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Detection and Genotyping of High-Risk Human Papillomavirus (HPV) Using Real-Time PCR in Cervical Samples: A Pilot Study

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Abstract

Human Papillomavirus (HPV) is a major etiological agent of cervical cancer, particularly high-risk (HR) genotypes such as HPV 16 and 18. This study aimed to detect and genotype HR-HPV in cervical samples using real-time polymerase chain reaction (RT-PCR). Cervical swab samples were collected and subjected to DNA extraction followed by amplification targeting the E6/E7 oncogenic regions of 14 HR-HPV genotypes. The assay utilized fluorescence-based detection for qualitative analysis. Among the analyzed samples, only one case (age group 60-70 years) tested positive for HR-HPV, specifically genotype 39/68. No positive cases were observed in younger age groups. The findings highlight the presence of HR-HPV in older age groups and emphasize the importance of molecular screening for early detection. The study underscores the utility of RT-PCR as a sensitive diagnostic tool and reinforces the need for vaccination and preventive strategies to reduce HPV-associated cervical cancer risk.

***Key words*:** Human papillomavirus, Real-time PCR, Cervical cancer, HPV genotyping, E6/E7 genes, Molecular diagnosis, High-risk HPV

Human Papillomavirus (HPV) is recognized as one of the most prevalent sexually transmitted infections worldwide and represents a major public health concern due to its strong association with cervical carcinogenesis. Among more than 200 identified HPV genotypes, a subset of high-risk (HR) types, particularly HPV 16 and HPV 18, are responsible for approximately 70% of cervical cancer cases globally. Persistent infection with these oncogenic HPV types is now well established as a necessary cause of cervical cancer, as first demonstrated by Walboomers *et al*. [1]. The carcinogenic potential of HR-HPV is primarily mediated through the viral oncogenes E6 and E7, which disrupt normal cell cycle regulation by targeting tumor suppressor proteins such as p53 and retinoblastoma (Rb), thereby promoting genomic instability and malignant transformation [2-3]. Despite being largely preventable, cervical cancer remains the fourth most common cancer among women worldwide and a leading cause of cancer-related mortality, particularly in low- and middle-income countries where organized screening programs are limited [4]. According to recent global cancer statistics, an estimated 604,000 new cases and 342,000 deaths occurred in 2020 [5]. The burden is disproportionately higher in developing regions, including India, where limited awareness, lack of screening programs, and socioeconomic barriers contribute to delayed diagnosis and poor prognosis [6-7].

Advancements in molecular diagnostics have significantly improved the detection and management of HPV infections. Real-time polymerase chain reaction (RT-PCR) has emerged as a highly sensitive and specific technique for HPV detection and genotyping, enabling early identification of HR-HPV infections before the onset of cytological abnormalities [8-9] (Cattani et al., 2009; Poljak et al., 2020). These assays allow rapid, simultaneous detection of multiple HPV genotypes with high analytical accuracy, making them indispensable tools in both clinical diagnostics and epidemiological studies [10]. Epidemiological evidence consistently demonstrates a strong association between persistent HR-HPV infection and the development of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer [11-12]. While most HPV infections are transient and cleared by host immunity, persistent infections with oncogenic types such as HPV 16, 18, 31, 33, and 45 significantly increase the risk of progression to malignancy [13-14]. Age-specific prevalence studies indicate that HPV infection is most common among younger women shortly after sexual debut, with a possible second peak in older women, emphasizing the importance of age-specific screening strategies.

Preventive strategies, particularly prophylactic HPV vaccination, have demonstrated high efficacy in reducing HPV-related infections and precancerous lesions. Currently available

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vaccines, including bivalent, quadrivalent, and nonavalent formulations, target the most common oncogenic HPV types and have shown substantial effectiveness in population-based immunization programs [15-17]. Integration of vaccination with regular screening is a key component of the global strategy proposed by the World Health Organization to eliminate cervical cancer as a public health problem [18]. In this context, accurate detection and genotyping of HR-HPV are critical for early diagnosis, epidemiological surveillance, and implementation of targeted interventions. The present study aims to detect and genotype high-risk HPV in cervical samples using real-time PCR and to analyze the distribution of HPV infection across different age groups. This study will contribute to a better understanding of HPV epidemiology and support improved screening and prevention strategies.

