Clinical Medicine & Pharmacology

press

https://doi.org/10.70731/nvj1rr15

The Effect of HPV Mixed Infection and HPV Single Infection on Vaginal Microecology and Their Correlation Analysis

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KEYWORDS

Human Papillomavirus (HPV);
Leukocyte Esterase (LE);
Sialidase (SNA);
Hydrogen Peroxide (H_2O_2);
N-Acetyl-β-Galactosaminase (NAG);
Vaginal Microecology

ABSTRACT

Human papillomavirus (HPV) is a major pathogen factor for cervicitis and cervical cancer, it mediates vaginal microecology changes to cause diseases occurrence. But its mechanism is full clear. This study investigated the effect of human papillomavirus (HPV) infection on vaginal microecology and analyzed its correlation. 300 female patients who visited in Xiangya Boai Rehabilitation Hospital from January 2023 to December 2023 were enrolled into this study. HPV of the patients was detected using PCR method. The patients were diagnosed by two clinical experts. According to the HPV infection, the patients were divided into HPV single model infection group and HPV mixed group, with 100 cases in each group. 100 cases of the healthy population without HPV infection and cervicitis served as normal control group. Subsequently, HPV and vaginal microecological detections were performed on all subjects, including vaginal secretion cleanliness, PH value, leukocyte esterase (LE), sialic acid (SNA), hydrogen peroxide (H_2O_2), and N-acetyl-β-galactosaminase (NAG). According to the test statistics, among the patients with HPV infection, the patients with HPV 51, 52, 53, 58 accounted for the majority. Compared with the normal control group and the HPV single infection group, the HPV mixed infection group had statistically significant differences in vaginal cleanliness, PH, positive rate of hydrogen oxidase, epithelial cells, LE, SNA, H_2O_2 and NAG in vaginal microecology ($P < 0.01$). In general, HPV mixed infection has a significant effect on vaginal microecology, while HPV single infection has little effect on vaginal microecology. These results can provide reference for clinical prevention and treatment of cervical lesions.

INTRODUCTION

Human papillomavirus (HPV) is a spherical deoxyribonucleic acid virus that infects humans mainly through direct or indirect contact with contaminated items or sexual transmission[1]. HPV not only has host-specific, but also has tissue-specific, and it can only infect human skin and mucosal epithelial cells. HPV infection can cause a proliferation of squamous epithelial cells in cervical mucosa, change growth pattern of the cells, and then infect innate immunity and adaptive immunity[2]. So far, more than 100 HPV genotypes have been identified[3]. According to the pathogenicity of HPV subtypes, HPV can be divided into low-risk and high-risk types[4]. More than 20% ~ 80% of sexually active people have a history of HPV infection, but the infection is usually transient[5]. Most of the infected people self-clear the virus within 12 ~ 24 months, whlie about 10% of patients will have a persistent infection[6]. Previous studies have shown that persistent infection of high-risk HPV is an important factor in

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Received 8 July 2025; Received in revised from 19 July 2025; Accepted 24 July 2025; Published online 30 July 2025.

the occurrence of cervical cancer[7]. Due to large genetic variation of the capsid protein encoded by variou subtypes of HPV, there are basically no cross-protective antibodies between different subtypes of HPV, which is easy to cause multiple or multiple infections with various types of HPV. Persistent HPV infection is one of the risk factors for cervical cancer, and the outcome after infection is closely related to vaginal microecology[8, 9].

Female vaginal microecology is a dynamic and relatively balanced system composed of normal vaginal anatomy, periodic endocrine changes, vaginal flora, and vaginal and cervical local immunity [10]. There are a large number of microorganisms in the vagina of normal women. These microorganisms grow and breed in the vagina and restrict each other to form a vaginal micro-ecosystem. Under normal circumstances, the content of Lactobacillus in these organisms is the highest, which is the dominant flora in the vagina. It produces lactic acid, hydrogen peroxide, and some enzymes to maintain stability of the vaginal environment. When the balance was broken, the incidence of female reproductive tract infections increased, such as bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and trichomonal vaginitis (TV)[11, 12]. In this study, five combined tests of vaginitis were used to identify whether there was vaginal disease and whether the vaginal microecology was unbalanced. Among them, N-acetyl-β-galactosaminase (NAG) had strong activity in candida albicans and trichomonal vaginitis. Sialidase (SNA) activity detection showed that Sialidase was a specific enzyme secreted by BV pathogens, such as Gardnerella and Campylobacter. Leukocyte esterase (LE) increased in bacterial and fungal vaginosis. Hydrogen peroxide (H_2O_2) was a marker of vaginal lactobacilli. pH reflects change of vaginal environment.

