ORIGINAL ARTICLE

Evaluation of Polymerase Chain Reaction (PCR) Method and Hybrid Capture II (HCII) Assay for the Detection of Human Papillomavirus in Cervical Scrapings

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SUMMARY

In order to investigate the reliability of detecting HPV DNA in cervical smears, we compared the performance of nested MY/GP PCR and FDA approved-Hybrid Capture II (HCII) using clinical cervical scrapings from 40 patients. It was found that PCR was more sensitive (81.8%) in comparison to HCII (36.4%) in detecting HPV although specificity of HCII was much higher (96.6%) than PCR (58.6%). The Negative Predictive Value (NPV) of both the techniques were quite similar but Positive Predictive Value (PPV) of HCII was much higher (80.0%) compared to PCR (42.9%). While the HCII method showed good specificity for HPV detection, its lack of sensitivity as compared to PCR may be a drawback for diagnostic use.

KEY WORDS:
PCR, Hybrid Capture II, HPV

INTRODUCTION

Cervical cancer remains the second most prevalent female cancer worldwide that kills more than 250,000 women around the world each year[1]. Human Papillomavirus (HPV) infection is the main cause of most cervical cancers and cervical intraepithelial neoplasias (CIN) worldwide[2,3]. For many years, cytology has been the gold standard test for cervical cancer screening. Cytological examination can only be carried out by pathologists and are liable for observer bias usually showing variable (poor to moderate) sensitivities [4]. Moreover, the Pap test is only suggestive of viral infection and is not a conclusive test for detecting HPV.

The essential component of any diagnostic test includes good sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Lately new molecular based methods for accurate detection of HPV have come into the market. These methods are said to have high degree of sensitivity and specificity. One of these include Hybrid Capture II (HCII) assay produced by Digene, which is United States Food and Drug Administration (USFDA) approved-commercially available kit. HCII assay is a nonradioactive, immuno-chemiluminescence method that is based on the hybridization of genotype specific-RNA probes to the HPVs genomic sequence.

Polymerase chain reaction (PCR) has been shown to be a very sensitive method for identifying HPV infection in clinical samples[5,6]. A number of different primer combinations amplifying DNA fragments from various regions of the HPV genome have been developed and used for the detection of HPV [7]. However, primers amplifying DNA fragments in the conserved L1 region are most widely used in clinical and epidemiological studies. These include MY09/MY11 primers (MY-PCR)[8] and the GP5+/GP6+ primers[9]. The first degenerate outer MY primer set amplifies approximately 450 bp within the HPV L1 structural gene[8] while the internal GP primers generate an approximately 140 bp long fragment from the HPV L1 region within the sequence amplified by the outer primer pair [9]. Therefore they can be used either as single primers or in the nested PCR after amplification with the MY primers [10]. Few studies suggest the application of nested PCR assay using MY9/11 primers and in second round semi-nested with MY11/GP6 increases the sensitivity approximately up to one log step [11]. This preliminary study was aimed to assess the performance of FDA approved-Hybrid Capture II (HCII) assay and Polymerase Chain Reaction for the detection of HPV DNA in clinical samples.

MATERIALS AND METHODS

Cervical scrapings from the patients were collected from Obstetrics and Gynecology clinics of Hospital Universiti Sains Malaysia, Hospital Kota Bahru and Hospital Kuala Terengganu (n = 40). Pap smear preparation was performed and evaluation was performed by pathologists based on Bethesda reporting system 2001 (TBS 2001). These results were taken as the gold standard in comparing the two molecular techniques.

Hybrid Capture II

Residual swabs were kept at 4°C in ThinPrep ®Test bottles. HPV DNA testing by the HCII assay method was performed with the automated HCII Assay system located in the Department of Pathology, Universiti Sains Malaysia, according to the protocol of the manufacturer. The samples were analyzed for the presence of High-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HPV type 16 DNA (1 pg/ml) was used as a positive control. Samples were classified as High-risk HPV DNA positive if the relative light

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unit (RLU) reading obtained from the luminometer was equal to or greater than the mean value for the positive control.

