RESEARCH NOTE

ESTABLISHMENT OF A MOLECULAR DIAGNOSTIC SYSTEM FOR DETECTING HUMAN PAPILLOMAVIRUS IN CLINICAL SAMPLES IN SRI LANKA

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Abstract. Human papillomavirus (HPV) is associated with variety of clinical conditions that range from innocuous lesions to cancer in both men and women. Consensus primer-mediated PCR assays have enabled screening for a broad spectrum of HPV types. We have established a molecular diagnostic system for detecting HPV DNA in clinical samples from STD clinics in Sri Lanka and compared the efficacy of three different primer sets, MY09/11, GP5+/6+ and CPI/IIG primer sets, to determine which primer set or combination of primers is most efficacious in screening for HPV. Cervical and urethral swabs were obtained from 51 patients who were suspected of having HPV. The presence of HPV DNA in swabs was detected by MY09/11 PCR (33%), GP5+/6+ PCR (72%) and CPI/IIG PCR (57%) primers. HPV DNA was detected in 23% of samples by all three methods, in 43% by any two methods, and in 6% only by GP5+/6+ primer set. GP5+/6+ PCR alone was capable of detecting the most number of HPV positives but, any single PCR method for the detection of HPV may underestimate the true prevalence of HPV in clinical samples. Nested PCR assay with MY09/11 and GP5+/6+ primer sets had higher sensitivity than singleplex PCR but, due to the risk of cross contamination in employing nested PCR, it was concluded that GP5+/6+ PCR is more suitable for HPV DNA detection in epidemiologic and clinical follow-up studies in Sri Lanka.

Keywords: human papillomavirus, molecular diagnosis, nested PCR, Sri Lanka

INTRODUCTION

Human papillomavirus (HPV) is the most prevalent sexually transmitted infection (STI) and is very common among young men and women in many parts of the world (Bosch *et al*, 1995; Qu *et al*, 1997; Furmoto and Irahara, 2002; Burd, 2003). More than 200 types of HPV have been recognized and 85 HPV genotypes have been characterized (Burd, 2003). Forty HPV types are spread through sexual contact and infect the cervix, vagina, vulva, penis and anus (Malloy *et al*, 2000; Szostek *et al*, 2006). High risk HPV can cause lesions leading to cancer; however low risk HPV produces lesions that do not evolve

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to cancer (Novaes *et al*, 2006).

The prevalence of genital HPV infection in the world is around 440 million (Epidemiological Unit, 2007). Cervical cancer accounts for 25.4% of all cancers in Sri Lankan women (Jordens *et al*, 2000). Worldwide, it is a common cancer of women, being second only to breast cancer (Garland and Tabrizi, 2006; Epidemiological Unit, 2007) and is known to claim more than 400,000 new victims each year (Hwang *et al*, 1995). In developing countries, cervical cancer is often the most common cancer and may constitute up to 25% of all female cancers (Harro *et al*, 2001). Estimates of the number of cervical cancer deaths are around 250,000 per year. International Agency for Research on Cancer (IARC) figures show an incidence of 492,000 new cases for 2002 worldwide with 409,000 (83%) occurring in developing countries. The prevalence of genital HPV infection in the world is around 440 million (Epidemiological Unit, 2007). Cervical cancer kills approximately 230,000 women annually, with the vast majority of deaths occurring in developing countries (Malloy *et al*, 2000).

Cervical cancer is the most common female genital cancer in Sri Lanka with 755 cases and an incidence rate of 6.9 per 100,000 population in the year 2000 (Epidemiological Unit, 2007). The incidence in Sri Lanka has greatly increased during the last two decades. Therefore, HPV diagnosis at early stages of the infection is of fundamental importance. A correct diagnosis will help to reduce the incidence of cervical cancer and its complications. The development of methods for simple, rapid and accurate detection HPV has a central role in many strategies designed to reduce the risk of cervical cancer.

