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Original Article

Microbiology Section

Molecular Evidence of the High-risk Human Papillomavirus 56 Genotype in Cervical Abnormalities using Multiplex Nested PCR Assay: A Cross-sectional Study from a Tertiary Care Center in Puducherry, India

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ABSTRACT

Introduction: Human Papillomavirus (HPV) is a group of Deoxyribonucleic Acid (DNA) viruses linked to both benign and malignant diseases. High-Risk (HR) HPV genotypes, especially HPV 16 and HPV 18, are key contributors to cervical cancer.

Aim: To determine the prevalence of HR HPV genotypes, including HPV 16, 18, 31, 45, 51 and 56, in cervical abnormalities in women by comparing cytological Pap smear results with in-house Multiplex Nested Polymerase Chain Reaction (PCR) to refine diagnostic protocols.

Materials and Methods: A cross-sectional study was conducted in the Department of Obstetrics and Gynaecology, tertiary care hospital and the Mahatma Gandhi Medical Advanced Research Institute, Puducherry, India over a period of six months, from November 2023 to May 2024. The study included 100 symptomatic women presenting with complaints such as vaginal discharge, abnormal bleeding and pelvic pain. OBG endocervical samples were cross checked against cytological pap smear and multiplex nested PCR. The PCR amplified products were further confirmed by bidirectional Sanger sequencing. The sequenced data were analysed and annotated using MEGA software version 10.0 and submitted to the National Centre for Biotechnology Information (NCBI) database.

Results: A total of 100 samples were analysed, with 14 (14%) detected as HPV DNA in symptomatic women. HPV 16 was observed in four patients (28.57%), followed by HPV 56 in four patients (28.57%). HPV 18 and HPV 31 were detected in two patients each (14.29%), while HPV 45 and HPV 51 were observed in one patient each (7.14%). Symptoms such as abnormal vaginal bleeding, pelvic pain and vaginal discharge were predominant with specific HPV genotypes. The detection limit of Multiplex Nested PCR was higher than that of the Pap smear. Co-infections with organisms like *Candida albicans* and *Trichomonas vaginalis* were noted in HPV-negative cases. The sequenced samples were published with GenBank accession numbers PQ518860 – PQ518863.

Conclusion: While nested PCR exhibited higher sensitivity compared to the Pap smear, this study concludes that routine HPV genotyping and cytology screening for HR HPV strains are essential to improve early diagnosis and prompt treatment outcomes in HR groups.

Keywords: Cervical cancer, Endocervical swab, Human papilloma virus 51, Pap smear, Sexually transmitted infection

INTRODUCTION

The HPV is a group of DNA viruses that comprises more than 200 genotypes, which predominantly infect squamous epithelial tissues, including skin and mucous membranes [1-3]. Most of the HPV genotypes are associated with genital infections that can be transmitted through sexual contact, and in some cases, they may be linked to several benign and malignant diseases [2,4,5]. HPV and its genotypes are classified based on their clinical severity: HR HPV 16 and 18 are oncogenic strains that contain viral early proteins E6 and E7, which are responsible for the progression of cervical, anal, oropharyngeal, vulvar and penile cancers globally. Low-risk types, such as HPV 6 and 11, are associated with genital warts that appear in the external genitalia, perianal area and mucosal surfaces [6,7]. Other predominant HR types include HPV 31, 33, 45, 35, 52, 56 and 58, although reports on these are relatively few [8-12]. In developing countries, HPV 45 and 52 are particularly emphasised as they are responsible for the progression of cervical cancer [12]. The clinical manifestation of HPV genotypes may depend significantly on the site of infection and the disease's progression. Clinical symptoms may include abnormal vaginal bleeding, pelvic pain, itching or discomfort during sexual intercourse and mass formation in the affected area [13-17]. Most low-risk HPV types tend to be asymptomatic and self-limiting.

In the Indian context, the incidence of cervical cancer is higher among middle-aged women, particularly at the age of 55 years, compared to other age groups [14]. To date, most medical institutes and multi-speciality hospitals use cytology-a Pap smear (Papanicolaou test)-as the conventional screening method for diagnosing cervical cancer [18-26]. Therefore, it cannot be claimed to directly diagnose HPV; however, advanced techniques such as Liquid-Based Cytology (LBC) and Immunohistochemistry (IHC) can increase sensitivity in detecting precancerous lesions in the cervical region [25]. During cytological examinations, most patients also have accompanying infections, like *Candida albicans* (candidiasis), *Gardnerella vaginalis* (associated with bacterial vaginosis), *Trichomonas vaginalis*, *Mycoplasma* spp., and *Ureaplasma* spp., which can increase the risk of cervical abnormalities and affect patient management [18]. Currently, HPV DNA testing is considered the gold standard for detecting HPV in the cervical region and plays a vital role in the treatment and management of the disease [13,21,22].