PCR

For PCR method, DNA extraction was performed by conventional phenol/chloroform method. The integrity of the extracted DNA was then checked by beta globin primers which were as follows:

B- GloRV 5' GAA GAG CCA AGG ACA GGT AC' 3
B- GloFW 5' CAA CTT CAT CCA CGT TCA CC'3

Plasmids for HPV genotypes 6, 11, 16, 18 were used as positive controls. These were obtained from Prof E.M deVilliers (Deutsches Krebsforschungszentrum, Heidelberg, Germany)

PCR Conditions

Two separate PCRs were performed. The first PCR directed at the HPV L1 region was performed using the MY09/MY11 outer primers, producing an amplicon of 450 bp[8]. The MY09/11-PCR was performed in 20 µl total reaction volume containing 1.5 mM $MgCl_2$, 200 µM, dNTPs, 10 pmole each of MY09 and MY11 primers, 1U of Taq polymerase and 5 µl of DNA template. Each PCR was carried out in DNA thermal cycler (Eppendorft Mastergradient Cycler) with first denaturation step at 95°C for 3 min and final extension step at 72°C for 7 min. Three steps of denaturation at 95°C for 30s, annealing at 53°C for 30s and extension at 72°C for 30s were repeated for 45 cycles. This was followed by GP5+/GP6+ PCR, producing an amplicon of 150 bp[9]. The GP5+/6+ PCR was performed in 20 µl total reaction volume containing 3.0 mM MgCl2, 200 µM each of dNTPs, 10 pmole each of GP5+ and GP6+ primers, 1U of Taq polymerase and 1 µl of DNA template which was obtained from the product of first PCR. The DNA amplification was carried out during 45 cycles with all parameters being same as MY09/11 PCR except the annealing temperature that was 42.3°C in this case.

RESULTS

The concordance of HPV detection results for the 40 study subjects, using cytology, PCR and HCII Assay are depicted in Table I, Table II and Table III.

Table I shows that seven samples negative with HCII were detected positive for viral changes by cytology. Only five samples (12.5%) were positive by HCII while 11 samples (27.5%) were suggestive of viral infection with cytology.

Figure 1 shows the result of PCR of Sample DNA. The amplicon size were compared with a 100 bp DNA ladder. An amplicon size of 450bp as seen in lane 2, 3, 4, 5 and 7 indicates a positive amplification of MY product and 140bp seen in all the lanes indicates a positive amplification of GP product.

Table II shows that more samples were positive by PCR (52.5%), as compared to cytology (27.5%). Twelve samples negative for any viral changes by cytology came out to be HPV positive by PCR. In total 19 samples (47.5%) were negative by PCR while 29 samples (72.5%) were negative for any cytopathological abnormalities suggestive of viral infection.

Table III compares the results of HCII Assay with PCR. Only five samples (12.5%) were positive by HCII while 21 samples (52.5%) were positive by PCR that included 17 samples, which were detected negative by HCII. In total, 19 samples (47.5%) were negative by PCR while 35 samples (87.5%) were negative by HCII.

The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) for PCR and HCII assay in comparison to cytology as the gold standard is displayed in Table IV.

Table I: HCII Vs Cytology

		Cytology		
		POS	NEG	TOTAL
HCII Assay	POS	4	1	5
	NEG	7	28	35
	TOTAL	11	29	40

Table II: PCR vs Cytology

		Cytology		
		POS	NEG	TOTAL
PCR	POS	9	12	21
	NEG	2	17	19
	TOTAL	11	29	40

Table III: PCR vs HCII Assay

		HCII Assay		
		POS	NEG	TOTAL
PCR	POS	4	17	21
	NEG	1	18	19
	TOTAL	5	35	40

Table IV: Evaluation of HCII Assay and PCR

	HCII Assay	PCR
Sensitivity	36.4%	81.8%
Specificity	96.6%	58.6%
Negative Predictive Value (NPV)	80.0%	89.5%
Positive Predictive Value (PPV)	80.0%	42.9%

