Acta Biomed 2024; Vol. 95, N. 2: e2024068 DOI: 10.23750/abm.v95i2.15405

Original article

Analysis of L1 gene sequence variations in high-risk and low-risk Human Papillomavirus (HPV) serotypes among Indian women: Implications for cervical cancer research

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Abstract. *Background and Aim:* Cervical cancer, ranking fourth globally and second in India among prevalent malignancies affecting women, poses a significant health challenge. Human Papillomavirus (HPV) infection, contributing significantly to cervical cancer, impacts about 5% of women worldwide annually. This study aims to comprehensively analyze sequence variations in L1 genes of high-risk (16, 18, 53, 58, 66, 73) and low-risk (6, 11, 70, 71, 84) HPV serotypes among Indian women. *Methods:* Liquid-Based Cytology (LBC) samples were systematically collected from diverse Indian regions. L1 variants were meticulously examined through Polymerase Chain Reaction (PCR) and DNA sequencing. Multalin software analyzed gene sequences, identifying alterations by comparing them with the NCBI Genebank's known HPV L1 sequences. *Results:* Mutation analysis revealed distinct alterations in various HPV serotypes, notably in HPV 6, 11, 16, 18, 31, 53, 58, 66, 70, 71, 73, and 84. High-risk serotypes 16 and 18, and low-risk serotypes 70 and 71, exhibited the highest number of mutations in the L1 region. *Conclusions:* This study provides crucial insights into genetic variations of prevalent HPV serotypes among Indian women. Identified mutations, particularly in high-risk HPV 16 and 18, and low-risk HPV 70 and 71, form a foundation for ongoing research in HPV-related cancers. These findings also open avenues for developing targeted HPV vaccines, promising advancements in cervical cancer prevention strategies. (www.acta biomedica.it)

Key words: Human Papillomavirus, L1 variants, cervical cancer, liquid based cytology

Introduction

Cervical cancer poses a significant global burden, with India contributing to more than one-third of the total cases (1,2). Human Papillomavirus (HPV) is a circular double-stranded DNA virus with a genome size of approximately 8,000 base pairs (3). The viral genome is encapsulated within a capsid protein and comprises three main functional regions: early proteins (E1-E7), late proteins (L1 and L2), and the Long Control Region (LCR). Notably, all developed vaccines target the major L1 capsid protein (4). From the observed 100 known HPV types, 30 to 40 are transmitted through sexual contact, primarily affecting the anogenital region and oropharynx. HPV serotypes 16 and 18 are responsible for about 66% of cervical cancer cases, while serotypes 31, 33, 45, 52, and 58 contribute to an additional 15% (5). High-risk HPV 16 and 18 account for approximately 70% of cervical cancers, while low-risk HPV strains like HPV 6 and 11 are responsible for around 90% of genital warts, which rarely progress to cancer (6). The HPV genome is functionally divided into three regions: the Long Control Region (LCR), which regulates DNA

replication by controlling the transcription of the Open Reading Frames (ORFs); the early region, encompassing ORFs E1, E2, E4, E5, E6, and E7, which play crucial roles in viral replication and oncogenesis; and the late region, encoding the L1 and L2 structural proteins responsible for viral capsid formation (7).

Epidemiological studies have demonstrated that the risk of contracting genital HPV infection and cervical cancer is influenced by sexual behaviors, parity, marital status and age (8,9). Individuals with multiple sexual partners and those engaged in early sexual activity are at greater risk. Additionally, individuals with a history of sexually transmitted diseases, genital warts, abnormal Pap smears, or cervical or penile cancer are predisposed to HPV infection. Preventive measures include HPV vaccination and cervical cancer screening (10). Vaccines such as Gardasil®, Gardasil® 9, and Cervarix™ have been developed to target specific HPV types. Cervarix™ protects against types 16 and 18, while Gardasil® protects against types 6, 11, 16, and 18 (11,12). The World Health Organization (WHO) recommends one or two doses of HPV vaccination for girls and women aged 9 to 20 years, with a two-dose regimen and a 6-month interval for women aged 21 years and older. It is recommended to administer the HPV vaccine at an early age, between 9 and 14 years (13).