Multiplex Nested PCR is highly effective for detecting multiple HPV genotypes simultaneously, but it is limited in its ability to detect viral

load [12]. Other methods, such as Hybrid Capture 2 (HC2) and the COBAS-4800 system, are also commonly used to detect HPV DNA in cervical samples [1,3]. Several commercial kits have been introduced to the market for the detection of HPV DNA using Real-Time PCR, such as PaxView HPV MPCR-ULFA, which allows for the simultaneous detection of 16 HR and eight low-risk HPV genotypes. Additionally, advanced technologies like Next-Generation Sequencing (NGS), GeneXpert HPV and Genotyping HPV are helpful in assessing the risk of cancer progression from different HPV types that carry oncogenic potential [27]. Understanding the prevalence of these HPV genotypes is critical for improving early detection, prevention and treatment strategies [12,21,22].

HPV DNA testing is lacking in most tertiary care hospitals. Hence, the primary objective of this study was to determine the presence of HR HPV genotypes, including HPV 16, 18, 31, 45, 51 and 56, in symptomatic women using multiplex nested PCR and cytological Pap smear. To the best of our knowledge, this was the first study to report molecular evidence of HPV 56 in the Puducherry region, India.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Obstetrics and Gynaecology of a tertiary care hospital and the Mahatma Gandhi Medical Advanced Research Institute, Puducherry, India over a period of six months, from November 2023 to May 2024. After obtaining written consent from the symptomatic women, the samples were collected with the approval of Institutional Human Ethics Committee (IHEC) (MGMCRI/2023/02/IHEC/101).

Inclusion criteria: Married women aged 18 years and above who exhibited any of the following symptoms—abnormal vaginal bleeding or discharge, pain during coitus, lower abdominal pain and clinician suspicion of cervical malignancy—were included in the study after being clinically assessed by a trained gynaecologist.

Exclusion criteria: Unmarried women, antenatal patients and vaccinated individuals were excluded from the study.

Study Procedure

Endocervical swabs were collected from 100 patients by a trained physician, who scraped the endocervix with a cervical swab. The swab was then placed into a tube containing 2 mL of Phosphate-Buffered Saline (PBS) solution and transferred immediately to the research facility for molecular analysis. For the preparation of the Pap smear (Papanicolaou test), samples were taken on slides with fixatives and were sent directly to the pathology department for routine analysis. The Pap smear report was retrieved from the cytology section of the pathology department for comparative analysis. The cytological findings revealed a spectrum of results ranging from Negative for Intraepithelial Lesion or Malignancy (NILM) to severe abnormalities, including Atypical Squamous Cells of Undetermined Significance (ASCUS), Low-Grade Squamous Intraepithelial Lesion (LSIL), High-Grade Squamous Intraepithelial Lesion (HSIL), adenocarcinoma and squamous cell carcinoma.

Molecular diagnosis: Genomic DNA was extracted from the endocervical samples using the commercially available QIAamp DNA Mini Kit (QIAGEN, Germany), following the manufacturer's instructions [28]. The genomic DNA was eluted in AE buffer and stored at -80ºC for further use.

Nested PCR: The Multiplex Nested-PCR (mN-PCR) assay was performed in two consecutive amplification reactions. The targeted outer primers MY09 (5'-GCGACCCAATGCAAA TTGGT-3') and MY11 (5'-GAAGAGCCAAGGACAGGTAC-3'), along with the inner primers GP5+ (5'-TTTGTTACTGTGGTAGATACTAC-3') and GP6+ (5'-GAAAAATAAACTGTAAATCATATTC-3'), were utilised for this research study [12]. The final reaction volume was 25 µL, which consisted of 12.5 µL of Taq DNA Polymerase 2x Master Mix RED with a final concentration of 1.5 mM $MgCl_2$, followed by 1 µL of each forward and reverse primer at 0.2 µM, 7.5 µL of PCR-grade water, and 3 µL of genomic DNA. This reaction setup was identical for both amplification sets, except that the first amplified product was used as a template for the second set of amplification.

