

Prevalence of Human Papillomavirus Infection and Genotype Distribution Determined via Real-Time PCR in a Korean Medical Check-up Population

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Received: March 6, 2018 / Accepted: March 29, 2018

Human papillomavirus (HPV) plays a critical role in the development of cervical carcinoma. This study analyzed the efficiency of multiplex real-time PCR in detecting and identifying HPV genotypes in samples from women who visited a Korean hospital for checkups. Cervical swab specimens were obtained from women who attended a checkup at the Health Improvement Center of Hospital in Dankook University Cheonan, South Korea and were referred for an HPV genotyping test between January and September 2014. A total of 1703 cervical swab specimens were collected consecutively during this period. PCR results were compared with those of the traditional cytological assay for the same population. Among the 1,703 specimens, 19.91% were HPV positive, of which 14.50% indicated a single infection and 5.40% indicated multiple infections. However, cytology identified only 2.52% of positive cases, including 1.23% cases of atypical squamous cells of undetermined significance, 1% of low grade squamous intra-epithelial lesion, and 0.29% of high grade squamous intra-epithelial lesion. The rate of high-risk and low-risk HPV in the abnormal cytology group was 48 and 23, respectively, and 274 and 136 in the normal group, respectively. HPV types 56, 52, 43 were the most prevalent in that order. Our results confirm the efficiency of the HPV DNA assay for the detection of 28 different HPV genotypes with reasonable sensitivity. A screening strategy that comprises the HPV DNA assay and cytology would help overcome the low sensitivity of a cytological diagnosis.

Keywords: Human papillomavirus, HPV, genotyping, real-time PCR

Introduction

Cervical carcinoma is the second most common malignancy among women worldwide, and the incidence rate of cervical cancer is below those of breast, gastric, and colorectal cancer, with over 5,000 new patients diagnosed every year in South Korea [1, 2]. Human papillomavirus (HPV) appears to be the most common sexually transmitted pathogen in the world [3, 4], and the critical role of HPV in cervical cancer has been clearly eluci-

dated [5–9]. Moreover, HPV has been found to cause cancer of the anus, penis, vulva, vagina, and oropharynx. Exposure to HPV is very common; the prevalence of infection is estimated to be 43–62% using genital samples, and the lifetime prevalence is certainly higher. Although transmitted primarily via sexual contact, HPV can also be transmitted by less intimate skin-to-skin contact, and most HPV infections are asymptomatic [10]. The majority of women are usually infected after they become sexually active, and most infections are cleared within 2–3 years; however, women with persistent infection are at high risk for developing high-grade cervical intraepithelial neoplasia (CIN) and invasive cancer years later [11, 12].

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HPV, a large genus of the Papillomaviridae family, is a nonenveloped virus with circular double-stranded DNA, measuring approximately 7.9 kb [13]. More than 100 genotypes of HPV have been identified, and around 20 distinct HPV types have been associated with cervical carcinoma [14]. Depending on the oncogenic potential, HPV genotypes are categorized into high-risk types, which include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, 73, and 82, and low-risk types, which include HPV 6, 11, 42, 43, and 44 [15]. High-risk types of HPV are considered the main risk factors for cervical cancer, with HPV 16 being the most prevalent type in women with normal cytology and women with cervical carcinoma or CIN, whereas low-risk types such as HPV 6 and 11 generally only cause benign lesions or have been rarely associated with cervical carcinoma [1, 16, 17]. Furthermore, HPV 16 and 18 mainly cause up to 70% of cervical cancers worldwide [18]. There is an accepted global classification of HPV types according to risk; however, the classification of high-risk HPV types still differs in each study owing to limitations in sensitivity and the diagnostic accuracy of cytology-based screening techniques [4, 19, 20]. Studies show that the sensitivity of a cytological test for high-grade CIN was only 40–80% [21–23]. Accurate identification of the correct HPV genotype is of great importance to ensure accurate diagnosis and prognosis, as well as effective monitoring and therapeutic options that are based on the risk type [24]. In addition, HPV genotyping can be used as a health screening test to prevent cervical cancer in asymptomatic women [25, 26].

