Detection of HPV DNA Using MY09/MY11 and G5/G6 Primer Sets in HIV Positive Patients with Positive Linear Array Genotype Assay Results

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ABSTRACT

Introduction: Human papillomavirus (HPV) is identified as the primary agent of cervical cancer and is the most common sexually transmitted disease in women especially in HIV infected women. Many studies have shown that Polymerase chain reaction (PCR) is the most sensitive method for the detection of HPV cervical samples as compared to conventional methods of detection of HPV infections.

Objective: This study aimed to compare the detection by of human papillomavirus (HPV) DNA in cervical specimens by using two different primers MY09/11 and G5/G6.

Methods: Two hundred and fourteen women who came to the department of Obstetrics and gynecology were enrolled in the study. In the first step all 214 samples were tested for the presence of HPV by Roche Linear array (RLA) genotype assay and thirty three samples which was positive for HPV in RLA were further tested for HPV DNA by using 2 different primers.

Results: From the 33 samples which were positive in RLA, HPV DNA was detected in 63.7% (21 of 33) and 72.73% (24 of 33) of the samples by using the MY09/11 and the G5/G6 primer sets, respectively. Among the 24 HPV-positive samples, 21 (63.7%) were detected by one or both methods and 3 (9.09%) were detected only by G5/G6 primer set.

Conclusion: This study suggests that more than one type of oligonucleotide primers should be used in clinical samples to increase sensitivity for the detection of HPV.

Key words: Cervical cancer, RLA, HPV-PCR, MY09/11, G5/G6.

INTRODUCTION

Cervical carcinoma is the fourth most common cancer of women worldwide which is due to Human papillomavirus infection. Current estimate indicate that 520 000 new cases are diagnosed every year and more than 270 000 deaths occur from this disease, more than 85% of these deaths are reported from less developed regions[1]. These estimates represent 7.5% of all female deaths due to cancer. The majority of cases are squamous cell carcinoma followed by adenocarcinomas[2-5].

Human papillomaviruses (HPV) belongs to DNA viruses whcih infect cutaneous, mucocutaneous and mucosal epithelial cells of human beings which most commonly causes subclinical infections without physical symptoms. Until now, more than 170 HPV genotypes have been identified[6] and these are characterized on the basis of the study of complete genomes, and the data shows that a larger number of unidentified genotypes still exists[7]. Out of this more than 45 types of which gets typically transmitted through sexual contact which infect the anogenital region

(anus and genitals) and mucosal regions[8]. These mucosal types have been further classified into 2 categories as High risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), and low-risk HPV types (6, 11, 40, 42, 43, 44, and 70).[9] It has been proved by several studies that infection with certain types of the human papillomavirus (HPV) is the primary risk factor for cervical cancer and its precursor lesions[10,11] and it has been shown that high risk HPV types are most commonly associated with several malignant diseases, of which cervical cancer is of prime significance. High risk HPV type DNA has been detected in 99.7% of cervical cancer tissue specimens[12], and also the persistent infections with an oncogenic HPV type, particularly HPV type 16 (HPV-16) or HPV-18, are recognized as an important cause of cervical cancer.

Several studies have shown that human papillomavirus (HPV) infection rate is higher in human immunodeficiency virus (HIV) infected women than HIV uninfected women, and these HIV

infected women are also at higher risk for persistent infection and further progression to malignancy[13-16]. In addition to this few studies have shown that HPV may be a cofactor in HIV acquisition[17,18].

Many studies have shown that HPV genotyping is of importance for the study of the natural history of infections with one or multiple HPV types and their role in persistence and progression of cervical lesions and also for the monitoring of success of vaccination and vaccine efficacy.

In developed countries, several screening programmes are in place which enables women to get screened regularly, making most of the pre-cancerous lesions identifiable at early stages which can easily be treated so as it can be prevented in approximately 80% of cases.

In developing countries, due to lack of access to effective screening methods and lack of awareness among the people, these diseases are often not identified until it is further advanced and further symptoms are developed. In addition, this makes prospects for treatment of such late-stage disease poor which results in a higher incidence of death from cervical cancer.

The high mortality rate from cervical cancer globally (52%) could be reduced by effective screening and treatment programmes[19].

Even though HPV infection can be detected by preventive examinations like a Pap smear test, or pathological test by colposcopy directed cervical biopsy, this is often not possible due to its less sensitivity and specificity. However, the presence of HPV can be confirmed with high accuracy in terms of high sensitivity and specificity, by identifying the viral genome present in cervical lesions by molecular methods[20].

Currently there are several techniques available for the molecular diagnosis of HPV, ranging from a conventional Polymerase Chain Reaction (PCR) to complex methods, such as real-time PCR, hybrid capture assay(HC2), cobas HPV test, Roche linear array[21,22]. The PCR technique is still considered the most reliable method for HPV diagnosis due to its simplicity and cost effectiveness as compared to high throughput sequencing tests which are in a way more expensive.

The PCR system commonly uses MY09/MY11 and G5/G6 primers that amplify the L1 region of viral genome. These primers are effective for amplifying a wide spectrum of HPV genotypes in cells obtained from cervical smears. The MY09-MY11 primer set is synthesized with several degenerate nucleotides in each primer and is thus a mixture of 25 primers with approximately 450bp[23,24]. The GP5+-GP6+ primer set consists of a fixed nucleotide sequence for each primer with approximately 150bp and detects a wide range of HPV types by using a lowered annealing temperature during PCR[24]. Several studies indicate that testing for HPV by any single method or technique may not give the true prevalence of HPV in cervical samples[25,26]. This study aimed to investigate HPV infection in HIV infected women with positive PCR-RLA assay results, to verify the performance of MY09/ MY11 and G5/G6 primer sets for HPV detection.

METHODS
Study population
This is a cross-sectional study of 214 HIV-infected women aged from 18- 45 years, attending department of Obstetrics & Gynecology at KLE's Prabhakar Kore hospital and medical research center, Belagavi, India. The study was approved by the human research ethics committee of the KLE University, Belagavi, India in June 2013, and informed consent was obtained from all the study participants after providing the study fact sheet.

Sample collection and methods
Cervical sample were collected from the transformation zone of the cervix using the DNA collection device (cytobrush) which were rinsed in 20ml of PreservCyt® vial (Hologic, Inc.). Samples were immediately stored at 4°C and transported to the laboratory at the department of Microbiology where they were further stored at -20°C until DNA extraction. Simultaneously, data collection, clinical examination, Papanicolaou smear (Pap-smear) sampling and colposcopy were performed during the same procedure whenever required.

Sample processing for DNA Extraction
DNA extraction of the cervical specimens was carried out at National Aids Research Institute (NARI), Pune. Cervical lavage samples which were collected in PreservCyt® and stored at -20°C were processed in a biosafety cabinet in a laboratory physically separated from where the PCR amplification was performed. DNA of both HPV and cellular, was released by lysing cervical cell specimens using lysis buffer supplied with the Roche Amplicor HPV kit under denaturing conditions at elevated temperatures in the presence of proteinase K followed by DNA purification in columns with a silica-based membrane using vacuum processing.

DNA Amplification
All the pre and post DNA extraction processes were carried out in a separate rooms and cabinets at National Aids Research Institute (NARI), Pune which are at different spaces so as to avoid further errors due to contamination. Blank controls were included during the extraction process to monitor contamination and errors.

1.   Linear array HPV (RLA)
All the extracted DNA samples were subjected to linear array genotyping assay (Roche Diagnostics, Indianapolis, IN, USA) for amplification and further identification of the genotype. The Linear array genotyping assay is based on PCR amplification of target DNA using HPV primers, hybridization of the amplified product to oligonucleotide probes and their detection by colorimetric reaction. Particularly, the master mix contains primers for the amplification of a 450-bp fragment of the L1 region of more than 37 HPV genotypes and a 268-bp fragment of the human β globin gene. The detection and genotype determination was performed using the denaturated amplified DNA and an array of oligonucleotide probes, located in the polymorphic region of L1 that permitted independent identification of individual HPV genotypes. Negative samples were included in each assay to establish the specificity of the test.

2.   Sample selection for testing with two different primers
Among the 214 women who attended Obstetrics & Gynecology clinic 33 samples yielded positive results in Linear array genotype assay and these samples were selected to test for HPV-DNA by PCR using the different primers MY09/11 and G5/G6.

3.   DNA Amplification using MY09/11 and G5/G6 primers
Two different pairs of oligonucleotides primers were used in this study to detect HPV in cervical samples that is MY09/MY11, G5/G6. In order to standardize the amount of material used equal volumes of DNA extract were used for PCR with two different primers. PCR reactions for both the primers were carried out separately by following the standardized protocol and also the manufacturer's instructions.

The PCR amplification was carried out separately using MY09/11 and G5/G6 primers to amplify L1-region of HPV genome. In one set of PCR, Consensus primers MY09/11 were used, in a mix containing Taq DNA Polymerase Master Mix (QIAGEN®), and 10 pmol of primers PC04/GH20 for the simultaneous amplification of a 248bp product of the human β-globin housekeeping gene. Amplifications were performed with an initial denaturation step of 15min at 95°C, followed by 40 cycles (1 min of denaturation at 94°C, annealing at 55°C/1 min and an extension step at 72°C/5 min) and a final extension of 72°C/10 min.

In another set of PCR, G5/G6 primers were used, in a mix containing Taq DNA Polymerase Master Mix (QIAGEN®), and 10 pmol of primers PC04/GH20 for the simultaneous amplification of a 248bp product of the human β-globin housekeeping gene. Commercially available positive controls were used during the PCR assay. Amplifications were performed with an initial denaturation step of 15min at 95°C, followed by 40 cycles (4°C/1 min, 55°C/1 min and 72°C/1 min) with a final extension of 72°C/5

min. Commercially available positive controls and water as a negative control for both the PCR reactions. After amplification the products were then visualized on 2% agarose gels containing 10 µL of ethidium bromide/100 mL agarose, under UV light.

Table 1: General primers sequences for HPV DNA detection

Primer	Sequences* (5'-3')	Size (bp)
MY11	GCMCAGGGWCATAAYAATGG	~ 450
MY 09	CGTCCMARRGGAWACTGATC	
G5	TTTGTTACTGTGGTAGATAC	~ 150
G6	GAAAAATAAACTGTAAATCA	

STATISTICAL ANALYSIS

Mc Nemar test was performed to assess the difference between MY09/11 and G5/G6 primer sers with positivity of HPV DNA. The data were analysed by using SPSS software (Chicago, IL, USA), 20.0 version. The statistical significance was set at 5% level (p <0.05).

Table 2: Percentage of DNA of HPV detection with oligonucleotide primers pairs

HPV DNA detection	MY09/ 11 (n=33)	G5/ G6 (n=33)	P value
Negative	12	09	0.2500
Positive	21	24	

Table 3: Comparison of G5/G6 and MY09/11 in assessment of HPV status

	Results	G5/G6			
		Positive	Negative	Total	%
	Positive	21	0	21	63.64
MY09/11	**Negative**	3	9	12	36.36
	Total	24	9	33	100.00
	%	72.73	27.27	100.00	
	Mc. Nemar, p=0.2500, NS				

Table 4: Sensitivity and specificity

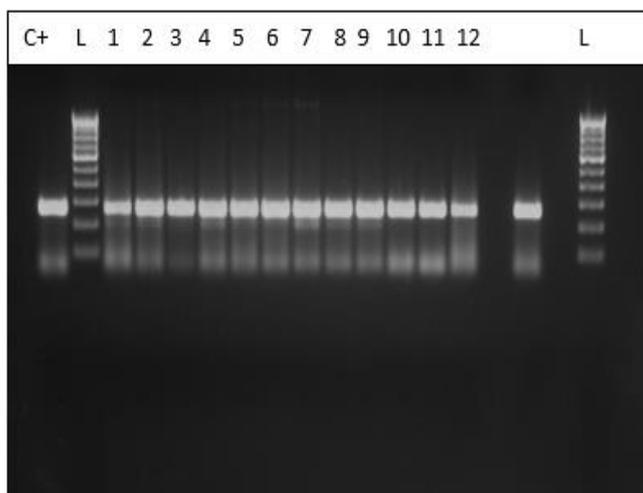|	
Sensitivity	87.50
Specificity	100.00
Positive predictive value	100.00
Negative predictive value	75.00
Disease prevalence	0.73

Figure 1: Agarose gel stained with blue green loading dye showing PCR electrophoretic profile using PCO3/PCO4 oligonucleotide primers for human ß-globin gene detection corresponding to ~110 pb
PCR: polymerase chain reaction; HPV: human papillomavirus; L: ladder;
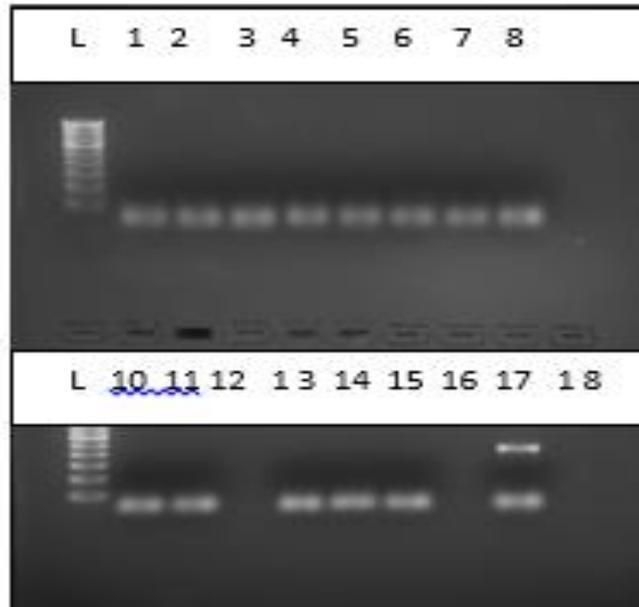**C+: positive control; C-: negative control.**

Figure 2: Agarose gel stained with blue green loading dye showing PCR electrophoretic profile using MY09/11 oligonucleotide primers for HPV DNA detection corresponding to ~450 pb
PCR: polymerase chain reaction; HPV: human papillomavirus; L: ladder;
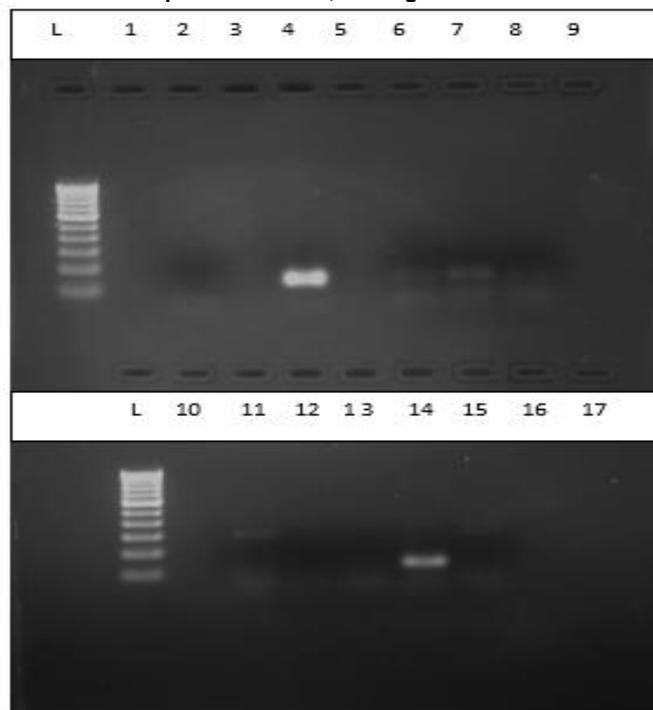**C+: positive control; C-: negative control.**

Figure 3: Agarose gel (1,5%) stained with blue green loading dye showing PCR electrophoretic profile using G5/G6 oligonucleotide primers for HPV-DNA detection corresponding to ~150 pb
PCR: polymerase chain reaction; HPV: human papillomavirus; L: ladder;
C+: positive control; C-: negative control.

RESULTS

All 214 samples were amplified for HPV DNA and genotypes were identified by Linear array genotype assay (Roche Diagnostics, Indianapolis, IN, USA), which yielded high risk HPV genotypes in 33 samples.

Thirty three samples which were positive in Linear array genotype assay were evaluated by using two different oligonucleotide primers MY09/11 and G5/G6 by HPV-DNA analysis by PCR. HPV DNA was detected in 63.7% (21 of 33) and 72.73% (24 of 33) of the samples by using the MY-PCR and the G5/G6 -PCR primer

sets, respectively. Among the 24 HPV-positive samples, 21 (63.7%) were detected by one or both methods and 3 (9.09%) were detected only by G5/G6 primer set. There was good agreement between the two PCR systems in the detection of HPV DNA. There was no statistical significance between the two oligonucleotides primers used in PCRs. The sensitivity and specificity in the detection of HPV DNA between two primer sets was found to be 87.50% and 100% respectively. The positive and negative predictive values are respectively 100% and 75%.
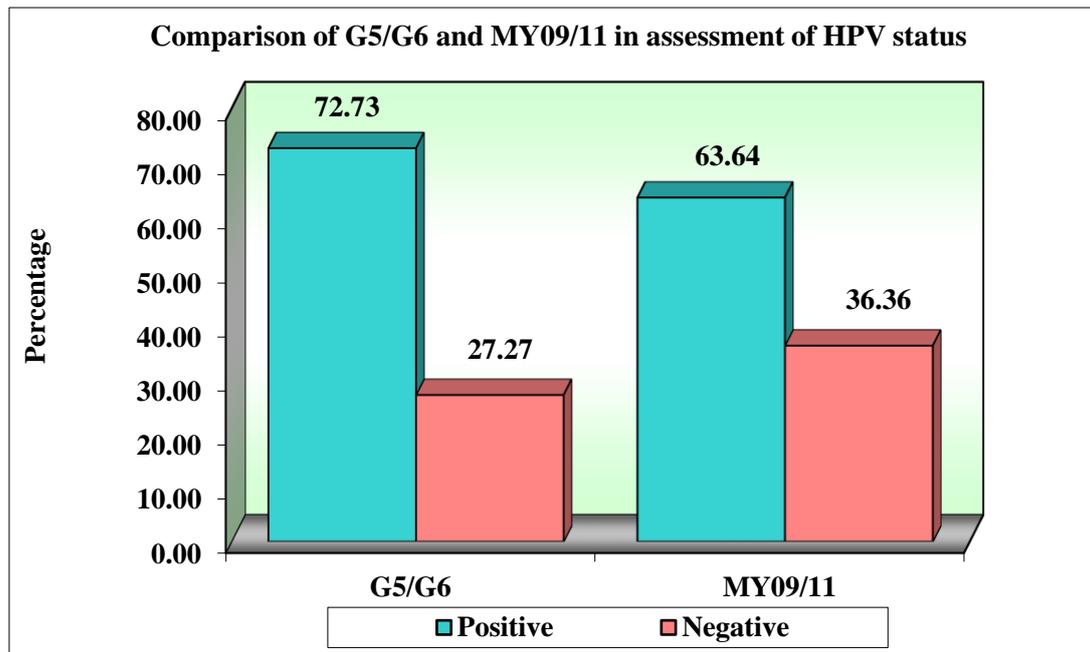
Figure 4: Comparison of G5/G6 and MY09/11 in assessment of HP status

DISCUSSION

Now a days PCR is most commonly used in clinical, research and diagnostic laboratories to detect HPV infections. For the L1 region, two oligonucleotides primers systems are commonly used: MY09/MY11 and G5/G6. Both of these primers detect a wide range of HPV DNA. Degenerated MY09/MY11 oligonucleotide primer uses annealing high temperature (55ºC) and can amplify multiple HPV infections.

However, G5/G6 oligonucleotide primer has lower annealing temperature (42ºC) compared to MY09/11 primer, allowing the best amplification by single genotype HPV infections[23].

In our study we have compared the two major primer sets used for PCR amplification for HPV DNA in a group of positive samples obtained from Liner array assay results from cervicovaginal samples obtained from HIV infected women.

Each PCR method showed a high level of reproducibility when compared to itself, as previously demonstrated with the MY09/11 and G5/G6 assays in different laboratories[27].

In our study we observed a greater number of positive samples using G5/G6 oligonucleotides, when compared to MY09/MY11. However, there was no statistical difference between the two oligonucleotides used in PCRs (p = 0.2500) but clinical significance is seen since G5/G6 primer set detected 3 more samples as positive compared to 21 from MY09/11 primer set. Our results were similar to walboomers study and Remmerbach studies[11,28].

According to Poljak et al.[29] and Vince et al.[30], one cannot rule out the possibility of false negative results in PCR due to low concentration of DNA in the clinical samples and the also due to the presence of PCR endogenous inhibitors or from faulty sample processing.

In a study by walboomers[11], they compared the sensitivity of these oligonucleotides primers for PCR in cervical biopsies where the authors demonstrated that the two oligonucleotides primers used were equally sensitive, with a correlation of 98%. Using G5/G6, the authors found HPV positivity of 95% with MY09/MY11 of 94%. Qu et al.[23], compared the detection rates of the same oligonucleotides primers used in our experiment (MY09/MY11 and G5/G6) in cervicovaginal washing sample, they found DNA of HPV in 45.5% and 42.8%, respectively, with no statistical difference between the two oligonucleotides used in PCRs, only in the evaluation of HPV types detected by each pair of oligonucleotide, result similar to ours, which also showed no statistical difference.

Remmerbach et al.[28], through oral mucosal smears analysis of patients suspected of carcinoma lesions, reported that HPV detection with G5/G6 oligonucleotides was higher than MY09/MY11 oligonucleotides.

CONCLUSION

Both MY09/MY11 and GP5/GP6 primer sets amplified a wide spectrum of HPV genotypes in cervicovaginal samples and detected similar results of HPV positivity but since G5/G6 primers detected additional 3 samples as positive our study suggests that more than one type of oligonucleotide primer should be used in clinical samples to increase the sensitivity in HPV detection.

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