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Research Article

# Molecular Detection of HPVs and their Clinical Application in Diagnosis of Cervical Cancer

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## Abstract

**Context:** Cervical cancer is caused by infection of carcinogenic human papilloma virus commonly known as HPV and that is very common in the world. In India, females above the age of 15 years are at the risk for developing cervical cancer. Among world records, 1,22,844 new cases and 67,477 deaths occur only in India. The present study describes the identification of HPV type by PCR and DNA sequencing, the prevalence of specific types of HPV and risk of cervical cancer in Gujarati population.

**Materials and Methods:** Total 89 samples were collected from the females of different age groups between 21 to 60 years for the diagnosis of the PAP test as per the criteria and transported to the lab. DNA was extracted, quantity and quality were verified. The HPV L1 gene was amplified by using sets of primers; MY09/11 and GP5+/6+. The product amplified by using GP5+/6+ was sequenced.

**Results:** Out of total 89 samples, two samples were found to be infected by HPV-16 and one each sample by HPV-51, 66 and 90. HPV-16, 51 and 66 are related to high risk of cervical cancer. However, HPV- 90 also carries risk of cervical cancer.

**Conclusions:** The present study describes the identification of specific types of HPV and risk of cervical cancer in the Indian Gujarati population. Early detection of high risk HPV types may prevent cervical cancer in the population.

**Keywords:** Cervical Cancer; Human Papillomavirus; PAP Test; Genital Warts; Nested PCR; L1 Gene; Genotype

## Abbreviations

HPV: Human Papillomavirus; CIN: Cervical Intra Epithelial Neoplasia; PAP Test: Papanicolaou Test; DNA: Deoxy Ribonucleic Acid; FFPE: Formalin-fixed; Paraffin-embedded; LSIL: Low-grade Squamous Intraepithelial Lesions; dNTPS: Deoxynucleotides; CBCC: Comprehensive Blood and Cancer Centers

## Key Messages

This study discovers the high risk HPV type-16, 51 and 66 and one more HPV type-90 that also equally carries risk for cervical cancer in the Indian population. The findings can be beneficial for practising Gynaecologists in preventing cervical cancer.

## Introduction

Cervical cancer is caused by infection of carcinogenic human papillomavirus commonly known as HPV. In India, females above the age of 15 years are at the risk for developing cervical cancer. Annually 5,27,624 new cases are registered in the world and 2,65,672 deaths registered because of cervical cancer, among these records, 1,22,844 new cases and 67,477 deaths occur in India only, which is close to $1/4^{th}$ of global incidence and death respectively. The main reason for incidence is because of poor awareness in people in the Indian states as per the survey by a volunteer of the American Cancer Society (ACS) and founder owner of Comprehensive Blood and Cancer Centres (CBCC) USA. Cervical cancer is also common in Gujarati population.

Cervical Cancer is the outcome of persistent infection with carcinogenic HPV strain. If HPV infection persists, it takes 5 to 20 years for progression of cervical cancer. Therefore, patients get a huge time to diagnose to avoid the progression to invasive cancer. Cervical intra epithelial neoplasia (CIN) is symptomless in the majority of women, therefore; regular screening is the only option to avoid cervical cancer. There are so many risk factors for HPV infection as well as the treatments. If cervical cancer is diagnosed with a pre-invasive state then it can be 100 percent curable**.**

The highly specific and sensitive diagnosis has the crucial role to prevent and cure cervical cancer. Information given on the Med-India reveals that the HPV DNA test is more accurate compared to the PAP test for cervical cancer detection. Eduardo Franco mentioned the accuracy of HPV DNA test is 94.6 and 55.4 for the PAP test for detecting the precancerous lesions, in the first round of Canadian cervical cancer screening program as per this Med-India published article (DNA Test More Accurate in Detecting Cervical Cancer Than Pap 2018). Molecular biology techniques are the most commonly used for HPV testing. There are various biomarkers which can be used for HR-HPV progression risk investigation. If conventional screening tests are found to be positive, HPV diagnosis based on molecular techniques is more accurate and reliable for detection and typing.