To the best of our knowledge, this is the first study that provides epidemiologic data on HPV genotypes in the general female population of a local area in Korea, by comparing the analytical results of HPV detection between the traditional cytological assay and the HPV DNA multiplex real-time PCR assay. In this study, we identified HPV genotypes in women from Cheonan, South Korea, with no obstetric or gynecologic symptoms, by using a multiplex real-time PCR-based assay that can specifically detect 28 different HPV types, to understand the overall and type- and age-specific HPV prevalence and extent of multiple infections in this population. In addition, we assessed and compared the efficiency of HPV detection between the cytological diagnosis and the multiplex real-time PCR assay. The results from our

analysis can provide basic information essential to the development of vaccination strategies for the prevention of single and multiple HPV infections and for use in public women health programs based on HPV testing.

Materials and Methods

Population

The present study was approved by the institutional review board (IRB) of Dankook University (IRB Approval No: 2016-08-009). The data were collected retrospectively. In this study, cervical swab specimens were obtained from women aged 21–73 years who attended a checkup at the Health Improvement Center of Dankook University Hospital in Cheonan, South Korea, and were referred for an HPV genotyping test between January and September 2014. A total of 1703 cervical swab specimens were collected from 1703 patients, consecutively during this period, and the subjects were enrolled in the study. They were all referred for cytological examination and HPV testing. Each subject's medical records were reviewed using an electronic medical records system to collect information on age and cytology results. The cervical swabs were collected using a cervical brush and stored in a specimen transport medium for PCR (Digene, MD, USA).

Cytology

The cytological classification of the clinical specimens was performed by experienced cytopathologists and reported according to the Bethesda System, which included normal, atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), and high-grade intraepithelial lesions (HSIL).

HPV DNA detection and quantification via multiplex real-time PCR

The nucleic acids from the clinical specimens were extracted from a 350- μ l sample using a QIAcube platform (Qiagen, Germany). DNA in the extracted nucleic acids was then amplified and tested for the detection and genotyping of HPV using the AnyplexTM II HPV28 Detection kit (Seegene, Korea) and CFX96 real-time thermocycler (Bio-Rad, USA) according to the manufacturer's instructions. The AnyplexTM II HPV28 Detection

kit adopted the Tagging Oligonucleotide Cleavage and Extension (TOCE) technology, which can simultaneously detect up to 19 strains of high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 strains of low-risk HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70) in a single specimen. The L1 gene of HPV DNA was amplified, and simultaneously, the human housekeeping gene (human beta-globin) was co-amplified as an internal control of the PCR process.

Each PCR reaction was briefly performed in a 20- μ l reaction mixture consisting of 5 μ l of extracted DNA, 1 \times HPV28 TOCE oligo mix, and AnyplexTM PCR Mix. The thermal cycling conditions consisted of denaturation at 95 $^{\circ}$ C for 15 min, followed by 50 cycles of denaturation (30 s at 95 $^{\circ}$ C), annealing (1 min at 60 $^{\circ}$ C), and elongation (30 s at 72 $^{\circ}$ C). Cyclic Catcher Melting Temperature Analysis was performed after PCR cycles 30, 40, and 50 by cooling the reaction mixture to 55 $^{\circ}$ C, holding at 55 $^{\circ}$ C for 30 s, and heating from 55 $^{\circ}$ C to 85 $^{\circ}$ C (5 s/0.5 $^{\circ}$ C) with continuous fluorescent monitoring.

Statistical analysis

The prevalence and 95% confidence intervals were calculated for the overall genotypes and each individual HPV genotype. HPV prevalence was analyzed by age group (20–29, 30–39, 40–49, and \geq 50 years), cytology results were based on the Bethesda system, and the semiquantitative levels of HPV DNA (grades 1, 2, and 3) were obtained via the AnyplexTM II HPV28 Detection kit. The semiquantitative levels were determined as the number of copies per reaction of each detected HPV type and were categorized as follows: grade 1, <102 copies/reaction; grade 2, \geq 102 but <105 copies/reaction; and grade 3, \geq 105 copies/reaction. All statistical analyses were performed using a χ^2 -test. All *p*-values <0.05 were considered statistically significant.