It is worth noting that the HPV vaccine, particularly the 9-valent vaccine, is produced using the yeast *Saccharomyces cerevisiae*. Individuals with a history of immediate hypersensitivity to yeast may experience allergic reactions and should avoid the vaccine. Additionally, HPV vaccination is not recommended during pregnancy, but it can be administered after childbirth. Overall, HPV vaccines have been proven to be safe, although some individuals may experience mild side effects, commonly localized reactions at the injection site (14).

Recognizing the importance of HPV vaccination in preventing cervical cancer, the Indian Ministry of Health and Family Welfare issued a circular in January 2023, urging seven state governments to include the HPV vaccine in their routine immunization programs. The initiative aims to start vaccinating girls aged 9 to 14 years, with subsequent expansion to cover other states and union territories within two years (WHO, 2022) (15). By 2025, it is projected that 68 million girls in India will be vaccinated against HPV, targeting approximately 11.2 million 9-year-old girls annually (16). In addition to vaccination, implementing primary prevention (vaccination) and secondary prevention (screening and treatment) strategies is crucial in reducing the incidence and mortality of cervical cancer (17). These combined efforts play a significant role in promoting women's health and well-being. In recent years, extensive research has focused on understanding the genetic variations within the L1 gene of HPV (18). The L1 gene encodes the major capsid protein, which is critical for viral entry, assembly, and immune recognition. Investigating the sequence variations in the L1 gene provides valuable insights into the potential impact on HPV infectivity, virulence, and immune evasion (19).

Materials and methods

The study was conducted on Liquid-Based Cytology (LBC) samples, and tissue biopsies were procured from individual patients at the diagnostic facility of LifeCell International Pvt Ltd, situated in Chennai, India. A total of 334 samples were (October-December 2021) randomly collected from diverse age groups of Indian women across different regions of the country. The region-wise distribution of samples is outlined in Table 1. Participants were included based on the following criteria: women of all age groups from diverse regions across India. Exclusion criteria comprised individuals who had previously undergone antibiotic treatment and those who expressed unwillingness to participate in the study. This sampling approach aimed to capture a representative population for the study

Table 1. Distribution of samples from various regions of India.

Region	No. of Sample	No. of Positive	Percentage of Positive
North	223	16	7.1
West	48	1	2.1
South	45	1	2.2
East	18	0	0
Over All	334	18	5

and enhance the generalizability of the findings. By incorporating samples from various geographic regions and age groups, the study sought to provide a comprehensive analysis of HPV sequence variations in Indian women.

Ethical considerations

This study received approval from the Institutional Review Board (IRB) of Sathyabama University, with reference number 141/IRB-IBSEC/SIST dated 10th August 2021. The approved study duration was one year (12th August 2021-11th August 2022). The study was developed in accordance with the Helsinki declaration (www.wma.net) and written informed consent was obtained from all the subjects included in the study.

Methods

The collected samples underwent a series of testing procedures to assess their characteristics. Firstly, Pap smear tests were performed to examine the cellular morphology and identify any abnormal changes indicative of potential HPV infection or cervical abnormalities. Subsequently, Polymerase Chain Reaction (PCR) analysis was conducted to detect the presence of HPV DNA in the samples.

For the samples that tested positive for HPV infection through PCR, genotyping sequencing was conducted. This sequencing process aimed to identify and classify the specific HPV serotypes present in the positive samples. By genotyping the HPV strains, the study could distinguish between high-risk and low-risk serotypes and further analyze the sequence variations within the L1 genes of these different serotypes.

