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Identification of Human Papillomavirus in Women with Genital Warts by PCR and Pap Smear in Sari

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ABSTRACT

Background: The most significant genital lesion caused by HPV infection is genital warts, which are benign. The importance of genital warts relates to cervical cancer risk factors. In fact, this virus has been identified as the cause of 99% of cervical cancers worldwide, necessitating non-invasive methods for disease management. Identifying an appropriate, accurate, and highly sensitive screening method is crucial. Therefore, we investigated the diagnosis of papillomavirus in women with genital warts using Pap smear and PCR.

Methods: From September 2019 to August 2020, 41 vaginal swab and cervical samples from females with genital warts were assessed for HPV DNA detection by PCR and Pap smear.

Results: Among 41 vaginal swab specimens from those with genital warts, 5 cases (12.12%) were detected by molecular method, while Pap smear analysis of the same 41 samples revealed 8 cases (19.5%) of abnormal findings, including 2 cases of ASC (4.9%), 6 cases (14.6%) of metaplastic cells, and 33 cases (80.4%) normal results, comprising 11 inflammatory cases (26.8%) and 22 normal cases (53.7%).

Conclusion: The incidence of HPV infection in females with genital warts was 12.12% by PCR. However, conventional Pap smear screening detected 19.5% cervical dysplasia cases attributable to papillomavirus infection. The discrepancy between PCR and Pap smear results stems from the specificity and low sensitivity of the Pap smear method. Therefore, it is recommended to use the PCR diagnostic method alongside Pap smear to enhance screening quality in individuals with genital warts.

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Introduction

Human papillomavirus (HPV) infection is associated with various genital lesions, with benign genital warts representing the most prevalent clinical manifestation (1). HPV has been definitively established as the primary causative agent in cervical carcinogenesis (2, 3). Globally, cervical cancer is the fourth most common malignancy among women and constitutes a notable etiology of cancer-induced mortality, particularly in developing nations, with about 570,000 novel cases and 311,000 deaths reported each year (2). In India specifically, cervical cancer accounted for 123,907 cases in 2020, representing 18.3% of all female cancer diagnoses, and resulted in 77,348 deaths, constituting 18.7% of cancer-related mortality among women (4).

According to reports from the International Agency for Research on Cancer Information Centre and the Institut Català d'Oncologia on HPV and Cancer (ICO/IARC) published in 2018, approximately 917 novel cervical cancers are diagnosed each year in Iran (5). Despite a decline in annual incidence from 7 to 5 per 100,000 women, cervical cancer remains one of the most prevalent malignancies with a high mortality rate (6, 7).

While several risk factors have been detected, including early sexual debut, multiple sexual partners, immunosuppression, sexually transmitted infections, and smoking, HPV infection remains the most critical etiological factor in cervical cancer development (8, 9).

To date, over 150 HPV genotypes have been identified, with approximately 40 genotypes known to be transmitted through sexual contact and capable of infecting the anogenital area (10-12).

Many countries worldwide, including Iran, have implemented prevention and early detection programs for cervical cancer through Pap smear screening tests, which represent the most cost-effective and accessible screening method (13). However, screening coverage remains inadequate in some populations; for instance, approximately 20% of women in the United States lack regular screening and have not undergone a Pap smear within the past 3 years (14).

The Pap smear test, first developed in 1947, has long been recognized as the most effective method for preventing and diagnosing cervical cancer. Since 1950, cervical cancer screening programs utilizing vaginal and cervical cytology, commonly known as Pap smear testing, have enabled the early detection of cervical cancer at stages amenable to effective treatment (15).

For approximately five decades, the Pap smear remained the sole available screening method for cervical cancer (16). However, efforts to enhance screening effectiveness have revealed limitations in Pap smear sensitivity. Recent studies have indicated that the relatively high rate of false-negative results is attributed to the inherent low sensitivity of conventional Pap smear testing (16-17). In recent years the prevalence of abnormal Pap smears in Iran is increasing to some extent, showing a 2-4-fold increase compared to previous reports (18,19).

Since it is not possible to detect this virus and its types using serological methods and cell culture, and given that accurate, definitive, and early diagnosis of this virus requires molecular methods such as PCR, which are of special importance, morphological studies—even with new liquid-based techniques alone—are not very sensitive for diagnosis (20). Therefore, we evaluated women with genital warts by Pap smear and PCR in the diagnosis of papillomavirus. The results of this study can be effective in the process of preventing, early diagnosis, and prompt treatment of cervical cancer.