Results

From January to September 2014, a total of 1703 female patients aged 21–73 years who attended a checkup at the Health Improvement Center of Dankook University Hospital were enrolled in this study. The average age of the cohort was 37.1 ± 8.3 years. HPV prevalence in the collected samples was analyzed via

Table 1. Prevalence of HPV infection diagnosed via cytology and the HPV DNA PCR test.

	Number of patients (%)	Average age (year)
Cytology		
Negative	1660 (97.48)	37.2 \pm 8.3
Abnormal	ASCUS	21 (1.23)
	LSIL	17 (1.00)
	HSIL	5 (0.29)
	Total	43 (2.52)
HPV DNA PCR		
Negative	1364 (80.09)	37.8 \pm 8.1
Positive	Single infection	247 (14.50)
	Multiple infection	92 (5.40)
	Total	339 (19.91)

HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade intraepithelial lesions.

cytological and multiplex real-time PCR assays.

HPV detection via cytological diagnosis

Among the 1703 specimens, 97.48% yielded negative cytology results, whereas 2.52% demonstrated positive cytology results. Table 1 shows the average age of the negative results group and the different HPV groups. HPV prevalence by age is shown in Fig. 1. The number of patients who tested positive for HPV according to the

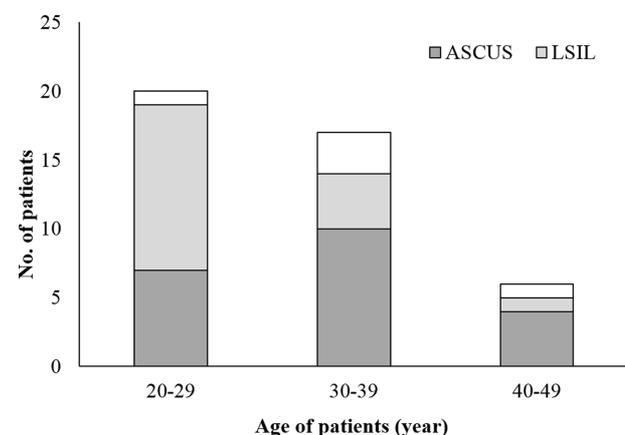


Fig. 1. Human papillomavirus (HPV) infection prevalence according to age. ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade intraepithelial lesions.

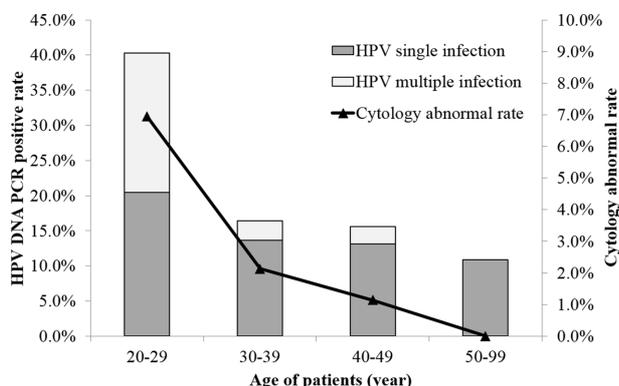


Fig. 2. Prevalence of single and multiple human papillomavirus (HPV) infections using an HPV DNA PCR assay in association with the results of the cytological diagnosis.

cytological classification (ASCUS, LSIL, and HSIL groups) and age group was as follows: 7, 12, and 1 (20–29 years); 10, 4, and 3 (30–39 years); and 4, 1, and 1 (40–49 years), respectively (Fig. 1).