MATERIALS AND METHODS

Materials and equipment

All reagents and consumables used in the present study were of molecular biology grade and handled under sterile conditions to prevent contamination. Adjustable micropipettes fitted with sterile filter or positive displacement tips were used for accurate liquid handling. Sterile bidistilled water, DNAse/RNAse-free 1.5 mL and 2 mL microcentrifuge tubes, and 50 mL conical tubes were employed throughout the experimental procedures. Personal protective equipment, including disposable powder-free gloves, laboratory coats, and protective goggles, was used to ensure biosafety. A vortex mixer, dry bath incubator, and cooling centrifuges (for both 1.5 mL and 15/50 mL tubes) were utilized for sample processing. DNA extraction was performed using a commercially available DNA isolation kit following silica membrane-based purification.

All PCR reactions were set up in a laminar airflow cabinet to maintain a contamination-free environment. Work surfaces and equipment were routinely decontaminated using DNA removal solutions such as Termi-DNA-Tor or equivalent. Amplification reactions were carried out in thin-walled 0.2 mL PCR tubes. Real-time PCR analysis was performed using the Himedia Insta Q96 system, which enables multiplex detection of HPV genotypes with high sensitivity and specificity.

General precautions

Strict laboratory precautions were followed throughout the study to ensure the accuracy and reliability of results. All reagents were transported and stored under recommended conditions, typically on dry ice, and checked for integrity upon arrival. Expired reagents were not used. All components were thawed at room temperature, mixed thoroughly, and briefly centrifuged prior to use.

The laboratory workflow was maintained in a unidirectional manner, starting from sample preparation and DNA extraction to amplification and detection, thereby minimizing cross-contamination. Samples were handled in a biosafety cabinet and treated as potentially infectious material. All contaminated materials and disposable plasticware were disinfected using 0.5% sodium hypochlorite or appropriate disinfectants before disposal, following institutional biosafety guidelines.

Personnel were trained in molecular techniques and adhered to standard laboratory safety practices, including avoiding eating, drinking, or handling contact lenses in the laboratory. Any spills were immediately cleaned and disinfected. In case of accidental exposure to reagents, affected areas were washed thoroughly with water and medical assistance was sought if necessary.

Sample collection and storage

Cervical samples were collected using sterile swabs and transferred into EziPrep liquid-based cytology medium. The samples were stored at 2-8°C until further processing to preserve nucleic acid integrity. Proper labeling and documentation were maintained for all samples to ensure traceability.

DNA extraction protocol

Genomic DNA was extracted from cervical cell samples using a silica membrane-based spin column method. Initially, the cervical swab samples were centrifuged at 6000 rpm for 10 minutes at room temperature to pellet the cells. The supernatant was discarded, and the pellet was washed with 1 mL phosphate-buffered saline (PBS) followed by centrifugation at 8000 rpm for 2 minutes. This washing step was repeated to remove contaminants. For pre-lysis, the pellet was resuspended in 25 µL proteinase K and 180 µL BT1 buffer, mixed thoroughly by vortexing, and incubated at 56°C for 1-3 hours until complete lysis was achieved. The lysate was then vortexed and mixed with 250 µL of buffer BB3, followed by incubation at 70°C for 10 minutes to ensure efficient lysis.

Subsequently, 250 µL of ethanol (96-100%) was added to the lysate to facilitate DNA binding. The mixture was transferred into a spin column and centrifuged at 10,000 rpm for 1 minute. The bound DNA was washed sequentially using wash buffers BBW and BB5 to remove impurities. A final centrifugation at 14,000 rpm ensured complete removal of residual ethanol. DNA was eluted by adding 50 µL of preheated buffer BBE (70°C) to the membrane, followed by incubation at room temperature for 5 minutes and centrifugation at 10,000 rpm. The purified DNA was stored at −80°C until further analysis.

Real-time PCR amplification

Real-time PCR was performed for qualitative detection and genotyping of high-risk HPV DNA. The reaction mixture was prepared using multiplex master mix and HPV primer-probe mixes specific for different genotype groups. Three separate reaction tubes (Tube 1, Tube 2 and Tube 3) were prepared for each sample.

Table 1 PCR (Polymerase chain reaction) reaction setup

Reagent	Tube 1	Tube 2	Tube 3
Multiplex Master Mix	10 µL	10 µL	10 µL
HPV Primer Probe Mix 1	10 µL	–	–
HPV Primer Probe Mix 2	–	10 µL	–
HPV Primer Probe Mix 3	–	–	10 µL
Total volume	20 µL	20 µL	20 µL

Each tube targets specific HPV (Human Papillomavirus) genotype groups, allowing multiplex detection. Separate reactions improve specificity and reduce cross-reactivity between probes.