The present studies have confirmed that in HPV infection, the richness and diversity of vaginal and cervical bacterial microorganisms increase, the richness of lactobacilli decreases, and the increase of anaerobic bacteria is particularly obvious, including Gardnerella and cilia. The results showed that vaginal microecological disorders increased the risk of HPV infection and cervical lesions[13]. In this study, PCR reverse dot blot hybridization was used to detect the genotype of HPV infection in cervical epithelium. The changes of vaginal microecology were detected by analyzing cleanliness of vaginal secretions, pH, LE, SNA, H_2O_2 and NAG. The effects of single HPV infection and mixed HPV infection on vaginal microecology were discussed.

OBJECTS AND METHODS

Object

300 cases of cervicitis patients who visited in Xiangya Boai Rehabilitation Hospital from January 2023 to December 2023 were enrolled this study. The age of the patients ranged from 16 to 64 years old, with an average age of (38 ± 1) years. All patients had a history of sexual life. According to whether HPV infection and infection occurred, the enrolled patients were divided into two clinical groups, including 100 cases of the single infection group and 100 cases of the mixed infection group 100 cases of healthy population with HPV infection served as the healthy control. There was no significant difference in age basic information of the three groups. All protocols were approved by the Ethics Committee of the Xiangya Boai Rehabilitation Hospital

Methods

HPV Detection

Cervical exfoliated cells were used as analysis samples. Before sampling, the medical staff first exposed the cervix with a vaginal speculum or a vaginal opener, and wiped away excessive secretions from the cervix with a cotton swab. The cervical brush was taken out and placed at the cervical orifice, and rotated in one direction for 4~5 weeks to obtain sufficient epithelial cell samples. Then the head of the cervical brush was placed in the eluent, and the cervical brush handle was broken along the crease of the brush handle. The eluting tube cover was tightened, the sample was marked, and the eluting tube was kept upright. After the samples were collected, they were sent for inspection as soon as possible. The samples were stored at room temperature for no more than 12 hours, and stored at 4°C for no more than 7 days. PCR in vitro amplification and DNA reverse dot blot hybridization were used to detect HPV genotyping. The amplified products were hybridized with the probes fixed on the membrane, including 17 high-risk HPV 16, 18, 31, 33, 35, 39, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 68, 73, and 82, and 6 low-risk HPV 6, 11, 42, 43, 81, 83. The presence or absence of hybridization signals was used to determine whether these HPV genotypes were infected.

Cleanliness Test of Vaginal Secretions

The secretions were taken from the posterior fornix of the vagina with a disinfected cotton swab, immersed in a test tube containing 1-2 ml of normal saline, and made into a thin smear for microscopic examination. According to the number

Table 1 | Standard of vaginal cleanliness

Cleanliness	Bacilli epithelial cells	Gonococcus	Epithelial cells	Pus cells or white blood cells (High power field)
I	more	-	field of vision	0-5
II	less	less	1/2 field of view	5—15
III	less	more	less	15—30
IV	-	might	-	>30

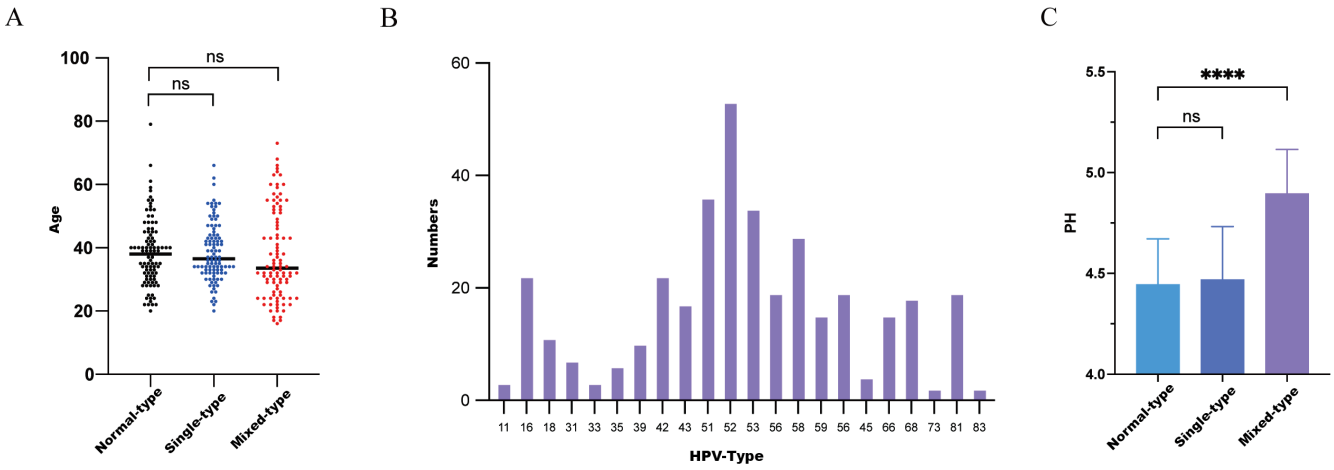

Figure 1 | Age and vaginal pH analysis of the patients with HPV mixed infection and HPV single infection.

