GENTAUR

Establishment of an efficient multiplex real-time PCR assay for human papillomavirus genotyping in cervical cytology specimens: comparison with hybrid capture II

J.-H. Lee*, N.-W. Lee , S.-W. Hong , Y.-S. Nam , J.-W. Choi* and Y.-S. Kim*

*Department of Pathology, and Department of Obstetrics and Gynecology, Korea University Ansan Hospital, Ansan, Korea, and Kogenebiotech Co., Seoul, Korea

Accepted for publication 10 May 2010

J.-H. Lee, N.-W. Lee, S.-W. Hong, Y.-S. Nam, J.-W. Choi and Y.-S. Kim

Establishment of an efficient multiplex real-time PCR assay for human papillomavirus genotyping in cervical cytology specimens: comparison with hybrid capture II

Objective: To establish an efficient multiplex real-time PCR assay for 15 human papillomavirus (HPV) genotypes, we designed multiplexing parameters and compared our PCR system with the hybrid capture (HC) II test using cervical cytology samples.
Methods: For preventing cross-reactive amplifications, variable HPV genes (E1, E2, E6, E7 and L1) were targeted. The melting temperatures of all primers and probes, and the size of the PCR product were optimized for the multiplex PCR. Our PCR system was compared with the HC II assays in the detection and genotyping of HPV infection using 173 cytology smears. Discordant cases between the two assays were verified by direct HPV DNA sequencing.
Results: Of 173 women, 93 (53.8%) were HPV-positive by the HC II assay and ⁄or the multiplex real-time PCR assay. The HPV genotypes were determined in 92 (98.9%) of 93 cases by the multiplex real-time PCR and ⁄or DNA sequencing. The agreement rate between multiplex PCR and HC II methods was 91.9% (kappa = 0.84). Although the sample size of this study needs to be increased to have epidemiological significance, multiple infections and HPV 16 were the predominant type. HPV 58, 52 and 18 accounted for 25% of HPV infections. HPV 52, 58 and 31 constituted 30% of CIN 2 ⁄3.
Conclusion: The multiplex real-time PCR system shows a good and reliable clinical performance. This in house PCR assay is fast and cost-effective for HPV genotyping and the detection of HPV co-infection in the post-HPV vaccination era.

Keywords: human papillomavirus, multiplex real-time PCR, genotyping, hybrid capture, cervical cytology

Introduction

Human papillomavirus (HPV) is the most significant risk factor for cervical cancer.[1] Cervical cancer is the second most common malignancy worldwide and the leading cause of cancer-related deaths among women in many developing countries.[2] More than 80 HPV genotypes are known, of which 40 infect the female genital tract.[1] These are classified into high-risk and low-risk types based on the oncogenic potential of each type. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are regarded as high-risk and HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81 as low-risk. HPV 26, 53 and 66 are considered probably carcinogenic.[1,3]

HPV testing for high-risk types has become an independent screening tool as well as an adjunct to cervical (Pap) smear cytology. Although various methods for detecting HPV are available, the most popular techniques are hybrid capture II (HC II) and polymerase chain reaction (PCR)-based assays.[4] To date, two HPV vaccines have been developed using HPV L1 virus-like

Correspondence:
Young-Sik Kim, Department of Pathology, Korea University Ansan Hospital, 516, Gojan-1 Dong, Danwon-Gu, Ansan-Si, Gyeonggi-Do, 425-707, Korea
Tel.: +82 31 412 5322; Fax: +82 31 412 5324;
E-mail: apysk@korea.ac.kr

