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Relationship between Hybrid Capture II Ratios and DNA Amplification of E1, E6 and L1 Genes Used for the Detection of Human Papillomavirus in Samples with Different Cytological Findings

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SUMMARY The hybrid capture II (HCII) assay is widely used in the detection of human papillomavirus virus (HPV). However, due to the limited number of HPV genotypes, it does not permit a comprehensive typing of viruses and "grey zone" (borderline negative or positive results) are often difficult to interpret. As such, polymerase chain reaction (PCR) should be used in parallel with HCII assays, and consensus PCR detection is capable of covering a wider detection range than with the HCII method. We examined the relationship between HCII relative light unit/cut-off (RLU/CO) ratios and PCR amplification results. This was done using previously described primer sets (MY/GP) as well as with our primers for HPV E1, L1 and E6 gene amplification, and performed on samples exhibiting different cytological findings**.** Together, 243 samples were divided into three groups having RLU/CO ratios of < 0.4 (n = 21), 0.4-4 (n = 64) and ≥ 4 (n = 158), respectively. All samples were subjected to PCR amplification using MY/GP and the newly designed E1, L1 and E6 primers. Results were verified by direct sequencing. PCR amplification sensitivities were higher when using the E1 primers than for the MY/GP, E6 or L1 primers. The E1 assay can be used for HPV detection with a sensitivity of 10^2 copies μl^{-1}. Samples with RLU/CO ratios exceeding 4, and grey zone samples of 0.4-4, were amplified using E1 primers in 79.74% and 26.56% of the total cases, respectively. Cytological data of grey zone samples were primarily found to be normal (77%) whereas those with RLU/CO ratios > 4 were found in any of the cytological data categories. We concluded that HPV screening by HCII for grey zone samples should be analyzed together with cytological data, as well as with a PCR screening tool that incorporates the E1 primers.

Cervical cancer is the most common cancer in women worldwide. A major risk factor in the development of this disease is infection with the human papillomavirus (HPV) and thus the biology and epidemiology of HPV infection has become a major area of research. HPV is a highly variable, non-enveloped, icosahedral DNA virus which replicates in the nucleus of squamous epithelial cells. Due to an inadequate availability of HPV screening tests and a high persistence of the virus, infections are often not diagnosed until later stages which can render therapy futile.

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Many millions of women are diagnosed each year with HPV. An aggressive management approach cannot be justified because almost all symptoms disappear without treatment.[1] However, these abnormalities cannot be ignored since most pre-cancers and cancers are diagnosed in women with minor abnormal cytological findings.[2] Cytological abnormalities are a less sensitive benchmark for HPV detection than molecular testing. Several molecular methods have been developed in order to identify HPV in liquid-base cytology (LBC) samples and tissue samples.[3,4] Molecular techniques that can be utilised for HPV DNA detection[5] include direct probe methods including Southern blotting and *in situ* hybridization, signal amplification methods such as hybrid capture II (HCII) assays,[6] and target amplification methods by PCR.[7] These are time-consuming and typically require large amounts of highly purified DNA. The HCII test has been designed to detect 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low risk HPV genotypes (6, 11, 42, 43 and 44).[8] However, it is incapable of identifying specific HPV genotypes. For this purpose, target products amplified by PCR must be subjected to DNA sequencing analysis,[9] RFLP analysis,[10] or hybridization with type-specific probes.[11-13] Results from samples acquired from patients who have contracted multiple infections may be difficult to interpret by RFLP and direct sequencing. Sub-cloning is an essential step taken prior to cycle sequencing. Hybridization assays are sensitive, but are also laborious and require downstream confirmation experimentation.

A commercially available HCII assay is widely used for HPV detection and routine screening. Due to the limited number of genotypes included in the hybridization probe mixtures, it does not allow for comprehensive typing of viruses. Furthermore, previous work has indicated that one crucial detail of the assay constitutes a "grey zone" between borderline negative and positive results which hampers the interpretation of the results. According to the manufacturer's guidelines, relative light unit/cut-off (RLU/CO) ratios that are $\geq$ 1 indicate a positive result (RLU/CO is 1.00 equivalent to 1 pg ml^{-1} HPV DNA), while a ratio that is below 1 is interpreted as a negative result. The FDA-approved cutoff for a positive test is 1.0 pg HPV DNA ml^{-1}, which corresponds to approximately 5,000 HPV DNA copies within the HCII assay. However, a previous study has shown that results that are between borderline negative and positive (grey zone) results are difficult to interpret.[14] Other research suggests that the grey zone or RLU/CO should encompass a range between 0.4 and 4.[8] These authors recommend that another method or combination of methods (including PCR) should be used concomitantly with HCII. Indeed, consensus PCR covers a broader range of detection values than HCII. The most widely published HPV-testing studies have been performed using PCR primer sets named MY09/MY11 and GP5+/GP6+.