A) Age analysis of the patients with HPV mixed infection, HPV single infection, and the normal control group. **B)** Population distribution of different types of HPV infection. **C)** Vaginal pH analysis of the patients with HPV mixed infection and HPV single infection, and the normal control group. Statistical analysis was performed using 1-way ANOVA.

* $P < 0.05$, * * $P < 0.01$, * * * $P < 0.001$, * * * * $P < 0.0001$, ns was not statistically significant.

of white blood cells or pus cells, epithelial cells, bacilli, and cocci, they were divided into I-IV degrees. The results are shown in the following **Table 1**.

Combined Detection of Vaginitis

LTS-V400 vaginitis detector and the vaginitis combined detection kit (chemical reaction method) were produced by Zhuhai Lituo Biotechnology Co., Ltd, they were used to detected *vaginitis*. Briefly, a sufficient amount of vaginal secretions were collected with a cotton swab, and then the cotton swab was inserted into the sample extract solution (the main component was normal saline) that was added to 0.3 ± 0.05 ml. The cotton swab was squeezed at the bottom of the sample extraction tube for about 10 times, the substances in the cotton swab were fully dissolved into the sample extract solution, and then the liquid in the cotton swab was squeezed to discard the cotton swab. The processed specimen extraction tube was placed at the specified position on the incubator plate of the detector, and then the detection card was placed. The color recognizer in the instrument uses LED white cold light source as the emission source. The color sensor in the recognizer received the color information of the reflected light of the detection card, and automatically calculated RGB value, so as to interpreted and analyzed the results. The detection items included pH, LE, SNA, H_2O_2 and NAG.

Statistical Methods

Mean difference and percentage were used to represent measurement data and count data in this study, and t test and x_2 test were used to represent the test tools of the two. $P > 0.05$ indicated that there was no significant difference in the comparison of results.

RESULTS

HPV Mixed Infection Mediates Vaginal pH Value Increase and Single HPV Infection Had Little Effect on Vaginal pH Value.

300 patients who were examined in Xiangya Boai Rehabilitation Hospital from January 2023 to December 2023 were investigated. The age of the patients ranged from 16 to 79 years, with an average age of 39 ±11 years. All patients had a history of sexual life. The enrolled patients were divided into two clinical groups, including the single infection group with100 cases and the multiple infection group with 100 cases. 100 cases of healthy population served as the healthy control group. There was no significant difference between three groups in the basic information including age, cervicitis, Disease status, etc (**Figure 1A**). The analysis results showed that, among all the people infected with HPV, the patients infected with HPV51,52,53,58 were the majority (**Figure 1B**). Subsequently, the vaginal pH values of the patients in the single infection group were compared with that in the multiple infection group or the healthy control. The results showed that the pH value of the mixed infection group was significantly increased when compared with single infection group and the healthy control *(P*<0.001) (**Figure 1C**), indicating that the internal environment in the vagina was destroyed, while the pH value in the vagina of patients with HPV single infection did not change when compared with the healthy control, indicating that the HPV mixed infection group was more likely to have a disorder in the vaginal environment.

Table 2 | Comparison of abnormal distribution of vaginal microenvironment indexes in each group [n (%)]

Peer Group	Vaginal cleanliness		Epithelial cell		LE		SNA		H_2O_2		NAG	
	III°～IV°	I°～II°	2^+	1^+	Positivity	Negative / weak positive	1^+	/	1^+	/	1^+	/
normal-type	29	71	67	33	0	100	0	100	59	41	0	100
single-type	25	75	76	24	0	100	4	96	60	40	4	96
chi-square value	0.22		1.57		0		2.29		0		2.29	
P value	0.63		0.21		1		0.12		1		0.12	

Table 3 | Comparison of abnormal distribution of vaginal microenvironment indexes in each group [n (%)]

Peer group	Vaginal cleanliness		Epithelial cell		LE		SNA		H_2O_2		NAG	
	III°～IV°	I°～II°	2^+	1^+	Positivity	Negative / weak positive	1^+	/	1^+	/	1^+	/
normal-type	29	71	67	33	0	100	0	100	59	41	0	100
mixed-type	94	6	44	56	26	74	21	79	93	7	22	78
chi-square value	86.49		9.79		27.63		21.28		29.85		22.52	
P value	<0.001		<0.01		<0.001		<0.001		<0.001		<0.001	

Table 4 | Comparison of abnormal distribution of vaginal microenvironment indexes in each group [n (%)]

peer group	vaginal cleanliness		epithelial cell		LE		SNA		H_2O_2		NAG	
	III°～IV°	I°～II°	2^+	1^+	positivity	Negative / weak positive	1^+	/	1^+	/	1^+	/
single unit type	25	75	76	24	0	100	4	96	60	40	4	96
mixed-type	94	6	44	56	26	74	21	79	93	7	22	78
chi-square value	95.94		19.57		27.63		11.70		28.48		12.77	
P value	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

Vaginal Cleanliness and Positive Rate of Catalase in the Vagina Increased After HPV Mixed Infection.