PAP smear examination

To collect the LBC samples, the BD SurePath kit was utilized following the recommended instructions for conducting a Pap smear test. The Pap smear samples were then subjected to the universal staining method known as the Papanicolaou stain. This staining technique allows for the differentiation of cells based on their maturity and metabolic activity. The Papanicolaou stain consists of three different stains, each serving a specific purpose. Harris' hematoxylin stain was used to color the cell nuclei by binding with the DNA present in the cells. Acid orange dye, on the other hand, reacts with mature squamous cells by binding with keratin. Eosin stain was employed to interact with immature squamous cells (basal and intermediate cells), glandular cells, and erythrocytes.

To prepare the samples for evaluation, the cells obtained from individual samples were enriched using density gradient centrifugation. Subsequently, the enriched cell suspension was transferred to a settling chamber mounted on a pre-coated microscopic slide. The cells settled within the chamber to form a thin layer, which was then stained and subjected to evaluation. For staining, a combination of hematoxylin and the BD EA-OG combo stain was used. Following the staining process, the slides were air-dried and mounted using D.P.X., a mounting medium commonly employed in histological preparations. The evaluation and reporting of the Pap smear samples were carried out based on the guidelines provided by The Bethesda System for Reporting Cervical Cytology specifically following the 3rd edition of the system (20). By employing the BD SurePath kit and the Papanicolaou stain, along with adherence to the standardized Bethesda System guidelines, the study ensured accurate and consistent evaluation of the cytological preparations obtained from the LBC samples (21,22). This approach allowed for the reliable assessment of the cellular morphology and identification of any abnormal changes associated with HPV infection or cervical abnormalities.

PCR and genotyping by sequencing

Viral DNA extraction and PCR

The extraction of viral DNA from the collected samples was performed using the MN kit (DNA/RNA Extraction) following the instructions provided by the manufacturer. The extracted DNA was then used as the template for PCR amplification. PCR was conducted using a concentration of 5-30 ng of the extracted DNA. To ensure the reliability of the PCR results, human β-globin was utilized as an endogenous

control. This internal control allowed for the verification of successful DNA amplification and the assessment of sample quality.

Specific primers targeting the L1 region of the HPV virus were designed for the PCR reaction. The sense primer PGMY09 (5'-CGTCCMARRGG AWACTGATC-3') and the antisense primer PGMY11 (5'-GCMCAGGGWCATAAYAATGG-3') were employed to detect the presence of HPV DNA. The PCR reaction was carried out under standard conditions, with an annealing temperature of 56°C and an extension temperature of 72°C. The amplification of the target DNA sequence using these primers allowed for the detection and identification of HPV infection in the samples. The primer sequence details performed in the assay is shown in Table 2.

To initiate the PCR amplification, a master mix containing all the necessary components for the reaction was prepared. The master mix included the DNA template, primers, nucleotides, and PCR enzymes. Care was taken to ensure proper mixing and accurate pipetting to maintain consistency across all samples. The prepared PCR tubes containing the master mix and the samples were securely placed in the thermal cycler block. The thermal cycler was programmed to initiate the activation program at 95°C, which facilitated the denaturation of the DNA template and the activation of the DNA polymerase enzyme.

Following the activation program, the amplification program commenced. This program involved a series of temperature cycles, including denaturation, annealing, and extension, which facilitated the replication of the target DNA sequence. These temperature cycles were repeated for a predetermined number of cycles to ensure adequate amplification. Upon completion of the amplification program, the PCR products were subjected to agarose gel electrophoresis. Following electrophoresis, the agarose gel was visualized and analyzed using a Gel Doc Quantity One Software. The gel was placed on a UV transilluminator, which emitted UV light at a wavelength of 254 nm.

Genotyping by sequencing

The respective L1 PCR (HPV-F and HPV-R) products were isolated from agarose gel and DNA was extracted. The sequencing reaction products were then processed using a Genetic Analyzer, specifically the ABI 3500 Dx instrument. This high-throughput sequencing platform enabled the automated and accurate determination of the DNA sequence.

The obtained sequencing data were further analyzed *in silico*, meaning the analysis was performed using computational methods and software. The sequences were compared with known sequences available in the Genbank database, which is maintained by the National Center for Biotechnology Information (NCBI). For sequence comparison, the NCBI-BLAST (Basic Local Alignment Search Tool) was utilized. The results were then compared with the sequences available in the Genbank database using the National Center for Biotechnology Information BLAST program (NCBI-BLAST).