Materials and Methods

Forty-one women with genital warts referring to the clinic of Sari Hospital for vaginal examination between September 2019 and August 2020 were

investigated. The research protocol was approved by the University Ethics Committee, and all subjects provided informed consent after answering the questionnaires. The inclusion criteria comprised women with genital warts or a history of genital warts, while exclusion criteria included women who were menstruating at the time of sampling, those with abnormal vaginal secretions, and pregnant women who declined participation due to concerns regarding potential abortion. Sampling was conducted by the treating physician under sterile conditions using sterile swabs to collect vaginal and cervical secretions from each patient. The samples were subsequently divided into two parts: one portion was immediately spread onto slide surfaces using spatula and brush techniques and transferred to the pathology laboratory, while the other portion was transported to the molecular laboratory for DNA extraction.

Molecular tests

For DNA extraction in this study, the thermosynthetic fermentase kit protocol manufactured in Lithuania was utilized. DNA was extracted both qualitatively and quantitatively (1.6 <OD <1.9) using primers PCO3: 5'-ACACAACTGTGTTCACTAGC-3' and PCO4: 5'-CAACTTCATCCACGTTCACC-3' targeting a fragment of the human β-globin gene. Amplification was performed simultaneously with multiplex PCR. Primer design for the MY09-MYO11 gene was accomplished using Oligo 7 software and sequence blast analysis on NCBI and primer 3 websites. Primer sequences were synthesized using HPLC technique with OD equal to 2. The primers presented in Table 1 were ultimately obtained for viral genome amplification. Five microliters of extracted DNA was mixed with the reaction mixture, while 5 µl of H2O was substituted for the template in one microtube to serve as a negative control. The UNI1400 program was implemented, with PCR cycle steps detailed in Table 2. The annealing temperature for the MY09-11 gene was set at 57.5 °C. Finally, PCR products were detected and photographed under UV light following electrophoresis on 2% agarose gel, which contained 1-0.5 µg/ml ethidium bromide for 45 minutes.

Pathology tests

For women who met the inclusion criteria and gave consent to participate in the study, samples for HPV testing were obtained during gynecological examinations. The cervix was visualized using a Cusco's speculum to ensure a clear view of the external os and the squamocolumnar junction for proper sampling. An Ayer's spatula was applied to the external os and rotated unidirectionally through 360 degrees. The specimen was then spread onto glass slides, which were placed in Coplin's jars containing a 95% ethanol solution. Cytological classification was conducted following the Bethesda System. To minimize the need for frequent follow-ups by patients, cervical washings were collected in DNA LBC cervical sample transport medium in accordance with the manufacturer's protocol (3B Blackbio Biotech India Ltd.), in conjunction with Pap smear collection for cases deemed suspicious. The collected samples were stored at 4 °C until further processing.

Statistical analysis

Data analysis was performed using SPSS 16 . To evaluate the simultaneous impact of multiple variables and identify significant predictive factors associated with HPV infection, logistic regression modeling was employed. This analytical approach allowed for the assessment of various demographic, clinical, and laboratory parameters as potential risk factors while controlling for confounding variables. The diagnostic accuracy of different testing methods was compared against

Table 1. Primers used for DNA template and PCR synthesis.

Primer	Sequence	Size (bp)
MYO9 (Forward)	5’-CGT CCM AAR GGA WAC TGA TC -3’	380
MYO11 (Reverse)	3’-GCM CAG GGW CAT AAY AAT GG -5’	380
PCO3 (Forward)	5’-ACACAACTGTGTTCACTAGC-3’	250
PCO4 (Reverse)	3’-CAACTTCATCCACGTTCACC-5’	250

Table 2. Cyclimg program of PCR program.

Steps	Temperature and time	Number of cycles
Primary denaturation	10 minutes at 95 degrees Celsius	1
Secondary denaturation	30 seconds at 95 degrees Celsius	45
Accession	30 seconds at 62 degrees Celsius	45

Table 3. Frequency distribution of wart patients according to PCR and Pap smear results in HPV diagnosis.