To further verify the changes of vaginal environment caused by mixed HPV infection, some evaluation indexes of vaginal environment was conducted, including vaginal cleanliness, positive rate of catalase in vagina, epithelial cells, LE, SNA, H_2O_2 and NAG. The results showed that there were significant differences in vaginal cleanliness, positive rate of hydrogen peroxide, epithelial cells, LE, SNA, H_2O_2 and NAG between the HPV mixed infection group and the normal control group and the single HPV infection group (P<0.01) (**Table 3**, **Table 4**). There was no significant difference in vaginal cleanliness, positive rate of hydrogen peroxide, epithelial cells, LE, SNA, H_2O_2 and NAG between the single HPV infection group and the normal control group (**Table 2**). These results further suggest that the HPV mixed infection is more likely to cause the disorder of the vaginal environment.

DISCUSSION

The female reproductive system has its specific microbial communities. These microbial communities play an important role in women's life processes and menstrual cycles. Their main functions are to maintain vaginal health and protect the vaginal environment from various urogenital infections. Vaginal microbiome interacts dynamically with the host and the environment to form a dynamic ecosystem called vaginal microbiome, which is mainly maintained by interaction with the local microenvironment. Normal vaginal microbiome plays an indispensable role in preventing female genital tract infection, and its changes are inextricably linked with the development of cervical lesions. If the vaginal microecological flora loses this dynamic balance and the immune system is impaired, foreign microorganisms are more likely to invade the reproductive tract and cause inflammation[14]. Some reports have shown that genital tract inflamma-

tion caused by HPV infection is closely related to tumorigenesis[15, 16]. This study also reported that HPV-positive women have more diverse vaginal microbial species when compared with HPV-negative women, and vaginal microecology may also be affected[17]. Previous literature has shown that vaginal microecology plays a vital role in preventing HPV infection and accelerating HPV virus clearance, and its homeostasis imbalance may be a synergistic factor for HPV infection. After HPV infection, the pH value in the vagina changes, which causes secretions to increase, resulting in an imbalance of the vaginal flora and inflammatory response. The reproduction of the bacteria would further damage the vaginal mucosal barrier and eventually cause vaginal microecological imbalance[18]. Our study showed that after HPV infection, single HPV infection did not cause changes in vaginal pH and vaginal cleanliness, but mixed HPV infection changed vaginal pH and vaginal cleanliness, indicating that mixed HPV infection is more likely to cause disorders of the vaginal environment, causing vaginal and cervical diseases.

Previous studies have shown that vaginal H_2O_2, SNA, NAG and other indicators can reflect the function and quantity of vaginal Lactobacillus. In this report, the status of the vaginal microenvironment was also evaluated[19], the results showed that the vaginal microbial flora in the population infected with HPV was changed, but the study did not distinguish between HPV single infection and HPV mixed infection. In the present study, we compared HPV single infection group with normal control group, the results showed that HPV single infection group had little effect on vaginal microenvironment, while HPV mixed infection group compared with the normal control group and the HPV single infection, its vaginal cleanliness, PH, hydrogen oxide enzyme positive rate, epithelial cells, LE, SNA. H_2O_2 and NAG have obvious abnormal changes, which indicates that patients with HPV mixed infection have a greater impact on vaginal microorganisms, and are more likely to quickly destroy the homeostasis of the vaginal microenvironment, resulting in the decompensation of the vaginal microenvironment, including the decrease of reproductive tract cell immunity, the destruction of epithelial cells, and the increase of bacterial susceptibility, which leads to the occurrence of cervical cancer[20]. In summary, patients with HPV mixed infection are more likely to lead to abnormal vaginal microecological regulation. For patients with HPV mixed infection, the disorder of vaginal microecology should be corrected as soon as possible. It is of great scientific significance to help patients restore a healthy vaginal microecological environment for the prevention and treatment of cervical cancer.

Acknowledgement We thank the members in Clinical Laboratory of Xiangya Boai Rehabilitation Hospital (Fuyuan District) for contributions.

Conflict of Interest The authors declare no competing financial interests.

Author Contributions Tang N and Zhang Y performed the experiments. Tang N prepared all the figures and wrote the manuscript. All authors read and approved the final manuscript.

Data Accessibility All data generated or analyzed during this study are included in this published article.

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