particle technology.[5–7] One is a bivalent vaccine against HPV 16 and 18 (Cervarix, GlaxoSmithKline, London, UK) and another is a quadrivalent vaccine against HPV 6, 11, 16 and 18 (Gardasil, Merck & Co, Whitehouse station, NJ, USA). Although immunogenic cross-reactivity of HPV types has been reported, these vaccines mainly prevent HPV infection by high-risk HPV 16 and ⁄or 18.[5] Therefore, type-specific testing for HPV has become essential for evaluating vaccine efficacy, determining the risk stratifications of cervical intraepithelial neoplasia (CIN) lesions, and monitoring re-infection or infection with multiple HPV genotypes. For HPV genotyping, commercial kits such as the Roche Diagnostics linear array HPV assay (Roche diagnostics, Basel, Switzerland) and the Innogenetics INNO-LiPA line probe assay (Innogenetics, Ghent, Belgium) are available. Both methods employ a reverse line blot technique, in which the DNA amplified by PCR is hybridized to HPV-specific probes on a membrane.[8–10] However, non-commercial methods of HPV genotyping have also been necessary because of economic or time-saving gains.

The objective of our study is to develop a fast and cost-effective multiplex real-time PCR for HPV genotyping and compare the clinical performance of this new system with the HC II test. To accomplish this, we designed and optimized the multiplexing parameters of the PCR assay using cervical cytology samples.

Methods

Clinical samples

One hundred and seventy-three consecutive cytology specimens were obtained from women visiting the gynaecology outpatient clinics at the Korea University Ansan Hospital for follow-up or routine screening. The study was conducted in accordance with the guidelines of the Institutional Human Tissue Committee. For each patient, cervical samples were obtained by cytobrush. The cervical cells were used both for preparing a conventional Pap smear and for collection in Cervical Sampler™ solution (Digene Co., Gaithersburg, MD, USA) for HPV detection and typing. The cytology samples were tested by the multiplex real-time PCR system and the HC II assay. Cases with discordant results by the multiplex real-time PCR and the HC II assay were further tested by direct sequencing. Women with atypical squamous cells of undetermined significance (ASC-US) or worse and ⁄or HPV-positive results were biopsied with colposcopy. Clinical performance was evaluated on the basis of cervical histology and Pap smear results.

HPV genotyping by multiplex real-time PCR

DNA was extracted from cytology specimens using QIAmp DNA mini kits (Qiagen, Hilden, Germany) and eluted into 200 μl of Tris-EDTA buffer. The primers and probes of the multiplex PCR assay were designed using the software programme Primer Express (Table 1). The computer programme was provided with an ABI 7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA). For real-time PCR amplifications, the target genes of the HPV genome were from the E1, E2, E6, E7 and L1 regions. All sequences of the primers and probes were verified by BLAST analysis. The melting temperatures (Tm) of all primers and probes and the amplicon size were designed to be very similar to each other to ensure optimum multiplexing. The Tm of the primers was 58–60 °C, while the Tm of the probes was 68–70 °C. The amplicon lengths were chosen between 60 and 80 bp. The TaqMan probe had a reporter dye at the 5′ end and a quencher dye at the 3′ end. We used 6-FAM, VIC, TET, JOE, HEX, Cy3, Cy5, ROX, Red 610, Texas Red, Red 670 or NED as the fluorescent reporter dye. The reporter was quenched by TAMRA, BHQ-1, BHQ-2, BHQ-3 or MGBNFQ (molecular-groove binding non-fluorescence quencher). For the multiplexing, five reaction tubes were used: tube 1 (HPV 16, 56 and 58), tube 2 (18, 52 and 33), tube 3 (39, 35 and 45), tube 4 (51, 31 and 59) and tube 5 (26, 68 and 66). Thus, each tube contained primers and probes for three HPV types, and one primer and probe for β-actin. The β-actin was used as an internal control for checking the DNA quality of the samples. The multiplex real-time PCR assay was performed in 20 μl of a reaction mixture containing 4 μl of DNA, 8 μl of 2 × PowerAmp real-time PCR master mixes, and 4 μl of each primer and probe mixture. The primer and probe mixture contained 500 nM of each primer, 100 nM of each probe, and an internal positive control. The PCR reactions were carried out in the ABI 7500 real-time PCR system under the following conditions: 2 minutes at 50 °C, 10 minutes at 94 °C, then 35 cycles consisting of 15 seconds at 95 °C and 1 minute at 60 °C. Uracil-DNA-glycosylase (UDG) digestion was carried out in the first step to prevent amplification of carry-over PCR products. Negative