The aim of the present study is to determine the relationship between HCII ratios (RLU/CO) and the results obtained by PCR amplification using previously described primer sets (MY09/MY11 and GP5+/GP6+) and of the E1, E6 and L1 genes, all of which will be conducted on samples that have shown a variety of different cytological findings. HCII is widely used in Thailand at present, since few laboratories are equipped for molecular testing by PCR. Therefore, this study describes the analysis of HPV data that is obtained using available test kits and compared with PCR approaches, in an attempt to provide insights into improved future HPV screening and detection methodologies.

MATERIAL AND METHODS

Sample collection

Female Thai patients from the Samitivej Srinakharin Hospital, Thailand and who reside in Bangkok province were included in the study. Samples were selected from specimens that had been obtained during routine check-ups, screening or investigation, and patients undergoing treatment for cervical cancer or other gynecological problems. All specimens were collected for cytology by LBC (ThinPrep®, Hologic, West Sussex, UK) and tested for HPV DNA using the Hybrid capture II assay (Digene, Gaithersburg, USA). All HPV positive samples were stored at -70^{o}C until required for PCR studies.

Permission was obtained from the Director of the hospital for the specimen use. These samples remained anonymous and were labeled with coding numbers. The Ethics Committee of the King Chulalongkorn Memorial Hospital and Faculty of Medicine

at Chulalongkorn University approved all study protocols.

DNA extraction and amplification

DNA was extracted using phenol-chloroform as previously reported.[15] All samples were subjected to HPV DNA amplification using the primer sets employed for large scale HPV testing (MY09/MY11), the primer set specific for the L1 gene as described in a previous publication (GP5+/GP6+)[16-18] and our specific primer sets for E1, L1 and E6 (Table 1).

HPV detection and PCR

PCR was conducted using the specific primer sets shown in Table 1. The MY/GP primer sets were used to amplify the L1 gene, while our primer sets were employed for L1, E1 and E6 gene amplification. The reaction mixture consisted of 2 µl of DNA template, 0.5 µmol of each primer, 10 µl of 2.5x MasterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reactions used in the first and second round were performed in a Eppendorf Thermocycler (Hamburg, Germany) under the following conditions (with the exception of L1 amplification using MY/GP primers, which was performed as previously reported[16-18]): initial denaturation at 94ºC for 3 minutes, followed by 40 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 45 seconds, and extension at 72°C for 80 seconds, with a final extension step at 72°C for 7 minutes. The PCR products sizes are indicated in Table 1. All amplicons were verified by direct DNA sequencing.

Housekeeping gene detection

β-globin served as an internal control for DNA extraction, using conventional PCR as a detection method. The primer sequences for the β-globin gene have been previously described.[19]

Agarose gel electrophoresis

The PCR products were mixed with loading buffer and electrophoresed on 2% agarose gels (FMC Bioproducts, Rockland, ME) at 100 V for 60 min-

Table 1 Primers specific for E6, E1 and L1 used for DNA amplification and sequencing of HR-HPV