The nested PCR cycling conditions for the outer primers MY09/11 were as follows: initial denaturation at 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 60 seconds and extension at 72°C for 60 seconds, with a final extension of seven minutes at 72°C. The cycling conditions for the second set of amplification (inner primers GP5+/6+) were the same as those for the first set, except the annealing temperature was set at 40°C for two minutes.

The amplified PCR products from MY09/11 and GP5+/6+ were separated by 2% agarose gel electrophoresis. The second set of PCR amplified products was sent for sequencing to Barcode Life Sciences, Bengaluru, Karnataka, India. All nested PCR amplifications were performed with appropriate positive and negative controls. Patient positive samples were confirmed by sequencing, which were then utilised as positive controls for the study. The sequenced samples were analysed and annotated using MEGA software version 10.0 and submitted to the NCBI database, a public domain.

STATISTICAL ANALYSIS

The mean and the standard deviation for the age group of the patients were analysed for categorical variables using Calculation.net online software.

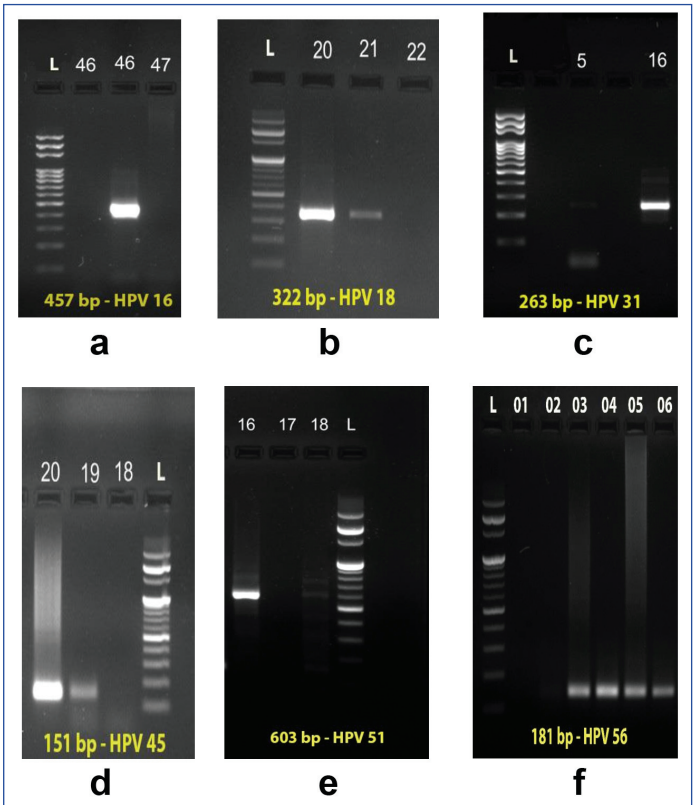
RESULTS

In the present study, a total of 100 endocervical swabs were collected from symptomatic women. The mean±SD age of the women was 41.44±10.93 years. In this study, a total of 100 samples were analysed, of which 14 were positive (14%) for different HPV genotypes using Multiplex Nested PCR. [Table/Fig-1] shows the results of symptomatic patients with cytological and molecular analyses of HPV-positive samples. Consequently, severe cytological changes were observed in the Pap smear, which correlated with specific HPV genotypes. [Table/Fig-2] presents the multiplex Nested

S. No.	Symptoms	Age (years)	HPV genotype	Base pair (bp)	Cytology reports*	Co - infections	GenBank accession number
1.	Vaginal discharge- watery	29	HPV 56	181 bp	NILM	-	PQ518860
2.	Abnormal vaginal bleeding	58	HPV 16	453 bp	ASC-US	-	-
3.	Vaginal discharge- odour	55	HPV 56	179 bp	Inflammatory cells seen	-	PQ518861
4.	Vaginal discharge- moderate	46	HPV 18	322 bp	LSIL	-	-
5.	Mucoid discharge	32	HPV 16	457 bp	AGUS	-	-
6.	Pain during sexual intercourse	45	HPV 45	251 bp	ASC-US	*Trichomonas vaginalis*	-
7.	Abnormal vaginal bleeding	54	HPV 31	263 bp	NILM	-	-
8.	Lower back pain	43	HPV 16	450 bp	Squamous cell carcinoma	-	-
9.	Pelvic pain	30	HPV 18	320 bp	HSIL	-	-
10.	Irregular vaginal bleeding and abdominal pain	61	HPV 16	451 bp	Squamous epithelial cells	-	-