 © 2010 Blackwell Publishing Ltd

Table 1. Primers and probes used for HPV genotyping

HPV type	Primer and probe sequences	Target gene	Amplicon size (bp)
16	F: 5¢-TTC GGT TGT GCG TAC AAA GC-3¢ R: 5¢-GCC CAT TAA CAG GTC TTC CAA A-3¢ P: 5¢-FAM-CAC ACG TAG ACA TTC G-MGB-3¢	E7	62
18	F: 5¢-AAG GCA ACA TTG CAA GAC ATT G-3¢R: 5¢-AGA AGG TCA ACC GGA ATT TCA TT-3¢P: 5¢-FAM-ATT GCA TTT AGA GCC CC-MGB-3¢	E7	65
26	F: 5¢-CTG TGC AGA GCA GTC GAC AGA-3¢ R: 5¢-AAG GAC ACG TCT TCC ATT AAC ATC T-3¢ P: 5¢-FAM-CGT TCG AGT GCT GGA GGB-3¢	E7	64
31	F: 5¢-ATG GCT GAT CCA GCA GGT ACA-3¢ R: 5¢-CTT CTA CAT AAA ACC AAC CAT TGC AT-3¢ P: 5¢-VIC-ATG GGG AGG GGA CGG -MGB-3¢	E1	64
33	F: 5¢-TGA TTT GTG CCA AGC ATT GG-3¢ R: 5¢-TTT GCA TTC CAC GCA CTG TAG-3¢ P: 5¢-NED-ACA ACT ATA CAC AAC ATT G -MGB-3¢	E6	64
35	F: 5¢-CAA GAA TTA CAG CGG AGT GAG GTA-3¢ R: 5¢-ATA TGG CTG GCC TTC TCT ATA TAC TAT ACA-3¢ P: 5¢-VIC-ATG ACT TTG CAT GCT ATG-MGB-3¢	E6	78
39	F: 5¢-AGT CTG CAG CCA TTA CAT GTC AA-3¢ R: 5¢-GTC AAC CGG CTG TAT TTC ATT GT-3¢ P: 5¢-FAM- AGG ATG CTC CAG CAC C -MGB-3¢	L1	66
45	F: 5¢-ATG GCA CAC AAT ATT ATT TAT GGC C-3¢ R: 5¢-CCA TCT GCA AAA AAA TAG GGA ATA C-3¢ P: 5¢-NED-TGG TAT TAT TAT TTT CCT AAA AAA CGT AAA-MGB-3¢	L1	82
51	F: 5¢-TGC CAC TGC TGC GGT TT-3¢ R: 5¢-CCC CAT GCC TAA TAT ATT GCT TAA A-3¢ P: 5¢-FAM-CCC AAC ATT TAC TCC AAG-MGB-3¢	L1	65
52	F: 5¢-GGA TTT GGT TGC ATG GAT TTT AA-3¢ R: 5¢-GCT ACA TAT ATC AAT GGG CAC ATC A-3¢ P: 5¢-VIC-ACC TTG CAA GCT AGT AAA-MGB-3¢	L1	69
56	F: 5¢-ATG CAT GGT AAA GTA CCA ACG CT-3¢ R: 5¢-TGT AGG TCA ATT TCT GTT TGA GGT G-3¢ P: 5¢-VIC- CAA GAC GTT GTA TTA GAA CTA -MGB-3¢	E7	71
58	F: 5¢-TCC AGG ACG CAG AGG AGA A-3¢ R: 5¢-TCC AAC GCC TGA CAC AAA TC-3¢ P: 5¢-NED-CCA CGG ACA TTG CA-MGB-3¢	E6	55
59	F: 5¢-AGC GAC CAA GAC AGT GTG GAT-3¢ R: 5¢-GTC CAC TGA CAC GCT GGT AGA C-3¢ P: 5¢-NED-CAC ACA GCA CCC TC-MGB-3¢	E2	59
66	F: 5¢-GAC AGG GAG ACA GCT CAA CAA TTA-3¢ R: 5¢-TTG CAA CGT CTG TGC ATC TG-3¢ P: 5¢-VIC-TGC AAG TAC AAA CAG C-MGB-3¢	E1	66
68	F: 5¢-GGC CAA TTG TGA AGG TAC CG-3¢ R: 5¢-CTT CTA CAA AAA ACC ATC CGT TAC AC-3¢ P: 5¢-NED- TGG GGA CGG GAC GG -MGB-3¢	E1	62
Control	F: 5¢-CTG GCA CCC AGC ACA ATG-3¢ R: 5¢-GCC GAT CCA CAC GGA GTA CG-3¢ P: 5¢-Cy5-ATC AAG ATC ATT GCT CCT CCT GAG CGC-BHQ2-3¢	Beta-actin	69