HPV prevalence via HPV DNA multiplex real-time PCR

HPV prevalence in the collected specimens was also tested and analyzed using HPV DNA multiplex real-time PCR. A total of 339 samples (19.91%) tested positive, and the average age of the patients was 34.1 ± 8.7 years. The prevalence of single and multiple HPV infections in this population and the average age of the patients are presented in Table 1. The distribution of single and multiple HPV infections associated with age is also shown in Fig. 2. The single infection rates according to age group in the HPV-positive patients were 20.49% (20–29 years), 13.66% (30–39 years), 13.14% (40–49 years), and 10.87% (50–99 years), whereas the

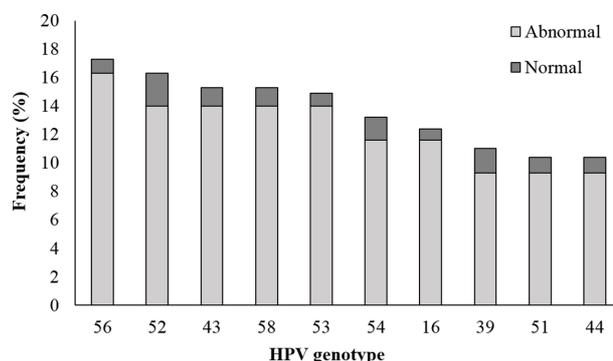


Fig. 3. Distribution of human papillomavirus (HPV) genotypes in HPV-positive samples including samples classified per a cytological diagnosis. The genotypes were determined using the Anyplex™ II HPV28 Detection kit. The error bar indicates 95% confidence intervals.

multiple infection rates were 19.79% (20–29 years), 2.76% (30–39 years), and 2.48% (40–49 years). In comparison with the multiplex real-time PCR results, the rate of abnormal cytology according to age group was as follows: 6.94% (20–29 years), 2.13% (30–39 years), and 1.14% (40–49 years). The ≥50-year-old age group showed no abnormality in the cytological diagnosis (Fig. 2).

HPV genotype analysis and viral load determination

Among the 1703 clinical samples, 481 HPV genotypes were isolated in 339 HPV-positive samples using multiplex real-time PCR. Regardless of multiple infections, the prevalence of high-risk HPV genotypes in the HPV-positive samples was 18.9% (322/1703), whereas that of low-risk HPV genotypes was 9.3% (159/1703). The most common HPV genotype was HPV 56 (17.3%), followed by HPV 52 (16.3%), 43 (15.3%), 58 (15.3%), 53 (14.9%), 54

Table 2. Isolated virus in the HPV-positive group.

Cytology		Number of patients (%)	HPV DNA multiplex real-time PCR		
			Virus type	Number of high-risk types (% of virus no.)	Number of low-risk types (% of virus no.)
Abnormal cytology	ASCUS	13 (61.9)	26	18 (69.2)	8 (30.8)
	LSIL	16 (94.1)	37	23 (62.2)	14 (37.8)
	HSIL	5 (100.0)	8	7 (87.5)	1 (12.5)
Abnormal Total		34 (79.1)	71	48 (67.6)	23 (32.4)
Normal cytology		305 (18.4)	410	274 (66.8)	136 (33.2)
Total		339	481	322 (66.9)	159 (33.1)

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade intraepithelial lesions.

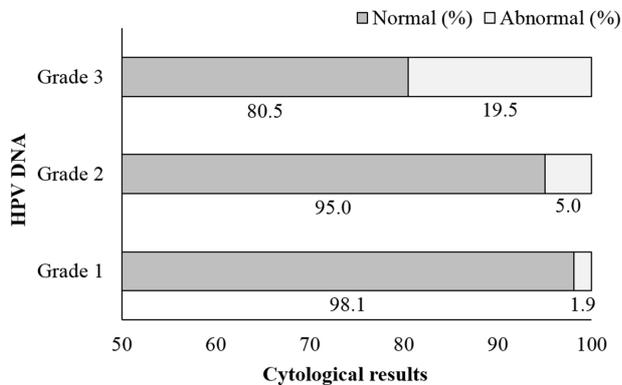


Fig. 4. Human papillomavirus (HPV) DNA load according to the cytological diagnostic result. The levels of HPV DNA were determined by the number of copies per reaction of each detected HPV type and were categorized as follows: grade 1, $<10^2$ copies/reaction; grade 2, $\geq 10^2$ but $<10^5$ copies/reaction; and grade 3, $\geq 10^5$ copies/reaction.