Result		N	Percentage	95% confidence interval (low limit)	95% confidence interval (upper limit)
PCR	Positive	5	12.2%	4.8%	24.7%
	Negative	36	87.8%	75.3%	95.2%
	Total	41	100.0%	.	.
Pap smear	ASC	2	4.9%	1.0%	14.7%
	inflammation	11	26.8%	15.2%	41.6%
	Meta plastic cell	6	14.6%	6.3%	27.7%
	Normal	22	53.7%	38.6%	68.2%
	Total	41	100.0%	.	.

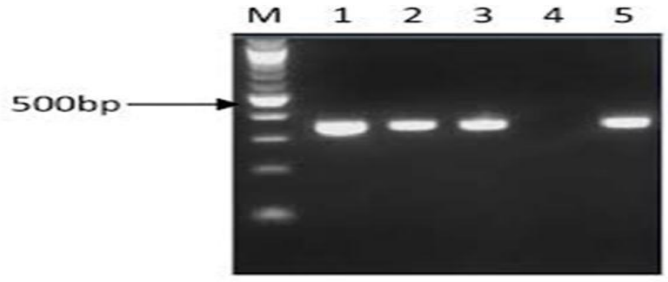

Figure 1. Electrophoresis of PCR products on 2% agarose gel. M represents the 100 bp marker, number 1 represents the positive control, number 4 represents the negative control, and numbers 2, 3, and 5 represent the papillomavirus infection with a band length of 380 bp.

Table 4. Pap smear diagnostic indicators based on PCR test results in the diagnosis of HPV in patients with genital warts.

			Pap smear		Total	Coefficient of agreement	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy
			Abnormal	Normal					
PCR	Positive	N	3	2	5	Kappa = 0.366 , P=0.015	60% (23.1% - 88.2%)	86.1% (71.3% - 93.9%)	82.9% (73% - 93%)
		Percentage	7.3%	4.9%	12.2%				
	Negative	N	5	31	36				
		Percentage	12.2%	75.6%	87.8%				
Total		N	8	33	41				
		Percentage	19.5%	80.5%	100.0 %				

HPV PCR results, which were considered the gold standard reference method due to their high sensitivity and specificity in detecting HPV DNA. The significance level was set at $p<0.05$, and confidence intervals were determined to indicate the precision of the estimated associations.

Results

The patients' mean age was 38.59±9.51 ranging from 20 to 50 years of age. Out of 41 vaginal swabs of patients with genital warts, 5 samples (12.12%) were positive and 36 cases (87.8%) were reported to be negative in terms of HPV DNA. Based on Pap smear samples of 41 samples, 8 cases (19.5%) were malignant and 33 cases (80.4%) were normal, of which 22 cases (53.65%) were normal and 11 cases (26.82%) were reported with inflammation, including 2 cases (4.87%) of atypical squamous cells with suspected high-grade lesion (ASCH) and 6 cases (14.63%) of metaplastic cells (Table 3).

In this study, the PCR method was considered as the gold standard compared to Pap smear. Only 3 cases out of 8 patients with papilloma were reported positive by both the Pap smear and PCR methods. Therefore, the Pap smear method comparing to PCR has Sensitivity (60%), Specificity (86%) table 4.

Positive Predictive Value (PPV) (0.375%) and Negative Predictive Value (NPV) (0.939%) where LR+ was 3.4 and LR- was equal to 0.465. As a result, with the exception of sensitivity and positive predictive value (PPV), other Pap smear diagnostic indicators in the diagnosis of HPV have been acceptable.

Discussion

HPV is responsible for one of the most prevalent sexually transmitted viral infections among women aged 15 to 44 worldwide, and its causal role in cervical cancer has been well established(21). Unfortunately, the incidence of this cancer is higher in developing countries(7). In our study, the participants' age range was between 20 and 50 years, and the prevalence of HPV DNA among individuals with genital warts was reported to be 87.8%. The high prevalence observed in our study may be attributed to the relatively small sample size and the focused inclusion criteria, which selected only individuals presenting with genital warts.

Haji Bagheri et al.(2018) investigated the frequency of human HPV virus and identified the HPV genome in 56% of studied samples, with the mean age of subjects reported as 34.07±8.7(22). Mirbehari and Sadeghi (2018) reported a prevalence of high-risk HPVs as 52.7% among 345 patients, with an age range of 16 to 72 years(23). Bidaki et al. (2022) conducted a study

where the age range was between 25 and 36 years, and among 345 samples, 93.2% were reported to be HPV DNA positive(24). In contrast, a 2020 Swedish study on dysplasia in older women reported an overall prevalence of 5.4% (25).