F, forward; R, reverse; P, probe.

 © 2010 Blackwell Publishing Ltd

controls without template DNA were added to each run. Fluorescent data were collected and automatically analysed with KoRAS software (Kogenbiotech, Seoul, Korea). Using plasmid dilutions of each HPV type, the dynamic range was determined to be at least five orders of magnitude. Cycle threshold-values were blotted against $\log_{10}$ of target DNA. A correlation coefficient of more than 0.98 for each HPV type could be obtained.

Hybrid Capture II assay

The HC II test is known to detect 13 oncogenic HPV types in aggregate and has shown good clinical performance for identifying CIN 2/3 in population-based studies.[11–13] The HC II high-risk HPV test was performed according to the manufacturer s instructions (Digene Co). Briefly, HPV DNA was denatured and then hybridized with a specific HPV RNA probe cocktail, resulting in the formation of RNA-DNA hybrids. The hybrids were captured by antibodies specific for the RNA-DNA hybrids. Immobilized hybrids were reacted with alkaline phosphatase-conjugated antibodies, and detected with a chemiluminescent substrate. The chemiluminescent light was measured using a luminometer and reported in relative light units (RLUs).

Direct HPV DNA sequencing

PCR products were purified and sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The reaction products were analysed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and DNA sequences were aligned with the basic local alignment search tool (BLAST). The GenBank accession numbers of the matched DNA sequences were FJ006723 (HPV 16), AF548831 (HPV 18), U45895 (HPV 33), U31793 (HPV 61), EF626592 (HPV 62), and EF177191 (HPV 66). HPV 51 was identified by singleplex real-time PCR because of its noise in sequencing peaks.

Immunohistochemistry

For accurate CIN grading of cervical biopsies, immunohistochemistry for $p16^{INK4A}$ (mtm lab. AG, Heidelberg, Germany) was performed using a ChemMate EnVision kit (Dako, Carpinteria, CA, USA).[14–16]

Statistical analysis

The degree of agreement between HC II and multiplex real-time PCR assays in detecting HPV infection was calculated by Cohen s kappa values. Kappa values of less than 0, 0–0.2, 0.21–0.4, 0.41–0.6, 0.61–0.8, 0.81–0.99 and 1.0 designate no, slight, fair, moderate, substantial, almost perfect and perfect agreements, respectively.

Results

We designed a multiplex real-time PCR assay for targeting 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and two intermediate-risk HPV types (26 and 66) (Table 1). The clinical performance of the multiplex real-time PCR assay was evaluated using clinical samples from 173 women. Of the 173 patients, 93 (53.8%) were HPV-positive by HC II assay and/or multiplex real-time PCR. The HPV-infected women had a mean age of 39.0 years (range, 20–76 years). In 92 (98.9%) of 93 cases, HPV genotype was determined by multiplex real-time PCR and/or direct DNA sequencing. Table 2 presents the multiplex real-time PCR and HC II assay results versus high grade CIN 2 or worse histology. The clinical sensitivity and specificity of the multiplex PCR system and HC II test were determined by comparing the assay results with histology and cytology results. Calculation was performed with respect to CIN 2 or worse histology. Of the 62 biopsy-confirmed specimens with CIN 2 or worse, 59 (95.2%) were positive on the multiplex real-time PCR system and 58 (93.5%) were positive in the HC II assay. Three cases that were negative by the multiplex PCR system were positive by the HC II assay

Table 2. Comparison of HPV detection by multiplex real-time PCR and HC II

	CIN 2 or worse		
	Positive	Negative	Total
Multiplex PCR			
Positive	59	30	89
Negative	3	81	84
Total	62	111	173
HC II			
Positive	58	25	83
Negative	4	86	90
Total	62	111	173

CIN, cervical intraepithelial neoplasia.