After preparation, 20 µL of reaction mixture was aliquoted into PCR (Polymerase chain reaction) tubes, followed by addition of 10 µL of extracted DNA (deoxyribonucleic acid). Amplification was performed in the real-time PCR (Polymerase chain reaction) instrument under optimized cycling conditions, ensuring precise thermal regulation and real-time detection of fluorescence signals for accurate quantification of target nucleic acid sequences.

Table 2 Channel configuration for detection

Tube	Target	Channel color	Reporter	Quencher	Gain
Tube 1	HPV (9 genotypes)	Green	FAM	None	Auto
Tube 2	HPV (3 genotypes)	Green	FAM	None	Auto
	HPV 18	Yellow	HEX/VIC	None	Auto
	Internal Control	Orange	ROX	None	Auto
Tube 3	HPV (2 genotypes)	Green	FAM	None	Auto
	HPV 16	Orange	ROX	None	Auto

Fluorescence channel settings

Different fluorescent channels enable simultaneous detection of multiple HPV (Human Papillomavirus) genotypes and internal control, ensuring assay reliability and validation of amplification, thereby facilitating accurate differentiation of target sequences and minimizing the risk of false-negative or false-positive results.

Table 3 High-risk HPV detection interpretation

Case	Internal control	Tube 1	Tube 2	Tube 3	Interpretation
1	Ct ≤27	+	–	–	Positive for HPV (16,31,33,35,52,58,51,56,66)
2	Ct ≤27	–	–	+	Positive for HPV (39,68)
3	Ct ≤27	–	+	–	Positive for HPV (18,45,59)
4	Ct ≤27	–	–	–	Negative for HR-HPV
5	Ct ≥27	–	–	–	Poor DNA quality
6	Absent	–	–	–	PCR inhibition

Qualitative result analysis

Presence of amplification signal (Ct ≤27) confirms valid DNA extraction and PCR performance. Absence of signal with valid IC indicates negative HPV (Human Papillomavirus) status, while failure of IC suggests poor sample quality or PCR (Polymerase chain reaction) inhibition.

Table 4 HPV Genotype Identification

Case	Tube 1	Tube 2	Tube 3	Interpretation
1	+	–	HPV16 +	HPV 16 detected
2	–	HPV18 +	–	HPV 18 detected
3	+	–	–	Other HR-HPV (31,33,35,52,58,51,56,66)
4	–	–	+	HPV 39 or 68
5	–	+	–	HPV 45 or 59

HPV genotyping (16 & 18)

Specific fluorescence signals correspond to individual HPV genotypes. Detection of HPV 16 and 18 is clinically significant due to their high oncogenic potential.

Quality control and data interpretation

Internal control (IC) was included in each reaction to monitor DNA extraction efficiency and PCR (Polymerase chain reaction) inhibition. A Ct cutoff value of ≤37 cycles was considered valid for HPV (Human Papillomavirus) detection. Amplification beyond 37 cycles was interpreted cautiously in conjunction with clinical findings. In samples with high viral load, IC (Internal control) amplification may be reduced or absent due to competition.

RESULTS AND DISCUSSION

Age-wise distribution of HPV infection

The age-wise distribution of HPV-positive cases in the study population is summarized in (Table 5).

Table 5 Age-wise distribution of HPV positive samples

Patient age group (Years)	Number of positive samples	Interpretation
30–40	Nil	No HPV infection detected
40–50	Nil	No HPV infection detected
50–60	Nil	No HPV infection detected
60–70	1	Positive for HPV genotype 39/68

The results demonstrate a very low prevalence of HPV infection in the studied cohort, with only one positive case identified among all tested samples. The positive case was observed in the 60-70 years age group and was associated with HPV genotypes 39 and/or 68, which are classified as high-risk HPV (HR-HPV) types. No HPV infection was detected in individuals aged 30-60 years. This age-specific distribution suggests that HPV infection in the present study population may be associated with persistent or latent infection that becomes detectable later in life, rather than newly acquired infection in younger age groups.

Human Papillomavirus (HPV) infection is a well-established etiological factor in cervical cancer and other anogenital malignancies. The present study revealed a low

detection rate of HPV, with only one positive sample identified in the 60-70 years age group. This finding is consistent with studies indicating that while HPV infection is more commonly acquired at a younger age, persistent infections that evade immune clearance may remain undetected and manifest later in life [14], [17]. The detection of HPV genotype 39/68 in an older individual underscores the importance of continued screening beyond reproductive age.