11.	Intermenstrual bleeding	37	HPV 56	183 bp	Adenocarcinoma	-	PQ518862
12.	Pelvic pain	48	HPV 31	374 bp	ASC-US	Candidiasis	-
13.	Vaginal discharge - odour	25	HPV 56	181 bp	NILM, Atrophic smear	-	PQ518863
14.	Unusual vaginal bleeding	52	HPV 51	603 bp	ASC-US	-	-

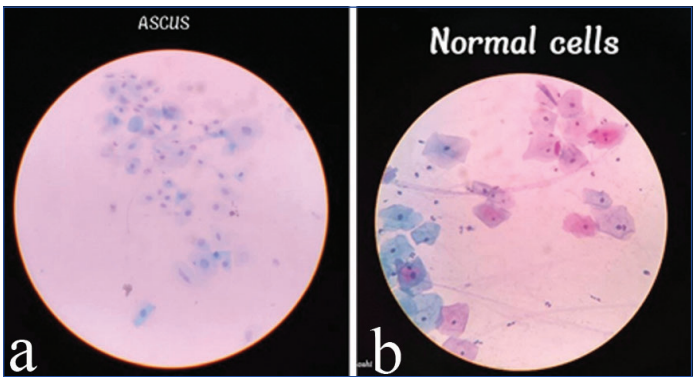
[Table/Fig-1]: Symptoms of women compared with HPV genotype and cytology reports (n=14).
*ASC-US: Atypical squamous cells of undetermined significance; AGUS: Atypical glandular cells of undetermined significance; and LSIL: Low-grade squamous intraepithelial lesion; NILM: Negative for intraepithelial lesion or malignancy; HSIL: High-grade squamous intraepithelial lesion



[Table/Fig-2]: The gel electrophoresis results show the PCR amplification of specific High Risk HPV genotypes. Lane L represents the DNA Ladder for base pair size reference. The observed bands match the expected band sizes such as a) HPV 16 457bp; b) HPV 18 322bp; c) HPV 31 263bp; d) HPV 45 151bp; e) HPV 51 603bp; f) HPV 56 181bp, confirming successful amplification of the respective various high-risk HPV genotypes.

PCR amplified results of HPV variants. HR-HPV 16 and HPV 56 were the most frequent genotypes, detected in four cases (28.57%) each, followed by HPV 18 and HPV 31, each appearing in two cases (14.29%). One case each tested positive for HPV 45 (7.14%) and HPV 51 (7.14%).

Co-infections, such as candidiasis and *Trichomonas vaginalis*, were identified in HPV-positive cases, as shown in [Table/Fig-1]. Two patients presented with pain during sexual intercourse and pelvic pain, respectively, exhibiting ASCUS [Table/Fig-3] and were co-infected with *Trichomonas vaginalis* and candidiasis, respectively. One of these two patients tested positive for HPV 45 (151 bp),



[Table/Fig-3]: Cytological examination- Pap smear report for the evidence of HPV positivity in symptomatic women. a) ASCUS-Atypical Squamous Cells of Undetermined Significance (10x) and b) NLIM-Negative for Intraepithelial Lesion or Malignancy (40x).

while the other tested positive for HPV 31 (374 bp). Other types of infections, including bacterial, fungal and parasitic infections, were observed in 45 HPV-negative cases, which included candidiasis (n=22), bacterial vaginosis (n=14), and *Trichomonas vaginalis* (n=9). The remaining 41 samples were negative for both HPV and any other types of co-infections.

In this study, authors included sequencing data for the identified HPV genotypes to provide a comprehensive molecular profile for the detected strains. The GenBank accession numbers for the four nucleotide sequences (PQ518860 to PQ518863) showed 100% identity to the HPV 56 genotype.

DISCUSSION

Screening for HPV infections is essential for both women and men in order to prevent secondary diseases in the future. HPV infection is widespread in India, although there are significant regional differences [6,8,9,11-16]. It is estimated that the prevalence in the general population is 7-10%, while in women with cervical cancer, it is 85% [15]. The HR types of HPV, namely HPV 16 and 18, play an important role in the development of cervical cancer and the occurrence of other HR HPV types. HPV infection is self-limiting and typically resolves spontaneously and the presence of HPV DNA does not necessarily lead to the development of precancerous abnormalities [13,14,16]. In contrast, the expression of E6 and E7 proteins occurs later in life and is more likely to be associated with neoplastic transformation [12,13]. Therefore, the presence of HR-HPV E6/E7 mRNA in cervical cells may more accurately determine the risk of developing cervical intraepithelial neoplasia and cervical cancer than the presence of HPV DNA [7,9]. Other HR-HPV 45, 51, and 56, have been associated with both squamous cell carcinoma and adenocarcinoma, which can lead to cervical dysplasia and precancerous changes, respectively [29].