Table 3. Discordant HPV results between HC II and multiplex real-time PCR

Case	Age (years)	Cytology	Histology	HC II (RLU)	Multiplex (HPV)	Sequencing (HPV)
1	58	HSIL	CIN 3	3.75	Neg	F
2	43	HSIL	CIN 1	664.49	Neg	16
3	23	ASC-US	CIN 2	2.19	Neg	18, 51
4	39	LSIL	CIN 2	13.31	Neg	18
5	32	ASC-US	CIN 3	Neg	52	61
6	46	HSIL	Koilo	Neg	33	33
7	36	LSIL	Koilo	Neg	18	18
8	40	LSIL	CIN 3	Neg	68	F
9	52	LSIL	CIN 2	Neg	56	NA
10	31	LSIL	CIN 1	Neg	16	16
11	35	WNL	CIN 3	Neg	16	16
12	26	ASC-US	Koilo	Neg	56	62
13	32	ASC-US	NA	Neg	66	66
14	22	ASC-US	CIN 1	Neg	33	16, 33

HSIL, high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; WNL, within normal limits; Koilo, koilocytic atypia; CIN, cervical intraepithelial neoplasia; NA, not available; F, failure to sequence.

while four cases that were negative by the HC II assay were genotyped by the multiplex real-time PCR (Tables 2 and 3). The clinical sensitivity and specificity of the multiplex real-time PCR system were 95.2% and 73.0% while the sensitivity and specificity of the HC II assay were 93.5% and 77.5%. The positive predictive value (PPV) and negative predictive value (NPV) of the multiplex PCR assay were 66.3% and 96.4%. The PPV and NPV of the HC II test were 69.9% and 95.6% for the detection of CIN 2+ lesions. Thus, the multiplex real-time PCR system was comparable to the HC II test for the significant histological lesions (Table 2).

The HPV results that were discordant between the HC II test and the multiplex real-time PCR assay are shown in Table 3. In four cases, HPV was detected by the HC II assay but not by the multiplex real-time PCR. Conversely, in 10 cases, HPV was identified by the multiplex real-time PCR assay but not by the HC II assay. Thus, the agreement rate of HPV results by the two methods was 91.9% (159 ⁄ 173 cases). The kappa value between HC II and multiplex real-time PCR assays in detecting HPV infection showed almost perfect agreement (k = 0.84). Multiplex real-time PCR failed to detect one case of HPV 16, one HPV 18, and a double infection by HPV 18 and 51. One case, which was considered to be an HPV 52 infection by multiplex real-time PCR, proved to be an HPV 61 infection by direct DNA sequencing (Table 3).

The most common HPV type was HPV 16, followed by HPV 52, 58, 33, 66, 18, 31, 51, 68, 56, 35, 45, 59 and 26 in descending order (Figure 1). However, considering infection with multiple HPV types, the prevalence was somewhat changed as follows. Infections with multiple HPV types and HPV16 were the most common, followed by HPV 58, 52, 18, 31, 33, 68, 35, 56, 66, 45 and 51. Interestingly, multiple HPV infections in an individual were the most common type. After HPV16 and multiple HPV infections, HPV 58, 52 and 18 accounted for 25% of HPV cases.