The epidemiology of HPV infection is strongly influenced by behavioral, biological, and socio-demographic factors. One of the प्रमुख risk factors is having multiple sexual partners, which significantly increases the likelihood of HPV transmission. The virus primarily infects the epithelial cells of the genital tract but can also spread to other mucosal sites. Additionally, vertical transmission from mother to neonate during childbirth has been documented, although its clinical significance remains limited in most cases. Studies have demonstrated that HPV transmission from infected women to men is more prevalent than the reverse, highlighting gender-specific transmission dynamics [19-20].

High-risk HPV genotypes, including HPV 16, 18, 31, 33, 39, 45, 52, 58, and 68, are strongly associated with the development of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. These oncogenic viruses express E6 and E7 proteins, which interfere with tumor suppressor pathways, leading to uncontrolled cell proliferation. In addition to cervical cancer, HR-HPV infections have also been linked to other gynecological conditions such as bacterial vaginosis and persistent inflammatory states [11-12]. The identification of HPV 39/68 in this study is clinically relevant, as these genotypes, although less common than HPV 16 and 18, still contribute to cervical carcinogenesis.

Age-related trends in HPV prevalence indicate that infection rates are highest shortly after sexual debut, followed by a decline due to immune-mediated clearance. However, a second peak in HPV prevalence has been reported in older women, which may be attributed to reactivation of latent infection or reduced immune function [14] [21]. The findings of the present study align with this observation, as HPV was detected only in the older age group.

Preventive strategies, particularly HPV vaccination, have significantly reduced the burden of HPV-related diseases in many populations. Prophylactic vaccines targeting major oncogenic HPV types have demonstrated high efficacy and safety. Current guidelines recommend routine vaccination for adolescents aged 11–12 years, with the option to initiate vaccination as early as 9 years. Catch-up vaccination is recommended up to 26 years of age, while vaccination for individuals aged 27–45 years is based on shared clinical decision-making [15], [17]. However, vaccination is generally not recommended for individuals older than 45 years due to limited benefit and lack of licensing.

Despite the effectiveness of vaccination, screening remains a critical component of cervical cancer prevention. Molecular diagnostic techniques such as real-time PCR offer high sensitivity and specificity for HPV detection and genotyping, enabling early identification of high-risk infections [22]. The integration of HPV testing with cytological screening can significantly improve early diagnosis and reduce cervical cancer incidence [23-24]. The presence of high-risk HPV genotypes emphasizes the need for continued screening and awareness, particularly among older women [25]. These findings support the importance of combining vaccination strategies with regular screening programs to effectively control HPV-related diseases.

CONCLUSION

The present study provides insight into the prevalence and age-wise distribution of high-risk Human Papillomavirus (HR-HPV) infection in cervical samples using a sensitive real-time PCR-based detection method. The findings revealed a very low prevalence of HPV infection within the study population, with only a single positive case identified in the 60–70 years age group. The detected genotype (HPV 39/68), classified among oncogenic HPV types, underscores the clinical relevance of screening even in older women, who are often overlooked in routine cervical cancer prevention programs. The absence of HPV detection in younger age groups (30–60 years) in this study may reflect a lower exposure risk, effective immune clearance of transient infections, or limitations related to sample size and population characteristics. However, the presence of infection in an older individual aligns with existing evidence suggesting that persistent or latent HPV infections may reactivate later in life due to age-related decline in immune function. This highlights the importance of extending HPV screening beyond the traditionally targeted reproductive age group. The study reinforces the critical role of HR-HPV in cervical carcinogenesis and emphasizes the need for early detection through molecular diagnostic tools such as real-time PCR, which offer high sensitivity and specificity for identifying and genotyping HPV infections. The detection of less common high-risk genotypes, such as HPV 39 and 68, further indicates the necessity of comprehensive genotyping approaches rather than focusing solely on the most prevalent types like HPV 16 and 18. Preventive strategies, particularly prophylactic vaccination, remain the cornerstone for reducing HPV-related disease burden. While vaccination programs primarily target younger populations before exposure to the virus, the importance of regular screening cannot be overstated, especially for unvaccinated individuals and older women who may harbor persistent infections. Integration of vaccination, awareness programs, and routine screening is essential for effective control and eventual elimination of cervical cancer as a public health problem, as advocated by the World Health Organization. In conclusion, although the study reports a low prevalence of HPV infection, the detection of high-risk genotypes in an older age group highlights the ongoing risk of cervical cancer and the need for sustained surveillance. Future studies with larger sample sizes and diverse populations are recommended to better understand HPV epidemiology and to strengthen prevention and control strategies.

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