The present study describes the identification of HPV type by DNA sequencing, the prevalence of specific types of HPV and risk of cervical cancer in Gujarati population.

## Materials and Methods

Total 89 brush samples were collected from the females of different age groups between 21 to 60 years for the diagnosis of the PAP test by the Gynaecologist. Sample collection criteria were irregular bleeding, pain after or during intercourse, bleeding after menopause, pelvic pain and abnormal vaginal discharge. Samples were transported in a container to the laboratory at room temperature and transferred at -$20^0$ C for further processing. Sample brush was mixed in normal saline by vortexing at high speed for 30 Sec. Brush was discarded and liquid portion was collected in 15ml centrifuge tubes. Supernatant was discarded and palette was washed twice with sterile phosphate buffer for the DNA extraction using DNA extraction kit (QiAmp DNA Blood mini kit). DNA Quantity and quality were verified by the Nanodrop-Lite and electrophoresis methods. PCR was performed by using the following sets of primers and Mastermix (includes dNTPS, Taq DNA pol., PCR buffer, $MgCl_2$ and loading dye) with a final volume of 25 µl (Takara Bio Inc.) as mentioned below in table 1.

| Primer Name | Targeted Gene | Primer Sequence | Roll of Primer Set |
|---|---|---|---|
| Beta-globin F | Beta-globin gene of Human | 5'-GAAGAGCCAAGGACAGGTAC-3' | DNA check for amplification |
| Beta-globin R | | 5'-CAACTTCATCCACGTTACACC-3' | |
| MY09 - F | L1 gene of HPV | 5'-CGTCCMARRGGAWACTGATC-3' | Outer primes of Nested PCR |
| MY11 – R | | 5'-GCMCAGGGWCATAAYAATGG-3' | |
| GP5+ - F | L1 gene of HPV | 5'-TTTGTTACTGTGGTAGATACTAC-3' | Inner Primers of Nested PCR |
| GP6+ - R | | 5'-GAAAAATAAACTGTAAATCATATTC-3' | |

**Table 1:** Forward and reverse primer sets of Beta-globin and L1 gene of HPV.

The study first used beta-globin gene primers to check the quality of DNA by amplifying. Positive samples for beta-globin gene amplification were then checked for detection of HPV infection by MY09/11 and GP5+/6+ primer sets separately. Once HPV (MY09/11 and GP5+/6+ amplification had been completed, all the samples were checked with these two primer sets (MY09/11 and GP5+/6+) by using the Nested PCR method. Reactions for all the primer sets were prepared as shown in table 2.

| Reagent | Stock concentration | Volume required for reaction preparation |
|---|---|---|
| Template DNA | 30 to 50 ng/µl | 3 µl |
| Mastermix | 2X concentrated | 12 µl |
| Forward Primer | 20 pM | 1 µl |
| Reverse Primer | 20 pM | 1 µl |
| Water | - | 8 µl |
| Total volume | - | 25 µl |

**Table 2:** Reaction ingredients and concentration for PCR.

Thermal cycler details for one set of primers targeting Beta-globin and two sets of primers targeting the L1 gene (GP5+/6+ and MY09/11) of HPV genome are given in table 3, 4, 5 and 6 respectively.

| For Primer set - Beta-globin | | | | |
|---|---|---|---|---|
| **Stage 1** | **Stage 2** | | | **Stage 3** |
| Initial Denaturation | Denaturation | Annealing | Extension | Final Extension |
| 72$^0$C | 72$^0$C | 72$^0$C | 72$^0$C | 72$^0$C |
| 10 min. | 45 sec. | 45 sec. | 45 sec. | 5 min |
| 1 Cycle | 35 Cycle | | | 1 Cycle |
| Product Size - 268bp | | | | |