Table 4 shows the distribution of high-risk HPV types according to cervical histologic lesions. CIN 2 ⁄ 3

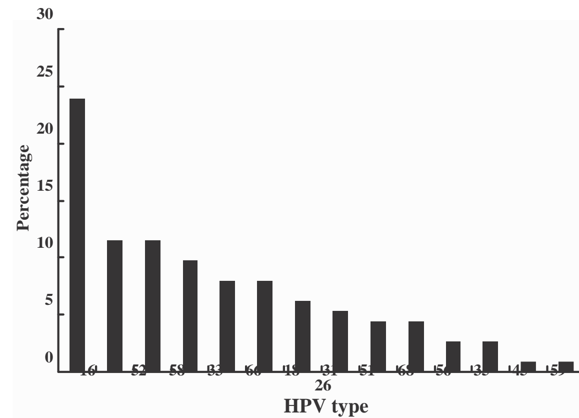

Figure 1. Distribution of HPV genotypes by the multiplex real-time PCR system and by direct HPV sequencing.

 © 2010 Blackwell Publishing Ltd

Table 4. Distribution of high-risk HPV genotypes according to different cervical lesions

Histology (HPV+ve)	HPV typing	High-risk HPV genotypes (%) 16	18	31	33	35	45	51	52	56	58	66	68	Multiple
Koilo (n = 4)	4	0	1 (25.0)	1 (25.0)	0	0	0	0	0	1 (25.0)	1 (25.0)	0	0	0
CIN 1 (n = 21)	21	2 (9.5)	2 (9.5)	1 (4.8)	2 (9.5)	1 (4.8)	0	0	1 (4.8)	0	1 (4.8)	2 (9.5)	3 (14.3)	6 (28.6)
CIN 2 (n = 14)	14	1 (7.1)	1 (7.1)	1 (7.1)	0	0	0	0	2 (14.3)	1 (7.1)	4 (28.6)	0	0	4 (28.6)
CIN 3 (n = 47)	46	15 (32.6)	2 (4.3)	2 (4.3)	2 (4.3)	1 (2.1)	2 (4.3)	2 (4.3)	5 (10.9)	1 (2.1)	4 (8.7)	0	1 (2.1)	9 (19.6)
SCC (n = 1)	1	1 (100)	0	0	0	0	0	0	0	0	0	0	0	0
NA (n = 6)	6	1 (16.7)	0	0	1 (16.7)	1 (16.7)	0	0	0	0	0	1 (16.7)	0	2 (33.3)
Total (n = 93)	92	20	6	5	5	3	2	2	8	3	10	3	4	21

HPV typing was performed by multiplex real-time PCR and /or direct DNA sequencing.
Koilo, koilocytic atypia; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; NA, not available.

Table 5. Multiple HPV infections in different cervical lesions

Histology	Cases	Multiple infection (No. of cases)
CIN 1	6	16/33 (1); 16/52 (2); 33/68 (1); 39/52 (1); 58/59/66 (1)
CIN 2	4	18/51 (1); 51/66 (1); 52/68 (1); 56/66 (1)
CIN 3	9	16/31 (1); 16/33 (1); 16/33/52 (1); 16/66 (1); 18/33/56/66 (1); 26/31 (1); 51/52 (1); 51/58 (1); 52/58 (1)
Unknown	2	16/33 (1); 26/45/66 (1)
Total	21	

CIN, cervical intraepithelial neoplasia lesion.

was diagnosed in 60 (65.2%) of 92 HPV-positive cases. HPV 16 and infection with multiple types were predominant in CIN 2/3. HPV 52, 58 and 31 comprised 30% of CIN 2/3. One HPV-infected case was invasive SCC. Table 5 represents the distribution of infection with multiple HPV genotypes. Multiple HPV infections were found in 21 (22.8%) of 92 cases. HPV 16, 66 and 52 were the types most commonly involved in multiple infections, with HPV 16 being found in seven and HPV 52 and 66 in six of the 21 cases.

