	Zheng et al. [13]
6	Bacterial infection, HPV infection	5 times increased risk of HPV infection in case of *Uu** infection	Biernat-Sudolska et al. [14]
7	Bacterial infection, HPV infection, CIN	Higher positive rate of *Uu** infection in CIN II (63%) than CIN I (33%) Significant difference in *Uu** infection between CIN I and II No significant difference in *Uu** infection between CIN II and III Consistent detection of *Uu** and HPV infection in CIN	Xiaolei et al. [15]
8	Bacterial infection, HPV infection	6 times increased risk of *Uu** infection in HPV positive woman	Camporiondo et al. [16]
9	Bacterial infection, HPV infection, CIN	Strong correlation between coinfection of *Up*§ and HPV and CIN1	Drago et al. [17]
10	Bacterial infection, HPV infection	Significant association between HPV and bacterial vaginosis such as, *Ct*‡ and *Uu**	Liu et al. [18]
11	Bacterial infection, Cytology	Significant relationship between *Uu** infection and cannonballs Significant relationship between *Uu** infection and coccoid bacteria	Okodo et al. [19]
12	Bacterial infection, HPV infection, Oncogene	Stimulation of *Uu**on the expression of HPV 16 E6 Increased risk of cervical cancer by overexpression of HPV E6 oncogene and *Uu** infection	Szostek et al. [20]

**Ureaplasma Urealyticum*
+Cervical intraepithelial neoplasia
‡*Chlamydia Trachomatis*
§*Ureaplasma Parvum*

Ct and *Mg* are known to cause STIs. According to Bhatla et al., *Ct* is not a direct cause of cervical neoplasia, but could be a causative factor in HPV infection.[22] Several reports showed that the presence of *Ct* negatively affects host cell immunity against HPV, furthermore, *Ct* induced micro-abrasion on the cervix that can increase susceptibility to HPV.[23,24] A recent study revealed that the incidence of asymptomatic *Ct* and *Uu* was 5.6% and 22.1%, respectively,[6] whereas in this study, the incidence was 2.3% and 36.0%, respectively. However, only 2 among 6 *Ct* infections and 3 among 5 *Mg* infections had HPV infection in our study. These cases were insufficient to determine statistical significance.

In our study, the incidence of asymptomatic *Uu* infection was 36.0%, whereas Zhang et al. reported that the incidence of symptomatic *Uu* infection was 58.7%.[25] The incidence rate of asymptomatic *Uu* infection was relatively low. However, asymptomatic *Uu* infection could be a cofactor in HPV infection.[2] Moreover, coinfection of *Uu* with HPV can contribute to development of CIN. Therefore, HPV testing should be performed in women with *Uu* infection, regardless of symptom status.

Our research showed that, HDC-*Uu*, an intracellular microorganism, was related to HPV infection in asymptomatic sexually active individuals. This important finding indicates that asymptomatic subjects with potentially harmful bacteria should be treated. We should note that high risk HPV infection is the most important risk factor for cervical cancer.[1] Therefore, asymptomatic infection with HDC-*Uu* combined with HPV should be eradicated for the prevention of CIN.

There has been persistently controversy regarding *Uu* as a pathogen in STIs. Therefore, it was unclear as to whether *Uu* colonization should be considered an infection. However, a recent meta-analysis suggested that *Uu* is an etiological agent in STIs.[25] A recent study also demonstrated that a high density of *Ureaplasma* may be associated with STIs.[26] When determining the clinical significance of *Ureaplasma* infection, the differentiation of colonization and infection is necessary because of the high prevalence in the healthy population (*Ureaplasma* 70-80%, *Mycoplasma* 30-40%). The presence of more than 10^4 CCU/mL in a sample is an additional criterion used to distinguish colonization from infection.[27]

Interestingly, our data also indicated that only *Uu* colonization with greater than 10^4 CCU/mL was significantly associated with HPV infection.

According to our results of antimicrobial susceptibility testing, *Uu* has a high rate of resistance to fluoroquinolones. Recently, Vargović et al. reported that 78.5% of *Uu* isolates were sensitive to ofloxacin, in contrast to 98.2% showing sensitivity to doxycycline.[28] Susceptibilities of specific species to antimicrobial agents show regional variation according to current trends of antimicrobial use in the community.[29] Our data showed that sensitivities to ofloxacin and ciprofloxacin were 40.0% and 10.5%, which were higher than the results reported by Xie et al. (sensitivities to ofloxacin and ciprofloxacin were 22.1% and 5.8%). They presented a decreasing trend of sensitivity of *Uu* to fluoroquinolones, and pointed out that topoisomerase IV ParE subunit plays a role in emerging fluoroquinolone resistance in *Uu*.[30]

Therefore, if *Uu* is detected in asymptomatic sexually active individuals in an area where susceptibility testing of *Uu* is not available, we recommend consideration of doxycycline or erythromycin (or its derivatives; eg, azithromycin, clarithromycin and pristinamycin) for empirical treatment.

A limitation of this study is that a partner's past sexual history could not be clearly determined. This could represent a strong risk factor for infection with *Uu* and HPV. During the study, it was very difficult to complete the interview about sexual history because all the participants were asymptomatic and some of were reluctant to provide a personal history.

In conclusion, HDC-*Uu* (>10^4CCU/mL) could be a risk factor for HPV infection. We recommend that women infected with *Uu* be treated with doxycycline or erythromycin derivatives as first line agents.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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