**Table 3:** Thermal cycler condition for Beta-globin PCR.

| For Primer set – MY09/11 | | | | |
|---|---|---|---|---|
| **Stage 1** | **Stage 2** | | | **Stage 3** |
| Initial Denaturation | Denaturation | Annealing | Extension | Final Extension |
| 95$^0$C | 95$^0$C | 55$^0$C | 72$^0$C | 72$^0$C |
| 10 min. | 45 sec. | 1 Min. | 45 sec. | 5 min |
| 1 Cycle | 40 Cycle | | | 1 Cycle |
| Product Size - 450bp | | | | |

**Table 4**: Thermal cycler condition for primer set of MY09/11 for L1 gene of HPV.

| For Primer set - GP5+/6+ | | | | |
|---|---|---|---|---|
| **Stage 1** | **Stage 2** | | | **Stage 3** |
| Initial Denaturation | Denaturation | Annealing | Extension | Final Extension |
| 95$^0$C | 95$^0$C | 50$^0$C | 72$^0$C | 72$^0$C |
| 10 min. | 45 sec. | 30 sec. | 30 sec. | 5 min |
| 1 Cycle | 40 Cycle | | | 1 Cycle |
| Product Size - 146bp | | | | |

**Table 5:** Thermal cycler condition for primer set of GP5+/6+for L1 gene of HPV.

| **Nested PCR - Primer set - MY09/11 and GP5+/6+** | | | | |
|---|---|---|---|---|
| **1$^{st}$ Step amplification with Primer set MY09/11** | | | | |
| **Stage 1** | **Stage 2** | | | **Stage 3** |
| Initial Denaturation | Denaturation | Annealing | Extension | Final Extension |
| 95$^0$C | 95$^0$C | 55$^0$C | 72$^0$C | 72$^0$C |
| 10 min. | 45 sec. | 30 sec. | 30 sec. | 5 min |
| 1 Cycle | 20 Cycle | | | 1 Cycle |

| 2nd Step amplification with Primer set GP5+/6+ | | | | |
|---|---|---|---|---|
| **Stage 1** | **Stage 2** | | | **Stage 3** |
| Initial Denaturation | Denaturation | Annealing | Extension | Final Extension |
| 95$^0$C | 95$^0$C | 58$^0$C | 72$^0$C | 72$^0$C |
| 10 min. | 45 sec. | 30 sec. | 30 sec. | 5 min |
| 1 Cycle | 30 Cycle | | | 1 Cycle |
| Product Size - 146bp | | | | |

**Table 6:** Thermal cycler condition for nested PCR for L1 gene of HPV.

All PCR products and their size were verified on 3% of Agarose gel which were purified for sequencing using a qiaquick PCR purification kit from Qiagen. Purified PCR products were sent for the Sanger sequencing (Applied Biosystem) with GP5+/6+ primer set. Sequencing results were received in the form of a FASTA file. Further, these results are analysed on NCBI. For genotyping of positive samples Chromas software (version 2.6.4 - 2017) was used. However, another tool MSA (Multiple sequence alignment viewer 1.9.1) of NCBI was used to find out new SNPs in sequence.

## Results

Out of 89 samples 7 samples were not suitable as they did not yield any information for the PAP smear test. Out of 82 samples, 42 (51.21%) found normal, 32 (39.024%) found mild inflamed, 3 (3.65%) moderate inflamed, 5 (6.097%) samples were found with mild dysplasia in PAP test results. PAP test results are shown in figure 1.

**Figure 1:** Distribution of PAP test results based on various age groups.

However, all 89 samples were passed through the DNA extraction method and DNA quantification. Quality and quantity of DNA were good for amplification of HPV genes except for a few samples.

Hence, in the circumstances, these samples were first checked by PCR of Beta-globin. The amplicons of Beta-globin revealed positive results in all the samples extracted. By confirming enough quality and quantity, all samples are then amplified for MY09/11 and GP5+/6+ primer sets. Out of 89 samples five samples detected positive for both the primer set figure 2 (A and B) and 3, which was confirmed by nested PCR.

**Figure 2:** (A&B) PCR results of positive sample with MY09_11 Primer set (Lane # 1, 4, 5, 6 and 7). Lane # 2 and 3 are DNA ladder of 100 bp.