Discussion

The present study suggests that the multiplex real-time PCR assay may be a suitable tool to replace the HC II test in pathology practice. In the post-HPV vaccination era, HPV genotyping has become an important procedure.[17] HPV genotyping should be cost-effective and time-efficient for clinical situa- tions.[17] For this purpose, the multiplex real-time PCR assay is the most suitable. The outcomes using this multiplex system are obtained in only 3 hours while commercial assay kits for HPV typing usually require 8 hours. The prices for commercial tests excluding DNA extraction range between USD 30 and USD 60. In contrast, the multiplex-real time system costs less than USD 3.

Previous studies have shown that multiplex real-time PCR is a highly sensitive and specific test for the detection and genotyping of HPV.[18,19] However, the multiplex real-time PCR assay also has a few limitations in view of the cross-reactivity among HPV genotypes. The target regions of HPV genomes in prior studies were L1[18] and LCR/E6/E7,[19] respectively. These multiplex PCR techniques used

molecular beacons[18] or Taqman probes.[19] To over- come the inherent disadvantage of multiplex real- time PCR, the PCR parameters of our system were designed for minimizing cross-reactivity and enhanc- ing PCR efficiency. For instance, primers were devised for amplifying the E1 ⁄ E2 ⁄ E6 ⁄ E7 ⁄ L1 regions of HPV genomes and the primers with at least two different targets were mixed in a reaction tube for multiplexing. The melting temperatures of the primers were adjusted to be very similar to each other. In addition, the PCR amplification sizes were optimized so that they ranged from 55 to 82 base pairs. Thus, we could perform multiplex real-time PCR effectively under the same PCR conditions.

To test the clinical performance of our system, we compared the multiplex PCR system with the HC II assay, which is known as a reliable and clinically validated test in pathology usage. The concordance rate between the multiplex real-time PCR and the HC II assay was about 92%, with a kappa value of almost perfect agreement. In fact, in terms of HPV detection for CIN 2+ lesions, the multiplex real-time PCR assay showed comparable clinical sensitivity and specificity to the HC II assay. Four cases with CIN 2+ histology were negative by the HC II but positive by the multiplex real-time PCR. The multiplex PCR system detected one case of HPV 16, one HPV 52, one HPV 56 and one HPV 68, which were missed by the HC II test. In contrast, the HC II assay identified one co-infected case of HPV 18 and HPV 51 and one case of HPV 18, which were scored as negative by the real-time PCR. HPV 18 was known to be the second most prevalent type in Korea and other countries, but this type accounted for no more than 8% of cases. The relatively low frequency of HPV 18 might be due to a regional variation of HPV genotypes in the country or a drawback of our multiplex real-time PCR system. The HC II results are represented in RLU and the threshold for a positive value is set at 1.0 RLU, corresponding to about 1.0 pg of viral DNA. In this study, one case with a high titre of HPV (approximately 665 RLU) was not genotyped by the multiplex real-time PCR. However, the high level of viral load did not appear to hinder the multiplex real-time PCR because the other 19 cases with a high titre of more than 665 RLU were all positive by the multiplex real-time PCR. To clarify these issues, more studies with large scale samples or adjusted multiplexing conditions of real-time PCR are needed. In addition, our multiplex system detects more HPV genotypes such as HPV 26 and 66 than the HC II assay. These HPV types are regarded as probably carcinogenic. To identify the discrepancy between the two assays, we sequenced HPV DNAs. As a result, we found that most HPV 66 or 26 infect cervical cells together with the other oncogenic HPV types as part of double or multiple infections. Thus, the HC II assay can also detect the HPV infection as a positive reaction.

Although our study has limitations regarding the sample size for gaining epidemiological significance, we found that multiple HPV infections are the most prevalent, occurring in about 20% of HPV-positive cases. Multiple HPV infections are known to be a significant risk factor for HPV persistence and the development of high-grade CIN.[20–22] Because HPV 16, 66 and 52 are the types most commonly involved in multiple infections, the current vaccination against HPV 16 and 18 may be limited in its ability to prevent the progression of cervical neoplasia completely. As HPV 52, 58 and 31 comprised 30% of CIN 2 ⁄ 3, type-specific typing for the second most prevalent HPV genotypes is required for primary screening of cervical cancer and precursors. Therefore, multiple HPV infections and regional variations of HPV genotypes should be considered for developing second-generation HPV vaccines.