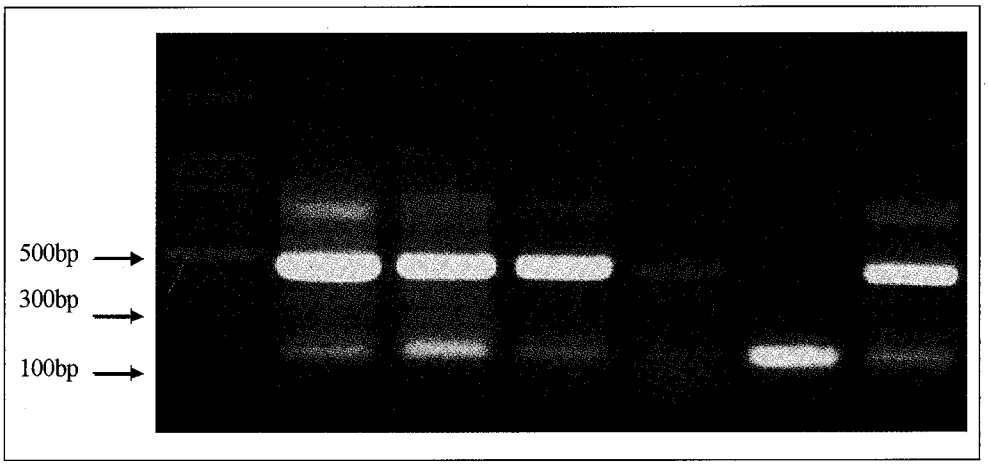

Fig. 1: PCR result of Sample DNA. Amplicon size of 450bp indicates a positive amplification of MY product and 140bp indicates a positive amplification of GP product.

DISCUSSION

About 3% of all women have been reported to have a morphological cervical lesion detectable by biopsy or cytology. Most of these are low-grade, 1.5% having high-grade pre-cancer and <0.1% having cancer [12]. High-risk HPVs have been identified in over 99% of a large series of cervical cancers collected and are the single most important risk factor for cervical cancer and its precursors [3]. High-risk HPV testing has been proposed as a method of identifying women with mild or borderline smear from the cervix uteri abnormalities screening programmes and in addition to cytology [13]. During the past 10 years, PCR has evolved as an imperative technique in HPV diagnostics. The recognized disadvantages of PCR are its extremely high analytical sensitivity and potential for contamination, leading to false-positive results. Commercially available HCII is widely used in routine analysis of cervical scrapings but it does not allow typing of viruses.

In our study, sensitivity of HCII was found to be low (36.4%) as compared to PCR (81.8%). This could have been because HCII identifies only High risk HPVs while PCR using degenerate primers can detect wide range of high risk and low risk HPVs. In one study, 200 specimens that were found negative by HCII, yielded 12.0% HPV DNA-positive results by nested PCR showing PCR to be more sensitive than HCII [14]. However, many studies have found HCII and PCR almost similar in sensitivity. In one study, the sensitivities for cervical intraepithelial neoplasia (CIN) III in cytology were 100.0% by both methods, and for CIN II, sensitivities were 80.0% by both methods. Similar results of comparable sensitivities with these methods have been reported in other studies as well [15-17].

We found an overall kappa value of 0.133 (95% confidence intervals, CI) of these two HPV DNA detection tests and the results were not statistically significant (p= 0.188). Nonogaki *et al* found a kappa of .733 (95% CI) with PCR performed using PGMY 09/11 L1 consensus primers[16]. Soderlund-Strand *et al* also found substantial agreement between the HCII and PCR-EIA (enzyme immunosorbent assay) (kappa, 0.70 before treatment and 0.72 after treatment)[18].

Overall, PCR identified more positive specimens compared to cytology. This result may be due to the high sensitivity of PCR in detecting DNA compared to any histology-based detection. Cases that were suggestive of viral infection by cytology but eventually turned out to be negative by either PCR or HCII assay could be due to the fact that cytology is an observer-biased method and the results are based entirely on subjective interpretation. Moreover, as cytology cannot identify HPV in particular, the features suggestive of viral infection could also have been due to other viruses e.g. HSV, which were not detected by either HCII or PCR. Bozzetti et al however found PCR and HCII results highly associated with cytology ($P < 0.0001$) [15].

Though sensitivity of HCII was found to be low, we found its specificity to be very high (96.6%) in comparison to PCR (58.6%). This was in contrast with another study where the specificities of these two methods were found to be quite similar[18]. In this study, the specificities for CIN II in the pretreatment cases were found to be 30.4% for PCR-EIA and 24.1% for HCII. The specificities for CIN II in the post treatment setting were 83.5% for PCR and 85.4% for HCII [18].

The negative predictive value for HCII was found to be a little lesser (80.0%) as compared to PCR (89.5%). In another study,

the HCII assay for HPV was found to be highly sensitive assay with a negative predictive value exceeding 99% [19]. In comparison, we found the positive predictive value of HCII (80%) to be much higher than PCR (42.9%).

CONCLUSION

In conclusion, it was found that HC II had much lower sensitivity than PCR but this was compensated by high level of specificity. In addition, these molecular methods had comparable negative predictive values though positive predictive value of HC II was much higher than PCR. Thus if false positive results obtained by PCR can be kept to minimum by following stringent laboratory procedures, PCR can be used as an ideal method for detecting HPV from clinical samples.

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