Results

A total of 334 LBC samples obtained from Indian women were subjected to comprehensive analysis to assess the presence of Human Papillomavirus (HPV) infection and identify specific serotypes. The mean values and standard deviations for 334 female participants, categorized by age, are presented in Table 3. Initial screening was performed using Pap smear study, which revealed that none of the samples exhibited intraepithelial lesion or cytological features indicative of malignancy. To further investigate HPV infection, PCR amplification targeting the L1 region of HPV was conducted using consensus degenerate

Table 2. Primer Sequences Used for HPV Amplification.

Region Name	Primer Name	Primer Sequence	Amplicon Size
HPV L1 gene	HPV-F	CGTCCMARRGGAWACTGATC	450 bp
	HPV-R	GCMCAGGGWCATAAYAATGG	

Table 3. Mean age and standard deviation of age group for female patients.

Female Participants (n=334)		
Age Range	**Mean**	**SD**
21-30	26.3	2.9
31-40	35.5	3.0
41-50	45.5	3.0
51-60	55.5	3.0
61-70	65.1	3.1
71-80	72.5	2.1
81-90	85	7.8

primers. Among the samples, 18 (5.4%) were found to be positive for HPV infection based on the detection of specific PCR amplicons. The positive samples were subsequently subjected to Sanger sequencing for serotype identification. The sequencing analysis revealed the presence of both high-risk (Hr-HPV) and low-risk (Lr-HPV) serotypes in the positive samples. The high-risk serotypes detected included HPV-16, HPV-18, HPV-31, HPV-53, HPV-58, HPV-66, and HPV-73. The low-risk serotypes identified were HPV-6, HPV-11, HPV-70, HPV-71, and HPV-84. Among the positive cases, 16.7% were infected with the low-risk type HPV-6, indicating its prevalence in the studied population. The high-risk serotypes HPV-18, HPV-58, HPV-66, and the low-risk serotype HPV-84 were detected in 11.1% of the positive cases. Additionally, 5.6% of the positive cases were associated with the serotypes HPV-11, HPV-16, HPV-31, HPV-53, HPV-70, HPV-71, and HPV-73. These findings demonstrate the presence of HPV infection in a subset of the studied Indian women population. The detection of both high-risk and low-risk serotypes emphasizes the potential risk for both cervical cancer development and the occurrence of genital warts. The identification of specific serotypes provides important insights into the prevalent HPV strains in the population under study.

Table 4. presents the HPV positive distribution. Table 5. outlined the HPV serotype mutation data, including the risk type associated with each serotype and the number of base pair mutations observed. The table provides a clear overview of the mutations identified in each HPV serotype. It shows that HPV serotypes 16, 18, 31, 70 and 73 had no base pair mutations detected. However, serotypes such as 71, 84, 11, and 58 exhibited varying numbers of base pair mutations.

Table 4. HPV positive distribution – serotype wise.

Risk Types	HPV Serotype	No. of Positive	% Positive
LR	6	3	16.7
LR	84	2	11.1
HR	18, 58, 66	2	11.1
LR	11,70,71	1	5.6
HR	16,31,53,73	1	5.6

Abbreviations: LR - Low-risk serotypes; HR - High-risk serotypes.

Table 5. HPV serotype mutation data.

HPV Serotype	Risk Type	Number of Base Pair Mutation
16	HR	0
18	HR	0
31	HR	0
70	LR	0
71	LR	4
73	HR	0
53	HR	0
58	HR	1
66	HR	2
84	LR	4
6	LR	2
11	LR	4

Discussion

Cervical cancer is the most frequent malignancy in women worldwide accounting for 17% of all cancer deaths among women aged between 30 and 69 years. Although, the incidence of cervical cancer is steadily declining in the developed world; it is most common in developing countries (23). Cervical cancer still causes more than 67,477 deaths annually in India due to the lack of organized screening programs and intervention approaches (24). Although, India is a diverse country

with extensive ethnicity, and socio-cultural diversity, the incidence of HPV infection may vary significantly in different regions so, it is required to study prevalence of HPV and its genotypes in every part of the country. The present study aimed to analyze sequence variations in the L1 genes of high-risk and low-risk serotypes of Human Papillomavirus (HPV) in Indian women. The results obtained provide valuable insights into the prevalence and mutation patterns of HPV serotypes in the study population. Additionally, comparing and correlating the findings with existing literature helps to contextualize the significance of the study.

The analysis of Liquid Based Cytology (LBC) samples using PCR and DNA sequencing revealed that 5.4% of the samples tested positive for HPV infection. This finding aligns with previous studies that have reported a considerable burden of HPV infection among women globally (25). The prevalence of HPV infection in the current study is consistent with the worldwide estimate of approximately 5% of women in the general population being affected by HPV infection annually (26).

Due to the high diagnostic value of L1, and its variability, L1 is often selected as a clinical diagnostic target. Mutation of different types of HPV are identified as follows. HPV 6-1 base pair mutation (2/3) and 1 base pair mutation (1/3), HPV 11- 3 base pair mutation (1/1), HPV16-22 base pair mutation (1/1), HPV 18- 24 base pair mutation (1/2) and 22 base pair mutation (1/2), HPV 31-28 base pair mutation (1/2), HPV 53- 12 base pair mutation (2/2), HPV 58- 2 base pair mutation (2/2), HPV 66-5 base pair mutation (2/2), HPV 70-28 base pair mutation (1/1), HPV 71-22 base pair mutation (1/1), HPV 73-16 base pair mutation (1/1), HPV 84-4 base pair mutation (2/2). Most high numbers of mutations in the L1 region are seen in HPV 16, 18, 70, 71 serotypes. An overall of 29 single nucleotide changes were observed in HPV-16 L1 sequences with 16/29 non-synonymous mutations and 13/29 synonymous mutations (six in alpha-helix and two in beta turns). 24 single nucleotide changes were observed in HPV-58 L1 sequences with 10/24 non-synonymous mutations and 14/24 synonymous mutations. (Eight in alpha helix and four in beta turn) (27). These findings are in line with previous studies indicating that HPV-16 and HPV-18 are the predominant high-risk serotypes associated with cervical cancer (28-30). The presence of these high-risk serotypes emphasizes the importance of HPV vaccination and early detection strategies for cervical cancer prevention in the Indian population.

Regarding the low-risk serotypes, HPV-6, HPV-11, HPV-70, HPV-71, and HPV-84 were identified in the positive samples. HPV-6 was found to be the most prevalent low-risk serotype, followed by HPV-84. These serotypes are commonly associated with genital warts and have a lower oncogenic potential compared to high-risk serotypes (31-33). The detection of low-risk serotypes highlights the need for comprehensive HPV screening programs that address both high-risk and low-risk infections to effectively manage and prevent HPV-related diseases. In terms of mutation analysis, the study identified specific nucleotide mutations in different HPV serotypes. The analysis revealed varying degrees of mutations in different serotypes, with some serotypes showing no mutations while others exhibited multiple mutations. Notably, high-risk serotypes such as HPV-58 and HPV-66 demonstrated one and two nucleotide mutations, respectively. These findings are consistent with previous studies highlighting the genetic diversity and mutation patterns within different HPV serotypes (34).

Comparing the obtained results with existing literature, it is evident that the distribution of HPV serotypes in the study population aligns with global trends. The predominance of high-risk serotypes, particularly HPV-16 and HPV-18, among the positive samples corresponds to their well-established association with cervical cancer (29). Furthermore, the identification of low-risk serotypes such as HPV-6 and HPV-11 in the positive samples is consistent with their known association with genital warts (35).

The mutation analysis provides valuable insights into the genetic variations within different HPV serotypes. While some serotypes exhibited no mutations, others displayed a varying number of nucleotide mutations. These findings are in line with previous studies that have reported genetic diversity and mutation patterns within HPV serotypes (36). The outcomes of this study have important implications for HPV-related cancer research and the development of targeted HPV vaccines. The identification of specific

serotypes and their mutation patterns can aid Vaccine targeting L1 only prevents infection by specific HPV subtypes due to lack of cross-protective epitopes in different HPV subtypes (37). Vaccination of young age girls prior to sexual debut appeared to be the most effective public health measure for the prevention of cervical diseases and cancer. In contemporary research, virus-like particles (VLPs) derived from L1 proteins are widely recognized as the most promising candidates for developing vaccines against HPV infections (38). Continuous mutation of L1 region HPV serotypes may lead to ineffective vaccines to the public. In the development of more effective diagnostic tools, therapeutic interventions, and HPV vaccines. The high prevalence of HPV-16 and HPV-18, known to be strongly associated with cervical cancer, underscores the importance of targeted prevention strategies, including vaccination against these specific serotypes.

The presence of multiple serotypes in the positive samples indicates the complexity of HPV infection and highlights the need for comprehensive screening methods that can detect both high-risk and low-risk serotypes. This finding is consistent with previous studies reporting the co-infection of multiple HPV serotypes in cervical samples (39). Co-infection with multiple serotypes may have implications for disease progression, treatment response, and the potential for the development of more severe lesions.

The mutation analysis conducted in our investigation provided insights into the genetic variability within HPV serotypes (40). The identified nucleotide mutations in certain serotypes suggest the existence of distinct viral strains with potential implications for virulence, transmission dynamics, and the development of more aggressive disease phenotypes. Understanding the genetic variations within HPV serotypes can contribute to a better understanding of the natural history of HPV infection, its association with disease progression, and the development of more targeted therapeutic strategies. Comparing our findings with existing literature, it is important to note that HPV serotype distribution and mutation patterns can vary across different populations and geographical regions. Factors such as population demographics, sexual behavior, and regional variations in HPV vaccination coverage can influence the prevalence and distribution of HPV serotypes. It is worth noting that this study has limitations, such as the relatively small sample size and the specific geographic region of India from which the samples were obtained. Further research involving larger sample sizes and diverse geographic regions would be beneficial to gain a more comprehensive understanding of HPV serotype distribution in Indian women. Overall, these results contribute to the existing knowledge on HPV prevalence and serotype distribution in Indian women, laying the groundwork for ongoing research in HPV-related cancer studies and the development of targeted HPV vaccines.

Conclusion

In summary, this study offers valuable insights into the prevalence, distribution, and mutation patterns of HPV serotypes within the Indian population. The detection of both high-risk and low-risk serotypes highlights the need for comprehensive HPV screening and vaccination programs to effectively prevent HPV-related diseases. The identification of specific serotypes and their mutation patterns contributes to our understanding of HPV diversity and can inform the development of targeted interventions for improved diagnosis, treatment, and prevention of HPV-associated cancers and other related conditions. Further research is warranted to explore the clinical implications of specific serotypes and genetic variations in HPV infection and to assess the long-term effectiveness of HPV vaccination programs in reducing the burden of HPV-related diseases.

Acknowledgements: The authors are thankful to Lifecell International Pvt. Ltd, Chennai, India for the research facilities and support.

Funding: This study was partially financially supported by grants from Lifecell International Pvt. Ltd, Chennai, India.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article. Each author confirms that there are no conflicts of interest between Lifecell International Pvt. Ltd. and the reported study.

Authors' Contribution: GS and KP spearheaded the study's design, actively participating in data collection, analysis, and interpretation. They led the initial manuscript drafting, leveraging their statistical expertise. CP and VS made key contributions, refining content and ensuring clarity in presentation during the revision process. All authors, CP, KP, GS, and VS, collaboratively reviewed and approved the final manuscript, addressing critical tasks like study design, analysis, drafting, statistical analysis, and editing.

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Received: 14 November 2023
Accepted: 31 January 2024
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