Most of the commercial kits available in the market primarily targeted HR-HPV 16, 18, and a few other genotypes in India and other countries. However, present study employed multiplex nested PCR for the successful amplification of various HR-HPV genotypes ranging in size from 100 bp to 1000 bp in endocervical swabs from 100 symptomatic women, whereas real-time PCR is limited to identifying genotypes below 200 bp [30]. Notably, HPV 16 and other genotypes exhibited variations in base pairs, suggesting different strains within the genotype, with base pairs observed at 457 bp, 405 bp and 451 bp [13].

In the present study, abdominal pain (general, lower and menstrual) was the predominant symptom, followed by an irregular menstrual cycle with or without abdominal pain. In a few cases, cervical erosions and vaginal discharge were also noted. In most hospitals and clinics, abnormal cytology reports are predominant for HPV cases; however, the results clearly indicated that most of present study participants were Negative for Intraepithelial Lesions or Malignancy (NILM). Co-infection was observed in only two cases: one patient had candidiasis with HPV 31, and another had *Trichomonas vaginalis* with HPV 45. This low rate of co-infection may indicate a limited prevalence of concurrent infections in this study.

Research conducted in South Andaman reported a prevalence of 5.9% for HPV, including 4.1% for HR HPV 16 and 1.8% for HPV 18 [8]. Another screening programme was recently conducted for cervical cancer at the Chennai Adyar Cancer Institute, where it was found that the prevalence of HR HPV in the Chennai population

is 5.73%, with HPV 16 being the predominant genotype [16]. Additionally, the National Cancer Registry Programme reported that 7.5% of all cancers in India are HPV-related, with cervical cancer accounting for 87.6% of these cases [11].

Arumugam P et al., identified the HR-HPV 16 (64%) and HPV 18 (3%) were the dominant types retrieved from biopsy and cytobrush samples of healthy women and cervical cancer patients in Puducherry, India. The overall prevalence was found to be 51% in healthy women and 87% in cervical cancer patients, respectively. Similarly, the current study focused solely on symptomatic women with various symptoms, rather than cervical cancer patients [31]. Research studies from India have shown that HPV 16 and 18 are the most predominant HR types associated with cervical cancer [16].

The present study highlights the first molecular evidence of the HPV 56 genotype in symptomatic women in the Puducherry region, compared to other studies conducted in India. The detection of HPV 56 genotype indicates regional differences among symptomatic women [16].

Among the 14 samples, four positive samples for sequencing based on the band size, which ranged between 100 to 200 bp. The amplified products were sequenced and assigned accession numbers for the four sequences: PQ518860, PQ518861, PQ518862, and PQ518863, each showing 100% identity to HPV 56. This genotype has not been reported in Puducherry to date. This sequencing data enhances the robustness of present study results and allows other researchers to access and compare these sequences in the GenBank database. These findings support ongoing public health efforts to implement comprehensive HPV prevention strategies, which are critical for improving women's health in South India and beyond [13].

The national HPV vaccination programme, introduced in some states, is expected to reduce HPV prevalence and the burden of cervical cancer in the coming decades. However, awareness and access to vaccination remain a challenge in many regions. Therefore, these findings contribute to more comprehensive genotypic HPV analyses and support further research involving larger populations and advanced molecular tools to detect HPV strain diversity and pathogenicity, thereby enhancing prevention and treatment strategies.

Limitation(s)

The study was limited by its sample size, the use of a single molecular technique (Multiplex Nested PCR), the generalisability of the findings to larger populations and the limited demographic diversity of the study participants.

CONCLUSION(S)

This study concludes the importance of molecular techniques, such as multiplex nested PCR, alongside traditional cytology for comprehensive HPV screening and early cancer prevention. These findings contribute to the epidemiological understanding of HR-HPV genotypes in South India, advocating for targeted HPV genotyping and regular screening programmes. To the best of our knowledge, HPV 56 has not been previously reported in the Puducherry region.

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