Exfoliative cytology, commonly known as the Pap test, is the main method used for the early detection of cervical cancer. It is recommended globally for mass screening because of its effectiveness in identifying premalignant lesions and its cost-efficiency. However, this test unfortunately exhibits numerous false-negative results (26-27). In our study, based on Pap smear analysis of 41 samples, 8 (19.5%) were abnormal and 33 (80.4%) were normal. Among the normal cases, 22 (53.65%) were classified as normal and 11 (26.82%) were reported with inflammation, including 2 (4.87%) cases of atypical squamous cells with suspected high-grade lesion (ASCH) and 6 (14.63%) cases of metaplastic cells.

Gupta et al. (2022) reported an HPV prevalence of 10.54% overall. According to Pap smear test results, 6.60% showed normal cytology and 81.5% exhibited atypical squamous cells with significant intraepithelial lesions (28). Mijtab et al. (2018) reported cervical Pap smear results based on the Bethesda system as 82.03% negative for malignancy and 17.97% with cervical epithelial abnormalities (29).

Various PCR-based diagnostic methods exist for identifying different HPV types, including conventional PCR, hybridization techniques, and real-time PCR. Probe-based real-time PCR methods demonstrate superior accuracy and sensitivity compared to other methodologies (30). Trieu et al. (2021) conducted a study aimed at detecting and determining the genotype of human papillomavirus using PCR testing, reporting 11.83% of samples as positive (31). Bidaki et al. (2022) reported 93.2% of samples as positive for HPV DNA using PCR testing (24). Saiki et al. (2020) reported 15.3% of samples as positive using PCR testing (32).

In Pap smear cancer screening methodology, two major errors reduce the sensitivity of this test. Sampling error may occur when the lesion is not adequately sampled or, if sampled, is not properly transferred to the slide. The second error involves diagnostic interpretation, wherein malignant cells are present in the sample but remain undetected, thereby compromising the overall sensitivity of this method (33-35).

Methods such as PCR, which detect viral DNA, actually identify the causative agent of infection. In contrast, cytological tests such as Pap smears determine the lesions and pathological effects of infection (36). The PCR technique is considered one of the most sensitive methods for detecting HPV in genital tissues due to its ability to amplify viral DNA using specific primers, and its combination with sequencing methods as a confirmatory test increases its accuracy to near 100%. Consequently, its use in conjunction with cytological methods can be significantly effective in enhancing the resolution and accuracy of clinical examinations of patients (37).

In our study, the prevalence of papillomavirus among 41 samples based on molecular testing (HPV PCR) was 12.19%, while conventional Pap smear screening detected 19.5% of cervical dysplasia cases, a discrepancy attributed to the specificity and low sensitivity of the Pap smear method (38). Additionally, in our study, the Pap smear method demonstrated a sensitivity of 60% and specificity of 86% compared to PCR, which was consistent with Fakhar et al.'s 2014 study (38). A 2016 Pap smear study in Thailand reported a sensitivity of 71.8% and specificity of 97% (39). Similarly, a 2018 Indian study involving 1500 women reported Pap smear sensitivity and specificity for papillomavirus diagnosis as 75.8% and 98%, respectively (39). Gupta et al. (2022) reported the sensitivity of PCR testing for papillomavirus detection as 81.48% and specificity as 93.40% (28). This observed difference may be attributed to racial variations,

high viral exposure rates, or the relatively small sample size in the present study (29).

Conclusion

It is recommended that all women over the age of 20 undergo routine screening. This screening should include both cytology (Pap smear) and HPV testing to identify suspected cases early. Simultaneous screening using both Pap smear and HPV PCR testing is strongly advised to enhance early detection and prevention efforts.

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Ethics approval and consent to participate

All procedures conducted in studies involving human participants adhered to the ethical standards set by the institutional and/or national research committee, as well as the 1964 Helsinki declaration and its subsequent amendments or comparable ethical guidelines. Furthermore, the ethics committee of Azad University of Medical Sciences in Iran approved this study (Code no: IR.IAU.SARI.REC.1398. 116).

Conflict of interest

The authors declare no conflict of interest.

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