(13.2%), 16 (12.4%), 39 (11%), 51 (10.4%), and 44 (10.4%) (Fig. 3).

Next, we analyzed the number of HPV genotypes detected in the normal and abnormal cytology groups. A total of 71 HPV genotypes were detected in the abnormal group, whereas 410 HPV genotypes were detected in the normal group (diagnosed as normal per the cytological assay). According to the multiplex real-time PCR results, the prevalence of HPV genotypes in the abnormal group was as follows: HPV 56 (16.3%), 52 (14.0%), 43 (14.0%), 58 (14.0%), 53 (14.0%), 54 (11.6%), 16 (11.6%), 39 (9.3%), 51 (9.3%), and 44 (9.3%). Meanwhile, the prevalence of HPV genotypes in the normal group was as follows: HPV 52 (2.3%), 39 (1.7%), 54 (1.6%), 43 (1.3%), 58 (1.3%), 51 (1.1%), 44 (1.1%), 56 (1%), 53

(0.9%), and 16 (0.8%).

Among the isolated HPV genotypes, high-risk HPV subtypes were found in 67.6% (48/71) of the abnormal group and 66.8% (274/410) of the normal group, while low-risk HPV subtypes were found in 32.4% (23/71) of the abnormal group and 33.2% (136/410) of the normal group. The distribution of high-risk and low-risk HPV genotypes in the ASCUS, LSIL, and HSIL subgroups is shown in Table 2.

After analyzing the results of HPV genotyping, the HPV load was determined and classified as grade 1, 2, or 3 according to the percentage of viral copies of HPV detected by the cytological assay. The distribution of HPV DNA grades 1, 2, and 3 was 98.1%, 95%, and 80.5% in the normal group and 1.9%, 5%, and 19.5% in the abnormal group, respectively (Fig. 4).

Comparing the diagnostic results between the cytological assay and the HPV DNA multiplex real-time PCR assay

Table 3 shows the prevalence of single and multiple HPV infections detected using HPV DNA multiplex real-time PCR in the normal and abnormal cytology groups. Overall HPV prevalence in the abnormal group was 79.1%, with 37.2% (16/43) of the patients harboring a single infection and 41.9% (18/43) harboring multiple infections. Among these HPV infections in the abnormal group, the rate of HPV-positive samples against each result of the cytology test was 61.9% for ASCUS, 94.1% for LSIL, and 100% for HSIL. Moreover, HPV DNA was detected in 305 (18.4%) of the 1660 samples that were reported as normal cytology, and the proportion of single and multiple HPV infections was 13.9% (231/1660) and 4.5% (74/1660), respectively.

Table 3. Cross analysis for HPV infection type between HPV DNA PCR and cytology.

Cytology	HPV DNA PCR				
	Number of patients	Single infection (%)	Multiple infection (%)	Total no. of HPV-positive cases (%)	
Abnormal cytology	ASCUS	21	8/21 (38.1)	5/21 (23.8)	13/21 (61.9)
	LSIL	17	6/17 (35.3)	10/17 (58.8)	16/17 (94.1)
	HSIL	5	2/5 (40.0)	3/5 (60.0)	5/5 (100.0)
	Total	43	16/43 (37.2)	18/43 (41.9)	34/43 (79.1)
Normal cytology	1660	231/1660 (13.9)	74/1660 (4.5)	305/1660 (18.4)	

HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade intraepithelial lesions.

Discussion

The prevalence rates of HPV infection in women of the general population have ranged from 1.6% to 41.9% depending on the region and nation [27]. Particularly, the HPV-positive rates in Korea were 19.2% and 16.7% in 2012 and 2014, respectively [7, 14]; this was similar to the 19.91% HPV-positive rate among 1703 subjects diagnosed using the multiplex real-time PCR assay in this study, whereas the cytology-based positive rate was only 2.52% using the same specimens (Table 1). Furthermore, HPV DNA was detected using multiplex real-time PCR in 305 (18.4%) patients with a negative diagnosis via the cytological assay (Table 2). Cases of HPV DNA detection in women with normal cytology have also been observed in previous studies [28, 29].