The Pap smear test is not efficient and adequate when used as the only method for diagnosis of HPV infection (Burd, 2003). However, it is still the most widely used method for primary screening of cellular abnormalities and some genital infections in most developing countries (Burd, 2003). PCR is a highly specific and sensitive diagnostic tool for the detection of HPV DNA and could be indicative for tracking HPV from cervicovaginal smears (Novaes *et al*, 2006). The sensitivity of PCR is more advantageous in studies relying on less than ample cervical swabs with low viral counts (Schiffman *et al*, 1991). Consensus primer-mediated PCR techniques are used to amplify a broad spectrum of HPV types in clinical specimens simultaneously in a single PCR amplification with high sensitivity and specificity (Hubbard, 2003; Szostek *et al*, 2006). L1 and E1 genes are suitable targets for consensus primers (Karlsen *et al*, 1996). MY09/11 and GP5+/6+ primer sets amplifying DNA fragments in the conserved L1 region have become most widely used in clinical and epidemiological studies and hence are able to detect all mucosal HPV types (Qu *et al*, 1997; Burd, 2003; Garland and Tabrizi, 2006). The CPI/IIG primer set amplifies DNA fragment in the E1 region of a broad spectrum of genital HPVs (Smits *et al*, 1995). The most commonly used method for detection of genital HPVs consists of nested PCR using the MY09/11 and GP5+/GP6+ primer sets (MY/GP+) (Haws *et al*, 2004).

Since there are no molecular diagnostic tests available in Sri Lanka so far to detect HPV, a low cost, reliable, reproducible, rapid, sensitive and specific molecular diagnostic assay was developed in this study for first time in Sri Lanka to provide a routine screening method for detecting HPV in clinical samples. This study also lays the foundation for epidemiological genotyping investigation of HPV in Sri Lanka.

Table 1
Primers used in the study.

Type	Primer	Primer sequence	Position on the genome	Amplicon length (bp)
MY	MY09	5′-CGT CCM ARR GGA WAC TGA TC-3′	7015-7034	450
	MY11	5′-GCM CAG GGW CAT AAY AAT GG-3′	6583-6602	
GP+	GP5+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′	6624-6649	150
	GP6+	5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′	6719-6746	
CP	CPI	5′-TTA TCW TAT GCC CAY TGT ACC AT-3′	1942-1964	188
	CPIIG	5′-ATG TTA ATW SAG CCW CCA AAA TT-3′	1777-1799	
MT	MT1	5′-TGA AGG AGA AGG TGT CTG GGG GA-3′	N/A	198
	MT2	5′- AGG ACG GTG CGG TGA GAG TG-3′	N/A	
TPOX	TPOXF	5′-ACT GGC ACA GAA CAG GCA CTT AGG-3′	N/A	224-252
	TPOXR	5′-GGA GGA ACT GGG AAC CAC ACA GGT TA-3′	N/A	

M, A or C; R, A or G; S, C or G; W, A or T; Y, T or C
MY09/11, GP5+/6+ and CPI/IIG primer sets were used to amplify L1 and E1 regions of HPV genome. MT1/2 and TPOX primers sets were included as internal PCR amplification controls.

MATERIALS AND METHODS

Clinical material

Samples were collected from men and women who were suspected of having HPV infection. The voluntary informed written consents of the participants were obtained as this was a part of a larger study to determine the epidemiology of HPV in Sri Lanka. The Ethical Clearance Committee of the University of Colombo approved the study as a part of the ethical approval process. A total of 51 cervical and urethral (dry) swabs were collected from the sexually transmitted disease (STD) clinic, National Hospital Colombo and Colombo Municipal Council dispensaries, Sri Lanka. Swabs were transported at room temperature provided that they are sent to the Genetech laboratory, Colombo, Sri Lanka within six hours after collection. Otherwise swabs were stored at 4ºC (less than 3 days) and at -70ºC (after 3 days).