Gene	Round	Primer name	Sequence (5'-3')	[a]Position	Product (bp)
E6	1	HPV-E6F1_27	AAA ACT AAG GGC GTV ACC GAA A	20-41	919
		HPV-E6R1_1019	CAC TAC AGC CTC HAC NDN AAA CCA	916-939	
	2	HPV-E6F1_27	AAA ACT AAG GGC GTV ACC GAA A	20-41	650, 707
		HPV-E6/E7R2_729	CAT CCT CMT CNT CTG AGC TGT	650-670	
		HPV-E6/E7R2_786	TGG TTC GGC YCG TCK GGC T	708-727	
E1	1	HPV-E1F1_1219	AGT ACA GGT TCT AAA ACG AAA GT	1110-1132	855
		HPV-E1R1_2119	CAT TAT CAA ATG CCC AYT GYA CCA T	1941-1965	
	2	HPV-E1F2_1383	GCGAAGACAGCGGNTATGGC	1249-1268	716
		HPV-E1R1_2119	CAT TAT CAA ATG CCC AYT GYA CCA T	1941-1965	
L1	1	HPV-L1F1_6153	CGT TTT CCA TAT TTT TTT HCA GAT G	5615-5639	1,008
		HPV-L1R1_7162	TAG TTG GTT ACC CCA ACA AAT RCC ATT	6596-6623	
	2	HPV-L1F1_6153	CGT TTT CCA TAT TTT TTT HCA GAT G	5615-5639	630
		HPV-L1R2_6804	AGT ATC TAC CAT ATC MCC ATC TT	6223-6245	
[b]MY	1	MY11	GCM CAG GGW CTA TAA YAA TGG	6582-6601	451
		MY09	CGT CCM ARR GGA WAC TGA TC	7014-7033	
[b]GP	2	GP5+	TTT GTT ACT GTG GTA GAT ACT AC	6624-6641	136
		GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C	6741-6760	

[a]Position based on reference sequence NC_001526
[b]Primer based on reference data (Manos *et al.* 1989; Evander *et al.* 1992; Jacobs *et al.* 1995)

utes. Following electrophoresis, DNA was visualized by ethidium bromide staining and UV transillumination (Gel Doc 1000, BIO-RAD, CA).

Preparation of positive controls for HPV

Samples found to be positive using Hybrid capture II assays were further tested for HPV DNA by PCR amplification of L1 using the MY/GP primer sets and of E1, L1 and E6 employing our primer sets. Reactions was performed as described above, and the PCR products separated on 2% agarose gels. Individual bands were then excised, purified using the Perfectprep Gel Cleanup kit (Eppendorf, Humburg, Germany) and subjected to direct DNA sequencing. The purified products were also subcloned into the pGEM-T Easy plasmid vector (Promega, Medison, WI) as described previously.[20] The resultant plasmids were extracted and purified using the FastPlasmid Mini kit (Eppendorf, Hamburg, Germany) and used as positive controls and for sensitivity tests.

PCR sensitivity and specificity tests

The recombinant DNA plasmids served as a positive control for HPV detection and were also used to determine the test sensitivity. DNA concentrations were measured via OD_{260} readings, and ten-fold serial dilutions were prepared (10-10^7 copies μl^{-1}) and used as a template for sensitivity tests. PCR specificity was evaluated by cross-reactivity with DNA and RNA that had been extracted from Hepatitis B virus (HBV), Parvovirus 4 (PARV4), Parvovirus B19, Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV) specimens.

RESULTS

HPV detection sensitivity and specificity

This project was designed to examine the relationship between HCII ratios (RLU/CO), the PCR amplification of the L1 gene using primer sets MY/GP, and the PCR amplification of the L1, E1 and E6 genes using our primer sets for samples of different cytological findings. The PCR specificities and sensitivities were analyzed and confirmed that none of the primer sets (Table 1) cross-reacted with HIV, Parv4, Parvovirus B19, HCV or HBV. The HCII sensitivity showed that RLU/CO $\geq$ 1 (equivalent to 1 pg ml^{-1}) was adjudged HCII positive. The sensitivity limit of the E1 primer sets was found to be 10^2 copies μl^{-1} and the L1 gene at 10^3 copies μl^{-1}, while for the MY/GP and E6 primer sets this value was 10^4 copies μl^{-1}.

HCII ratios and PCR detection

All samples in this study were subjected to HCII assays. The ratio was determined which reflected relative light units divided by positive cut off (RLU/CO), where a ratio < 1 indicates a negative HCII finding and ratios $\geq$ 1 indicating a positive HCII result. A previous report, however, has shown that ratio values between 0.4 and 4 may be difficult to interpret as positive or negative, thus defining them as falling in a "grey zone".[8] Specifically, this refers to results between borderline negative and positive. In contrast, RLU/CO values < 0.4 can be conclusively interpreted as negative, and those greater than 4 as positive.

Table 2 HPV detection using HCII ratios (RLU/positive cut off) and PCR using our primers (E1, L1 and E6 gene primers) and primers published previously (MY09/MY11 and GP5+/GP6+)

		Number of samples with negative HCII ratios (< 0.4)	Number of samples with HCII ratios		
			0.4-4	>4	Total
	All	21	64	158	222
PCR categories	MY/GP	-	11 (17.18%)	98 (62.03%)	109 (49.1%)
	E1	-	17 (26.56%)	126 (79.74%)	143 (64.41%)
	L1	-	7 (10.94%)	99 (62.66%)	106 (47.73%)
	E6	-	5 (7.81%)	97 (61.39%)	102 (45.95%)
	Any primers	-	17 (26.56%)	131 (82.91%)	143 (64.41%)

Together, 243 samples were tested for RLU/CO ratios, and the results were divided into three groups: ratios of < 0.4 (n = 21), 0.4-4 (n = 64) and > 4 (n = 158). All of the 20 samples with RLU/CO ratios < 0.4 proved to be HCII negative and displayed normal cervical cytology, except for one sample which displayed ASC-US but tested negative for E1, E6 and L1 by PCR with the previously published primer sets. The remaining samples (with RLU/CO ratios > 0.4) comprised of 109 (49.10%), 143 (64.41%), 106 (47.73%) and 102 (45.59%) where successful amplification with MY/GP, E1, L1 and E6 PCR primer sets was achieved, respectively (Table 2). The E1 primer pair yielded the highest abundances of PCR product as compared to all other primer sets used in the study. The HPV E1 gene nucleotide sequences obtained from this study were submitted to the GenBank database under designated accession numbers FJ610146-52, GQ161244, GQ-161246, GQ161248-50, GQ161253-83, GQ161285-359, GQ161361-66, GQ161368-81, GQ161384-89, GQ161396, GQ161609 and GQ161629.

HCII positive samples were divided into two groups with ratios of 0.4-4 (n = 64) and exceeding 4 (n = 158). As the E1 primers yielded the highest percentage detection rates, positive PCR results denotes successful E1 amplification (Table 2). Results comparing HCII ratios, L1 PCR amplification (using a previously published primer set) and L1, E1 and E6 PCR amplification using our primer sets are shown in Table 2. Samples with HCII ratios exceeding 4 contain detectable E1 using PCR at a rate of 79.74%, which is greatly lowered to 26.56% for samples with HCII ratios of 0.4-4. Across all HCII ratio values, the percentages obtained with our E1 primer sets were higher than those obtained by the primers reported in previous studies (Table 2).[16-18] Comparison of our findings for L1, E1 and E6 revealed highest percentages of amplification when using the E1 primer set. These results demonstrate that the PCR amplification is directly proportional to HCII ratios. Interestingly, DNA amplification using the MY/GP primers was not observed in some samples despite their HCII ratio value, but could be achieved using our primer sets (data not shown).

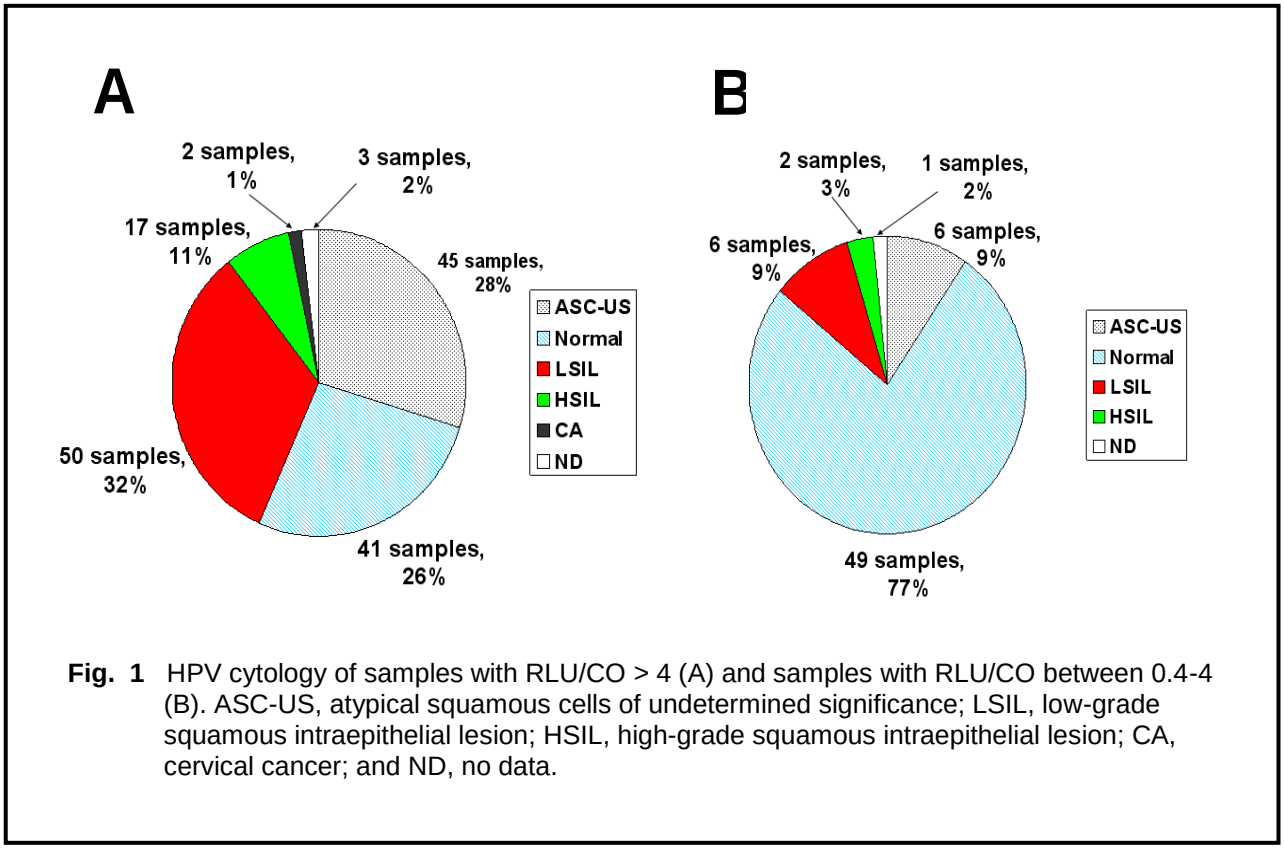

Fig. 1 HPV cytology of samples with RLU/CO > 4 (A) and samples with RLU/CO between 0.4-4 (B). ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CA, cervical cancer; and ND, no data.

Cytological data and grey zone analysis

Cytology analysis revealed that most samples containing HCII ratios > 4 were observed across all cytological category groups, and most predominantly in LSIL (32%), ASC-US (28%) and normal (26%) groups (Fig. 1A). HPV samples with HCII ratios between 0.4-4 (grey zone) primarily displayed normal cytology (77%) with ASC-US and LSIL ranked equal second at 9% each (Fig. 1B). According to the manufacturer's guidelines for the HCII assay, samples producing a negative result contain ratios lower than 1.00. In this study, 9 samples contained HCII ratios ranging between 0.4-0.99 and were therefore classified as negative. Despite this, HPV could be detected in two of these samples using the E1 primers (2/9; 22.22%). One sample was diagnosed as ASC-H, whereas cytological data was not available for the other sample. All HCII negative specimens showed normal cytology. Our study also examined the proportions of positive and negative samples falling within the grey zone as detected using E1 primers. These proportions for HCII ratios of 1-1.99, 2-2.99 and 3-3.99 were 4/31 (12.90%), 7/18 (38.89%) and 4/6 (66.67%), respectively (Fig. 2A). Furthermore, samples within the grey zone can be inferred as HCII positive while displaying normal cytology data (Fig. 2B). We recommend that these patient groups are subjected to HPV detection tests in follow-up visits.

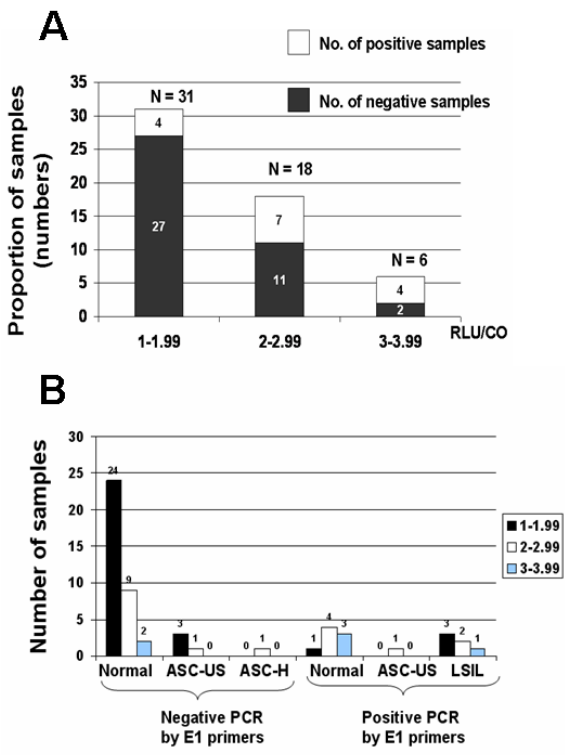

Fig. 2 Proportion of positive and negative samples detected by using E1 primers at an RLU/CO range of 1-1.99, 2-2.99 and 3-3.99. Positive samples are depicted as the upper (unfilled) band of the bars (A). Classification of cytological data as numbers of positive and negative PCR experiments using the E1 primers, and compared to RLU/CO ranges of 1-1.99, 2-2.99 and 3-3.99 (B).

DISCUSSION

The present study focused on the most essential HPV genomic regions. PCR primers were designed to amplify the following genes: L1, which is widely used in commercially available test kits; E6, found to play roles in cervical cancer development, and; E1, which encodes functional proteins that are essential for viral replication, and is a gene that has recently been implemented in classifying HPV genotypes (Papillo-Check, Frickenhausen, Germany). Amplified products were confirmed by direct sequencing. Previously published primer sets as well as our newly designed primers specific for the E1, L1 and E6 gene were incorporated into the study. The use of either primer set revealed that gene amplification using samples containing high HCII ratios was more readily obtained than those with low ratios (Fig. 1). Moreover, it became apparent that PCR amplification of E1 was indicative of a more accurate and sensitive diagnosis than the amplification of L1 and E6. All samples that could be PCR-amplified with MY/GP primers were from the same patients as the samples amplified with the E1 primers. E1 amplification was achieved in more samples with the E1 primers than when using the MY/GP primer set.

Nucleotide alignments of high-risk category HPV genomic DNA showed high diversity among the sequences, containing only a few short stretches of conserved regions to permit the design of primers. However, some E1 gene segments are more conserved than L1, and so this region was selected for primer design. Also, the E1 amplified fragments were longer (855 bps in the first round of PCR and 716 bps in the second round) than those previously

reported for L1 (approximately 450 bps with MY09/MY11 and 150 bps after nested PCR with GP5+/GP6+ primers). Therefore, the E1 PCR product is more amenable to direct sequencing than L1, which would be essential for an accurate determination of HPV genotypes. We should note that PCR amplification may produce false negative results, particularly when using ASC-US and ASC-H samples (Fig. 2B). Indeed**,** PCR screening using E1 primers on samples found to be within the "grey zone" (from the HCII assays) should then be analyzed together with cytological data.

We found that HCII ratios are directly proportional to successful PCR amplification. However, the HCII grey zone introduces speculation into HPV detection. Hence, a combination of the methods should be employed. Our study also showed a correlation between HCII ratios and the cytological data. HCII ratios of 0.4-4 often displays a normal cytology, whereas ratios exceeding 4 typically display several different cytological findings including LSIL, ASC-US and normal results with HCII ratio of 0.4-4 are difficult to interpret. Therefore, cytological data and PCR should be incorporated into HPV screening tests. Future studies should encompass the verification of our findings in this study through a larger project with increased numbers of samples that fall within grey zone HCII ratios. We also suggest here that data correlating the HCII assay and PCR methods are used as preliminary screening tools for the establishment of a standardized HPV detection method within Thailand.

In conclusion, comparisons between HCII ratios and PCR detection based on amplification of E1, L1 and E6 showed a relationships between the two methods. PCR provides greater sensitivity for samples containing higher HCII ratios than those with lower ratios. Finally, primer specificity proved to be greater for the amplification of E1 than for MY/GP, L1 or E6. Combining the data that is acquired using HCII, cytology and PCR methodologies should provide useful additional information for patients and for future HPV therapies.

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