In summary, this study indicates that the multiplex real-time PCR assay shows as reliable a clinical performance as does the HC II test. Given the frequent occurrence of multiple infections and the regional variations in HPV genotypes in high grade CIN, HPV genotyping is indispensable to cervical cancer screening and the triage of women with equivocal cytology. Thus, the multiplex real-time PCR system will be useful in the practise of pathology in the post-HPV vaccination era.

Acknowledgments

The authors thank Sang-Ju Lee, Mi-Ran Jeong, Eun-Ja Lee, Sung-Su Lee and Jee-Hye Ok for their technical assistance.

Competing interests

None to declare.

References

1. Munoz N, Bosch FX, de Sanjose S et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003;348:518–27.

 © 2010 Blackwell Publishing Ltd

2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
3. Schiffman M, Herrero R, Desalle R et al. The carcinogenicity of human papillomavirus types reflects viral evolution. Virology 2005;337:76–84.
4. Molijn A, Kleter B, Quint W, van Doorn LJ. Molecular diagnosis of human papillomavirus (HPV) infections. J Clin Virol 2005;32(Suppl. 1):S43–51.
5. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. Nat Rev Immunol 2004;4:46–54.
6. Zhou J, Sun XY, Stenzel DJ, Frazer IH. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology 1991;185:251–7.
7. Kirnbauer R, Taub J, Greenstone H et al. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J Virol 1993;67:6929–36.
8. Gravitt PE, Peyton CL, Apple RJ, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol 1998;36:3020–7.
9. Gravitt PE, Peyton CL, Alessi TQ et al. Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000;38:357–61.
10. Kleter B, van Doorn LJ, Schrauwen L et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol 1999;37:2508–17.
11. Cuzick J, Clavel C, Petry KU et al. Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J Cancer 2006;119:1095–101.
12. Castle PE, Wheeler CM, Solomon D et al. Interlaboratory reliability of Hybrid Capture 2. Am J Clin Pathol 2004;
122:238–45.
13. Carozzi FM, Del Mistro A, Confortini M et al. Reproducibility of HPV DNA testing by Hybrid Capture 2 in a screening setting. Am J Clin Pathol 2005; 124:716–21.
14. O Neill CJ, McCluggage WG. p16 expression in the female genital tract and its value in diagnosis. Adv Anat Pathol 2006;13:8–15.
15. Hu L, Guo M, He Z et al. Human papillomavirus genotyping and p16INK4a expression in cervical intraepithelial neoplasia of adolescents. Mod Pathol 2005;18: 267–73.
16. Mulvany NJ, Allen DG, Wilson SM. Diagnostic utility of p16INK4a: a reappraisal of its use in cervical biopsies. Pathology 2008;40:335–44.
17. Meijer CJ, Snijders PJ, Castle PE. Clinical utility of HPV genotyping. Gynecol Oncol 2006;103:12–7.
18. Takacs T, Jeney C, Kovacs L et al. Molecular beacon-based real-time PCR method for detection of 15 high-risk and 5 low-risk HPV types. J Virol Methods 2008;149: 153–62.
19. Schmitz M, Scheungraber C, Herrmann J et al. Quanti- tative multiplex PCR assay for the detection of the seven clinically most relevant high-risk HPV types. J Clin Virol
2009;44:302–7.
20. Spinillo A, Dal Bello B, Gardella B et al. Multiple human papillomavirus infection and high grade cervical intraepithelial neoplasia among women with cytological diagnosis of atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions. Gynecol Oncol 2009;113:115–9.
21. Lee SA, Kang D, Seo SS et al. Multiple HPV infection in cervical cancer screened by HPVDNAChip. Cancer Lett 2003;198:187–92.
22. Cuschieri KS, Cubie HA, Whitley MW et al. Multiple high risk HPV infections are common in cervical neoplasia and young women in a cervical screening population. J Clin Pathol 2004;57:68–72

GENTAUR

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium