Comparison of Three Different Commercial Kits for the Human Papilloma Virus Genotyping

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Background: High-risk type human papilloma virus (HPV) is the most important cause of cervical cancer. Recently, real-time polymerase chain reaction and reverse blot hybridization assay-based HPV DNA genotyping kits are developed. So, we compared the performances of different three HPV genotyping kits using different analytical principles and methods. **Methods:** Two hundred positive and 100 negative cervical swab specimens were used. DNA was extracted and all samples were tested by the MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPV DNAChip. Direct sequencing was performed as a reference method for confirming high-risk HPV genotypes 16, 18, 45, 52, and 58. **Results:** Although high-level agreement results were observed in negative samples, three kits showed decreased

interassay agreement as screening setting in positive samples. Comparing the genotyping results, three assays showed acceptable sensitivity and specificity for the detection of HPV 16 and 18. Otherwise, various sensitivities showed in the detection of HPV 45, 52, and 58. **Conclusions:** The three assays had dissimilar performance of HPV screening capacity and exhibited moderate level of concordance in HPV genotyping. These discrepant results were unavoidable due to difference in type-specific analytical sensitivity and lack of standardization; therefore, we suggested that the efforts to standardization of HPV genotyping kits and adjusting analytical sensitivity would be important for the best clinical performance. *J. Clin. Lab. Anal.* **30**:1110–1115, 2016. © 2016 Wiley Periodicals, Inc.

Key words: comparison; genotyping; human papilloma virus

INTRODUCTION

Cervical cancer is known as the second most common cancer and third leading cause of cancer death among females. In 2012, about 527,600 of new cervical cancer cases were diagnosed and 265,700 deaths (1). The major cause of cervical cancer is infection of oncogenic human papillomavirus (HPV), and more than 100 genotypes are identified. Among them, approximately 10–20 genotypes, including HPV genotypes 16 and 18, have been classified as high-risk types for cervical cancer (2). Although vaccination for these types of HPV was widespread through the world, cervical screening programs still remain the most important strategy for cervical cancer (3) because it is far from now that the effects of vaccination show effectiveness on morbidity of cervical cancer (4).

Papanicolaou test was introduced for screening of cervical cancer in the 1950s (5); however, several drawbacks of the Pap test such as clinical sensitivity or inter-observer variability have been known (6). As various molecular diagnostic tools for the detection of

HPVs received the approval by US Food and Drug Administration and available on the market, these new assays have settled down as screening tool for supplement of Pap test. Now, many cervical screening guidelines recommend that women aged between 30 and 65 should be screened by cytology with HPV test together (7, 8).

Various HPV tests have been developed to not only determine samples as positive for high-risk HPV types but also specify the HPV genotypes; especially, single genotyping of HPVs has become important in the prediction of risk in cervical cancer and follow-up of specific genotype HPV infection. For this reason, several commercial assays were successfully introduced for

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HPV screening purpose as well as HPV genotyping and proved to be of equivalent performance compared with previously used assays only for screening purpose (9, 10). However, due to diversity of detection methods between manufacturers, it was inevitable that the difference exists in genotype inclusivity and type-specific sensitivity or specificity among HPV genotyping kits (10).

Recently, two new HPV genotyping kits have been introduced in the market lately, and these assays are based on different principle for genotyping HPVs. MolecuTech® REBA HPV-ID (YD Diagnostics Corp., Seoul, Korea) is a recently developed reverse blot hybridization assay designed to detect 32 HPV genotypes, and Anyplex™ II HPV28 Detection (Seegene, Seoul, Korea) is a new multiplex real-time PCR assay with the capability of distinguishing 28 unique HPV genotypes. In this study, to compare the performance of screening and genotyping capacities, we genotyped 300 clinical samples with the following three commercially available HPV genotyping kits: MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPV DNACHIP™ (BioMedLab Co., Seoul, Korea).

MATERIALS AND METHODS

Samples

Over a 5-month period, between March 2014 and July 2014, we collected 1,356 cervical swabs requested to HPV screening or genotyping. These samples were from 1,356 patients aged 20 to 60 years who visited the Chung-Ang University Hospital for routine screening smear or outpatients. To evaluate the HPV genotyping kit as a screening setting, we selected randomly 200 positive samples and 100 negative samples. Samples were collected using the Digene cervical sampler (Qiagen, Hilden, Germany) or the HPV DNACHIP Sampler (Bio-MedLab Co.) and were stored at -70°C until test date. The collection of all cervical samples was approved by Chung-Ang University Hospital ethics committee.

DNA extraction

Total DNA was extracted using the MICROLAB Nimbus IVD system (Hamilton, Reno, NV, USA) with STARMag96 Virus kit (Seegene) following the manufacturer's instructions. Three aliquots of the extracted DNA were used with the MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPV DNACHIP, respectively.

Genotyping of HPV

HPV genotyping using the MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPV DNACHIP was

carried out according to the manufacturer's recommended protocols.

MolecuTech REBA HPV-ID is PCR-based reverse blot hybridization assay that can identify the 18 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, and 73), 1 probable high-risk HPV (34), and the 13 low-risk HPV genotypes (6, 11, 32, 40, 42, 43, 44, 54, 70, 72, 81, 84, and 87). The extracted DNA was taken to be used in nested PCR by GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) to amplify the target region using MY11 and MY9 primers, followed by the GP5/GP6 primer pair. Then, PCR amplicon was denatured with a denaturation solution and the denatured PCR product was added into membrane strip labeled with genotype-specific probe for HPV genotyping. After hybridization step was over, PCR product without hybridization was washed, the alkaline-phosphatase enzyme reaction and chromogenic reaction were performed to develop detection signals. Then, the developed strips were scanned by REBASCAN (YD Diagnostics Corp.).

Anyplex II HPV28 Detection uses HPV-specific dual priming oligonucleotides (DPO) and Tagging Oligonucleotide Cleavage and Extension (TOCE) technologies. With these technologies, real-time PCRs were performed in two tubes and can detect 19 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, and 82) and 9 low-risk HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, and 70), simultaneously. The assay was conducted with the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and data were recorded and interpreted with the Seegene viewer software.

HPV DNACHIP is a PCR-based DNA microarray system for HPV genotyping and detects simultaneously 15 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 69) and 7 low-risk HPV genotypes (6, 11, 34, 40, 42, 43, and 44). With DNA extracted from cervical swab sample, target L1 regions were amplified by GeneAmp PCR System 9700 and DNA was labeled by a single dye indocarbocyanine-dUTP during PCR. Then, the sample was applied onto the DNA chip and hybridization was performed following the washing step. In a slide glass, 22 type-specific oligonucleotide probes were immobilized. After hybridization, hybridized DNA was visualized using a ScanArray Gx Microarray Scanner (PerkinElmer, Waltham, MA, USA). When HPV amplicons showed double-positive spots on DNA chip slide, the sample was positive for HPV corresponding type-specific oligonucleotide probes.

Type-specific PCR and sequencing analysis

To evaluate the analytical sensitivity and specificity for genotyping, we selected five major high-risk HPVs

(16, 18, 45, 52, and 58) that are known as the most common HPV genotypes in the cases of invasive cervical cancer in Asian population (11). A total of 94 samples showed positive results in five major high-risk HPVs with at least one kit, and the type-specific PCR was carried out with previously reported method (12). All type-specific primers are designed to target the L1 gene region. Then, the PCR products were electrophoresed on a 2% agarose gel at 100 V for 40 min and the DNA was stained with ethidium bromide. To confirm the HPV genotypes, nucleotide sequence of PCR products was analyzed by ABI 3730xl DNA analyzer (Life Technologies, Carlsbad, CA, USA) and compared with GenBank BLAST database.

Statistical analysis

To evaluate the agreement of genotyping results from three genotyping kits, the Fleiss' kappa (κ) statistics and absolute agreement were calculated. Agreement between tests was assessed according to κ -values, where values in the range 0.81–1.00 indicate almost perfect agreement, 0.61–0.80 substantial, 0.41–0.60 moderate, 0.21–0.40 fair, 0.00–0.20 slight, and <0.00 poor (13). Sensitivity and specificity were calculated using conventional contingency tables. All statistical analyses were performed using SPSS v.19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Due to difference in categorization of carcinogenicity between three kits, we categorized high-risk type HPV according to the previously reported classification (2); HPV 6, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 were classified as high-risk type HPVs and the rest of genotypes were regarded as low-risk HPVs. The HPV genotypes of detection between three kits are summarized in Table 1.

The positive rate of MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPVDNACHip was

TABLE 1. Inclusivity of HPV Genotypes in the Three Commercially Available HPV Genotyping Kits

	Oncogenic potential		
	High-risk group	Probable high-risk group	Low-risk group
MolecuTech REBA HPV-ID	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73	26, 53, 66	6, 11, 32, 34, 40, 42, 43, 44, 54, 69, 70, 72, 81, 84, 87
Anyplex II HPV28 detection	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73	26, 66	6, 11, 40, 42, 43, 44, 53, 54, 61, 69, 70, 82
HPVDNACHip	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	66	6, 11, 34, 40, 42, 43, 44, 69

shown in Table 2. Among the 200 positive samples, the MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPVDNACHip detected 154 (77%), 171 (85.5%), and 178 (89%) HPV-positive cases, respectively (72% agreement, $\kappa = 0.311$). The positive rates were 55% (110/200) for MolecuTech REBA HPV-ID, 67.5% (135/200) for Anyplex II HPV28 Detection, and 63.5% (127/200) for HPVDNACHip in high-risk HPV infection (74% agreement, $\kappa = 0.632$), and 28% (56/200) for MolecuTech REBA HPV-ID, 35% (70/200) for Anyplex II HPV28 Detection, and 34.5% (69/200) for HPVDNACHip in low-risk HPV infection (62.5% agreement, $\kappa = 0.430$). Among the 100 negative samples, the positive rates were 15% (15/100) for MolecuTech REBA HPV-ID, 11% (11/100) for Anyplex II HPV28 Detection, and 11% (11/100) for HPVDNACHip in overall HPV infection (84% agreement, $\kappa = 0.507$). According to HPV risk group, the MolecuTech REBA HPV-ID, Anyplex II HPV28 Detection, and HPVDNACHip detected 12, 8, and 7 high-risk HPV infections (87% agreement, $\kappa = 0.471$) and 3, 3, and 4 low-risk HPV infections (95% agreement, $\kappa = 0.483$), respectively.

The genotype-specific agreements are summarized in Table 3. The percentage of agreement ranged between

TABLE 2. Positive Rate between Positive Samples (a); Negative Samples (b)

	MolecuTech REBA HPV-ID		Anyplex II HPV28 Detection		HPVDNACHip		Absolute agreement	κ -value
(a) Positive samples								
Overall HPV DNA	77.0%	154/200	85.5%	171/200	89.0%	178/200	72.0%	0.311
High-risk genotypes	55.0%	110/200	67.5%	135/200	63.5%	127/200	74.0%	0.632
Low-risk genotypes	28.0%	56/200	35.0%	70/200	34.5%	69/200	62.5%	0.430
(b) Negative samples								
Overall HPV DNA	15.0%	15/100	11.0%	11/100	11.0%	11/100	84.0%	0.507
High-risk genotypes	12.0%	12/100	8.0%	8/100	7.0%	7/100	87.0%	0.471
Low-risk genotypes	3.0%	3/100	3.0%	3/100	4.0%	4/100	95.0%	0.483

TABLE 3. Type-Specific Agreement for Each HPV Genotype Detected by Three Kits

Genotype	Number of positive samples				Absolute agreement (%)	κ -value	Meaning
	MolecuTech REBA HPV-ID	Anyplex II HPV28 Detection	HPVDNAChip				
High risk	16	42	31	37	95.7	0.865	Almost perfect
	18	15	11	11	98.3	0.859	Almost perfect
	31	1	6	3	98.0	0.393	Fair
	33	5	5	3	99.0	0.766	Substantial
	35	3	7	6	98.7	0.745	Substantial
	39	8	13	9	97.3	0.724	Substantial
	45	4	1	1	99.0	0.497	Moderate
	51	0	14	13	95.3	0.465	Moderate
	52	9	20	19	95.7	0.714	Substantial
	56	11	13	19	96.3	0.731	Substantial
	58	14	18	9	97.0	0.770	Substantial
	59	0	5	5	98.3	0.494	Moderate
	66	7	15	13	97.3	0.762	Substantial
	68	2	11	12	95.3	0.424	Moderate
Low risk	6	5	6	5	99.7	0.936	Almost perfect
	11	1	3	1	99.3	0.598	Moderate
	40	4	11	19	94.0	0.450	Moderate
	42	0	16	16	93.7	0.384	Fair
	43	2	4	3	99.3	0.776	Substantial
	44	0	15	12	94.7	0.389	Fair
	69	0	0	0	100.0	1.000	Almost perfect

93.7% and 100.0% and most of them showed more than moderate agreement. However, the level of discordance between three assays was relatively high in HPV 31, 42, and 44. Furthermore, MolecuTech REBA HPV-ID could not detect any HPV 42, 44, 51, and 59 infection compared with other commercial kits.

To examine the performance of the assay in the detection of the five common HPV genotypes in invasive cervical cancer, the type-specific PCR with direct sequencing analysis was performed as a reference method. The sensitivity and specificity of three assays in HPV 16, 18, 45, 52, and 58 are summarized in Table 4. All three assays showed acceptable sensitivity (73.3% to 100.0%) in the detection of HPV genotypes 16 and 18. Otherwise, various sensitivities were showed for the detection HPV 45, 52, and 58 that the most sensitive assay was MolecuTech REBA HPV-ID for

HPV 45, and Anyplex II HPV28 Detection for HPV 52 and 58.

DISCUSSION

In this study, we compared two newly developed HPV genotyping kits with the previously used DNA microarray system. Although high-level agreement results were observed in negative samples, there were discordant results in positive samples among three assays; especially, the strength of the interassay agreement decreased in the detection of low-risk group HPV infections. It was well known that the analytical performance of three kits was affected by the storage condition of cervical swab samples or DNA extraction method (14). However, because the samples were processed under the same storage condition and because of the same DNA extraction method, we assumed that the discrepancy of screening capacity was more strongly influenced by other factors such as inclusivity of HPV genotypes and type-specific sensitivity.

Currently, there is no consent about inclusivity of HPV genotypes between HPV genotyping kits. Obviously, HPV 16 and 18 are considered as the most important genotypes for carcinogenicity (15), and some HPVs except HPV 16 and 18 are also categorized as carcinogenic, probably carcinogenic, or possibly carcinogenic according to the World Health Organization International Agency for Research on Cancer (16) and frequently found in cancers (17). Therefore, these HPV

TABLE 4. Diagnostic performances for the detection of major 5 high-risk HPV genotypes

HPV DNA type		16 (%)	18 (%)	45 (%)	52 (%)	58 (%)
MolecuTech REBA HPV-ID	Sensitivity	100.0	100.0	100.0	42.9	82.4
	Specificity	100.0	100.0	100.0	100.0	100.0
Anyplex II HPV28 Detection	Sensitivity	73.8	73.3	25.0	95.2	100.0
	Specificity	100.0	100.0	100.0	100.0	99.6
HPVDNAChip	Sensitivity	83.3	73.3	25.0	85.7	52.9
	Specificity	99.2	100.0	100.0	99.6	100.0

genotypes are classified as high-risk group HPVs and almost all HPV genotyping kits can detect these HPV genotypes due to clinical significance. However, there is a great discordance about inclusivity of other genotypes categorized as low-risk group. In this study, while 14 genotypes overlap in the detection of high-risk group HPVs between three genotyping assays, there are only 7 genotypes that overlap in the detection of low-risk group HPVs. These differences between HPV genotyping kits would probably affect the performance of assays as screening tool, especially in the test for low-risk type HPV infections. In addition, no uniform classification of carcinogenicity was applied among HPV genotyping assays that categorized high- or low-risk HPV genotypes in different ways, and this problem could also lead to discrepancy between HPV screening results. Therefore, to minimize the difference in performance as HPV screening tool, the efforts to standardization would be needed for inclusivity of HPV genotypes and classification of genotypes.

Analytical sensitivity is another issue to consider. Although there are more literatures insisting the reliable and accurate performance of all three assays included in this study (9, 12, 18–21), it was also well known that the analytical sensitivity differs among different kits due to the difference in their applied methods (20). These differences may lead to contradicted screening results between assays and make confusion in decision-making by clinicians. In addition, it is important that the previously reported papers about performance evaluation focused on the analytical sensitivity; however, this index was not validated with pathologic findings. Thus, analytical sensitive test could make a large number of clinically insignificant positive results and contribute to psychological distress in healthy women (9). No test could be perfect and the reasonable efforts would be needed to improve analytical sensitivity to detect specific HPV genotypes; however, too much is never enough. The analytical sensitivity for all HPV genotypes included in each assay should be adjusted to the result of cervical cytology and both clinical sensitivity and specificity should be considered most important (22).

In the genotype-specific agreement between three assays, at least substantial agreements were observed for most of HPV genotypes and especially almost perfect agreement for HPV 16 and 18 which are the most important carcinogenic genotypes. Compared with the reference method, all three kits showed appropriate sensitivity and specificity for the detection of HPV 16 and 18. However, for HPV 31, 42, and 44, there were fair agreement between assays and the MolecuTech REBA HPV-ID detected no HPV 42,

44, 51, and 59 infection when compared with other genotyping kits retrospectively. We made an inquiry about these problems to manufacturer and received replies that the most the of MolecuTech REBA HPV-ID kits that we used in this study had a problem during purification of genotype-specific probe which were embedded in membrane strip. After that, manufacturer announced us that new assays solved probe problems by changing the purification method, but unfortunately we could not perform the MolecuTech REBA HPV-ID due to insufficient amount of clinical samples.

Quality assurance has been a very important role in HPV genotyping kit (23). Because enormous genotypes are detected simultaneously in one run of test, the quality control of kit makes up a large portion of the reliability of the test. All three kits include internal control to ensure the successful steps of DNA extraction to amplification. Nevertheless, each commercially developed three kits have their own positive controls according to different policies. While Anyplex II HPV28 Detection includes 3 positive control tubes that cover all HPV genotypes detected in Anyplex II HPV28 Detection, MolecuTech REBA HPV-ID and HPV-DNAChip provide only one positive control tube that covers only one HPV genotype (HPV 33 and 16, respectively). Due to lack of positive control in other HPV genotypes, one cannot find out the false-negative result until the test result is compared with other laboratories such as external quality assessment/proficiency test (EQA/PT) (24). In this study, we could not recognize the false-negative results of several HPV genotypes in MolecuTech REBA HPV-ID until the genotyping results with other assays had been accumulated. To resolve this problem, anticipating EQA/PT would not be a proper solution because EQA/PT could not cover all HPV genotypes for all commercially available HPV genotyping kits. However, manufacturer should be responsible for this problem and it is necessary to provide positive controls of all HPV genotypes for each HPV genotyping kits.

In conclusion, we evaluated the recently developed reverse blot hybridization assay and real-time PCR-based assay for the detection of HPV genotypes in comparison with PCR-based DNA microarray assay. These three assays showed dissimilar performance as a screening tool for HPV infection and moderate concordance in HPV genotyping. Due to lack of standardization and no optimization of clinical sensitivity and specificity, discrepancy between kits would be inevitable. Therefore, we suggested that the effort to standardization of HPV genotyping kits would be important for the best clinical performance.

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