**Figure 3:** PCR results of positive sample with GP5+_6+ primers set (Lane # 2, 3, 5, 6 and 7). Lane # 4 is a DNA ladder of 50 bp.

Precisely, out of 89 samples, 5 samples show the positive results of HPV infection by both primer sets separately for which simple PCR and nested PCR methods were performed. The results reveal that the specificity and sensitivity are equal for the primer sets (MY09/11 and GP5+/6+) and PCR methods (conventional PCR and Nested PCR) used in the study. As per the results noted in this study, the overall prevalence of HPV is 5.617% in Gujarat. HPV DNA positive samples were further processed by the Sanger sequencing to confirm the genotype of infectious HPV. Sequences were aligned with reference sequences available on the NCBI database by using the BLAST tool. Out of five positive samples, two samples confirmed for HPV-16, and remaining three were confirmed for HPV - 51, HPV - 66 and HPV - 90.

## Discussion

HPV type-16, 51, 66 are high risk types whereas, type 90 is unclassified and low risk. Studies during 2013 indicated a prevalence of HPV 90 and its association with cancer in the North-American population. As per their findings HPV 90 is less studied and underestimated for its oncogenic potential. Because of its less prevalence and unknown carcinogenic potential in early studies, it is left unattended and without follow-up. Now Quiroga-Garza reported the increased prevalence of HPV 90 in their studies. Their findings show an association of HPV 90 with low-grade squamous intraepithelial lesions (LSIL). The present study also reported one HPV 90 genotype out of five positive samples that supports the data of Quiroga-Garza. Present study has not reported any HPV type-18 which is also a highly prevalent high risk type of HPV after type-16. The finding of the present study is supported by Patel., *et al*. as they also concluded the lesser prevalence of HPV 18 in the Gujarati population.

The present study reveals prevalence of HPV (5.617%) in a small sample size is also more or less equal to the observations by Schiffman and Kjaer where they reported 6.3% prevalence of HPV in Mumbai, 4.8% in Trivandrum, and 7.8% and 5.2% in two cities in West Bengal respectively. However, Sowjanya., *et al.* and Sankaranarayanan., *et al.* observed higher percentages of HPV (10.4% and 10.3%) respectively. This variation may be the result of a large sample size and restriction for enrolment of females over 30 years in their studies. Sometimes cervical infection of HPV can also be transferred by oral sex from their male partner having an oral HPV infection.

The present study used Nested PCR that can improve the sensitivity and specificity of conventional PCR methods. Similar to our studies Tawe., *et al.* used the double nested PCR for the diagnosis of HPV from very low quantity DNA extracted from Formalin-fixed, Paraffin-embedded (FFPE) specimens by using SB01/02 primer set as outer primers followed by MY09/11 and GP5+/6+ as an inner and inner most primer sets respectively. Thus, they used a double nested PCR method which was found to be good for very low quality of DNA and high rate of detection of HPV. However, our present studies do not show different sensitivity and specificity between conventional and nested PCR as DNA was extracted from sufficient and fresh samples.

Naucler., *et al.* stated that the most feasible screening method should be cervical cytology and molecular diagnosis of HPV types. Authors are of the opinion that early detection of high risk HPV types may prevent cervical cancer in the population as PCR and sequencing methods are cost effective and rapid. More studies are also needed with large sample sizes to evaluate the significance of HPV types in regards to cervical cancer.

## Conclusion

The present study describes the molecular characterization and the prevalence of high risk HPV type-16, 51 and 66, which are associated with cervical cancer in Gujarati Indian population. Whereas, it is recommended to reclassify HPV type-90 based on more research. Authors are of the opinion that early detection of

high risk HPV types may prevent cervical cancer in the population as PCR and sequencing methods are cost effective and rapid. More studies are also needed with large sample size to validate the significance of HPV types in regards to cervical cancer [1-19].

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## Conflicts of Interest

The authors have no conflicts of interest relevant to this article.

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