PCR amplification and detection of amplicons

HPV DNA from swabs was extracted using guanidium thiocyanate DNA extraction method (Boom *et al*, 1990). HPV DNA was stored at -20ºC until used. Three primer sets (MY09/11, GP5+/6+ and CPI/IIG) were used to amplify L1 and E1 regions of HPV genome (Karlsen *et al,* 1996; Baay *et al*, 1996) (Table 1). MT1/2 and TPOX primers sets were included as internal PCR amplification controls (Table 1).

For amplification of L1 gene, MY primers MY09 and MY11 were used in 25 l solution containing 5.0 l of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.6 mM $MgCl_2$, 0.1 g/ l bovine serum albumin, 0.2 mM (each) dNTP, 10 pmol of each primer mix and 2.5 U of *Taq* polymerase (Promega, Madison, WI). The PCR

thermocycling program consisted of 94ºC for 5 minutes; 40 cycles of 94ºC for 30 seconds, 50ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 10 minutes.

For amplification of L1 gene using GP+ primers GP5+ and GP6+, the above PCR conditions were used, but with the following changes: 2.5 l of DNA, 1.5 mM $MgCl_2$, 12.5 pmol of each primer and PCR thermocycling program consisting of 94ºC for 5 minutes; 35 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 10 minutes.

For amplification of E1 gene using primers CPI and CPIIG, the above PCR conditions were used as with MY primers, but with the following changes: 2.5 l of DNA, 3.0 mM $MgCl_2$, 8.5 pmol of CPI primer, 13 pmol of CPIIG primer and PCR thermocycling program consisting of 94ºC for 5 minutes; 35 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 10 minutes.

Nested PCR was performed with MY09/11 and GP5+/6+ primer sets because the target region of GP5+/6+ primer set is located within the target region of My09/11 primer set on the HPV genome (Table 1). The first round of nested PCR was performed with MY09/11 primer set under the same conditions used for singleplex PCR with MY primers, but with the following changes: 2.5 l of DNA, 1.5 U of *Taq* polymerase (Promega, Madison, WI) and PCR thermocycling program consisting of 94ºC for 5 minutes, 40 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute and a final heating at 72ºC for 10 minutes. Then the second round of nested PCR was performed with the GP5+/6+ primer set under the same conditions used for the singleplex PCR with GP+ primers, but with the following changes: no addition of bovine serum albumin, 2.5 l of 1st round PCR product, 1.5 U of *Taq* polymerase (Promega) and PCR thermocycling program consisting of 94ºC for 2 minutes, 40 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute and a final heating of 72ºC for 10 minutes.

Amplicons (12 l) were applied to 2% agarose gel, and electrophoresed at 100 V for 2-3 hours. Gels were stained with ethidium bromide, and the DNA bands were visualized under UV illumination. A 50 bp DNA molecular weight marker (Promega) was used for estimation of band size.

RESULTS

All samples were positive in the TPOX-PCR (224-252 bp amplicon). Of 51 samples, 37 (72%) were positive for HPV DNA, 17 (33%) with MY09/MY11 primer set, 37 (72%) with GP5+/GP6+ primer set and 29 (57%) with CPI/CPIIG primer set. In 32% of samples HPV DNA was detected by all three methods, 59% of samples by two methods and 8% only by the GP5+/GP6+ primer set (Table 2). The efficiency of the primer pair was inversely correlated to the length of the amplicon: GP5+/GP6+ (150 bp, positivity 72%), CPI/CPIIG (188 bp, positivity 57%) and MY09/MY11 (450 bp, positivity 33%). Fig 1 shows agarose gel-electrophoresis of amplicons.

DISCUSSION

The GP5+/GP6+ primer set was markedly better than the MY09/MY11 and CPI/IIG primer sets at detecting HPV. The number of positive results with CPI/IIG primer set was more than that of MY09/MY11 primer set. More samples were positive with GP5+/GP6+ and CPI/CPIIG primer sets. None of the samples was

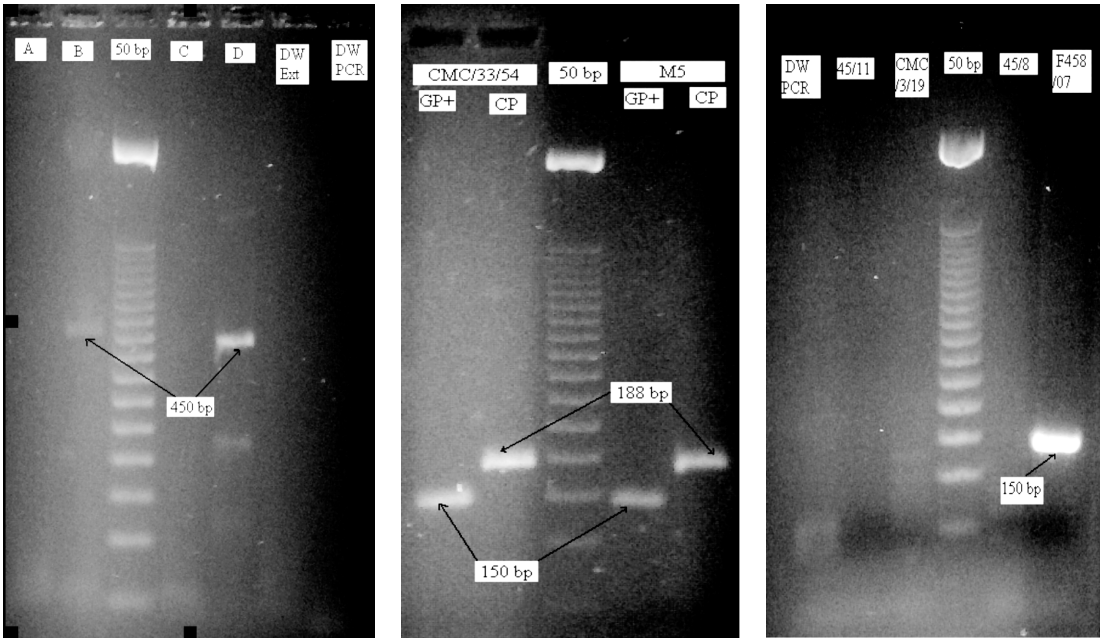

Fig 1–Agarose gel electrophoresis of MY, GP+, CP and nested PCR amplicons. Left panel: HPV DNA was amplified using MY09/11 primer set. Lanes B and D are positive; lanes A and C are negative; lanes DW Ext and DW PCR are negative PCR controls; lane 50 bp contains DNA molecular weight markers. Center panel: HPV DNA was amplified using GP5+/6+ and CPI/IIG primer sets. Lanes CMC 33/54 and M5 are positive for HPV DNA using GP+ and CP primer sets. Lane 50 bp contains DNA molecular weight markers. Right panel: Nested PCR was performed with MY and GP+ primer sets to detect HPV genome. Lane F458/7 is positive; lanes 45/11, 45/8 and CMC/3/19 are negative; lane 50 bp contains DNA molecular weight markers.

found positive with MY09/MY11 primer set but negative with GP5+/GP6+ primer set and none was positive with CPI/CPIIG primer set but negative with GP5+/GP6+ primer set. Twenty samples were positive with GP5+/GP6+ and CPI/IIG primer sets and negative with MY09/MY11 primer set.

Karlsen *et al* (1996) and Szostek *et al* (2006) found variations in the HPV detection rate with these three sets of primers due to differences in specificity of these primer sets. Van den Brule *et al* (1990) and Karlsen *et al* (1996) found differences in HPV positivity among the consensus primers, showing that there may be deletions or mutations of the L1 or E1 templates. Matsukura *et al* (1986) and Wagatsuma *et al* (1990) found deletions in the L1 and E1 genes from cell lines. In this study, 20 samples tested with GP5+/GP6+ primers for L1 were PCR positive, while they were negative with the MY primer set, indicating either a possible deletion/mutation in L1 between the annealing area of the MY primers.

Szostek *et al* (2006) reported that PCR with the MY09/MY11 primer set is a good method for HPV screening. Soltar *et al* (2004) suggested that the nested PCR assay with MY09/MY11 and GP5+/

Table 2
Concordance of HPV positive results obtained by PCR with different sets of primers.

No. of primer sets	Primer set used	No. of HPV (+) samples (%)
Three	MY09/MY11 + GP5+/GP6+ + CPI/CPIIG	12 (32)
Two	MY09/MY11 + GP5+/GP6+	5
	MY09/MY11 + CPI/CPIIG	0 (59)
	CPI/CPIIG + GP5+/GP6+	17
One	MY09/MY11	0
	GP5+/GP6+	3 (8)
	CPI/CPIIG	0

GP6+ primer sets is more sensitive than the conventional PCR with either of these two primer sets. Baay *et al* (1996) suggested that the efficiency of a primer pair is inversely correlated to the length of the amplicon and even the combination of the three most widely used primer sets, MY09/MY11, GP5/GP6 and CPI/CPIIG, still results in an underestimation of HPV prevalence. It is possible that a similar effect occurred in the present study where the maximum number of positive samples was 37 out of 51 samples. This number may be an underestimation.

Infection with multiple HPV types is frequent in many populations (Tozetti *et al*, 2006). The MY09 and MY11 primers are degenerate consensus primers with 8 to 16 variant primers for each strand. For this reason, MY-PCR detects a wide range of HPV DNA types. The primers for GP+-PCR are general primers with one primer for each HPV DNA strand. GP+-PCR amplifies different HPV types by the use of relatively low annealing temperature, a non-stringent condition. Thus, the GP+-PCR general primers may be dominated by the amplification of one or two types of HPV, which cause inaccurate results in the detection of multiple HPV types (Tucker *et al*, 1993). Therefore, it was decided to perform nested PCR with MY09/MY11 and GP5+/GP6+ primer sets in this study.

Qu *et al* (1997) found differential amplification sensitivities for different HPV types between the MY-PCR and GP+-PCR primer systems. The GP+-PCR can amplify femtogram levels of HPV DNA. Differences in the detection of specific HPV types in clinical samples by different PCR systems can result in a potential difference in the ability of the primers to amplify specific types. The most marked differences were seen in the ability of MY-PCR to amplify HPV-35 and GP+-PCR to amplify HPV types 53 and 61. The differences in amplification of HPV types 35, 53 and 61 are generally, but not absolutely, reflected in the extent of primer mismatches. The GP+-PCR primers are relatively inefficient in the amplification of HPV types 53 and 61 compared to MY-PCR, whereas MY-PCR was inefficient in the amplification of HPV-35. This effect may have caused the differences in detection sensitivity among the three primer sets as observed in the present study.

Karlsen *et al* (1996) suggested that the samples positive for HPV but whose PCR products produce faint signals upon stain-

ing might have a low viral load. Strong PCR signals in the gel may indicate a high viral load. In the present study it was also observed that some PCR amplicons gave much fainter bands when stained with ethidium bromide suggesting low viral load in the samples or small sampling amounts.

Different DNA isolation methods, the accuracy of taking of sample size and tissue quality, the amount of DNA in the PCR solution and PCR conditions may also explain the variations in HPV detection rates with different sets of primers (Karlsen *et al*, 1996). Choosing the optimal PCR system for use in clinical and epidemiological studies should take into consideration such factors as the source of the clinical material, the size of the PCR amplicon, the spectrum of HPV types to be amplified, the ability and availability to amplify multiple HPV types and the availability of type-specific probes for the identification of different HPV genotypes and variants (Qu *et al*, 1997). In this study, GP5+/GP6+ primer set alone detected the most number of the positive samples (72%) and this is a potentially useful tool for HPV DNA detection in epidemiologic and clinical follow-up studies in Sri Lanka.

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