HPV infections are shown to have a common age-specific distribution with a sharp peak in prevalence among young women following sexual activity [30]. In this study, the cytological positive rate and HPV detection rate were higher in women aged 20–29 years than in the other age groups by 6.9% and 40.3%, respectively, and the prevalence and distribution of single and multiple HPV infections also peaked in women aged 20–29 years by 20.5% and 19.8%, respectively. Moreover, the rate of single infection was two times higher in women aged 50–59 years, and that of multiple infections was eight times higher in women aged 40–49 years. Generally, the frequency of multiple infections is high in women aged <35 years [31], and young age is one of the main risk factors for HPV infection [4]. Similarly, the HPV positive rate in Korea has been highest in women aged <39 years [32], and women in their twenties demonstrate shown twice the rate of HPV infection compared with women aged >30 years [14]. In this study, the HPV positive rate also decreased with advancing years and showed a very similar tendency with that in other previous reports.

HPV genotypes are divided into high-risk and low-risk groups depending on their oncogenic potential [33, 34]. Among the high-risk HPV group, HPV 16 has the highest infection rate worldwide [35]. However, several studies on HPV distribution in Asia, including Korea, have shown different results from those in the USA or Europe [1, 5, 6, 16, 25]. HPV detection rates in China in decreasing order of prevalence [15] has been HPV 52, 16, and 58, whereas that in Korea has been HPV 70, 16, and 33

[36]; HPV 18, 16, and 58 [32]; or HPV 16, 53, 56, and 58 [7]. As shown in Fig. 3, the order of HPV detection rates in this study performed in Cheonan, Korea, was HPV 56, 52, 43, 58, 53, 54, and 16, which is quite different from the reported HPV genotype distributions in the USA and Europe. The difference in HPV prevalence in this study might be caused by ethnicity-dependent sensitivity, geographical variation in HPV genotypes, and HPV detection methods. Overall, HPV 56, 52, and 58 were thus classified as potential high-risk types, but their detection frequency is relatively lower in Europe and the USA than in Asia. These results indicate that diverse and continuous research on HPV prevalence and genotyping in Asia, including Korea, is necessary.

The viral load in HPV infection is correlated with the risk of cervical dysplasia [14]. Persistent HPV infection induces a high viral load, and is more likely to lead to HSIL, 200 times more than in HPV-negative patients; moreover, persistent HPV infection plays a critical role in cervical carcinoma [37–41]. In this study, 481 HPV genotypes were detected in the specimens using multiplex real-time PCR, including 71 and 410 types in the abnormal and normal cytology groups, respectively. The results in our study also showed that the proportion of abnormal rates increased with the grade of viral load, from 1.9% with grade 1 to 19.5% with grade 3 (Fig. 4).

The clinical importance of multiple infections has been reported with varying clinical results [42–44]. Chan *et al.* [45] and Bosch *et al.* [33] reported that multiple HPV infections did not show any significant association with cervical cancer or uterine cervical dysplasia, as opposed to single infection. Meanwhile, Sasagawa *et al.* [46] reported that multiple HPV infections were associated with CIN and cervical cancer. Multiple HPV infections have been also shown to weaken the immune response and increase the severity of cervical neoplasia to a greater extent than single HPV infection [46, 47]. Furthermore, several HPV types such as HPV 18 and 51 in combination as concurrent or recurrent infections act synergistically in cervical carcinogenesis [48, 49]. In our study, the prevalence rate of multiple HPV infections, which was 27.1% among the HPV-positive group and 5.4% among the total samples, was higher than those reported in previous studies, such as 10.6% and 19.6% [45], respectively. Additionally, the rate of multiple HPV infections increased in a cytological severity-dependent

manner. In the abnormal cytology group, the rate of multiple HPV infections was higher than that of single infection. In contrast, the rate of single infection was higher than that of multiple infections in the normal cytology group.

We analyzed the efficiency of HPV DNA multiplex real-time PCR in detecting HPV infection and identified HPV genotypes in 1703 swab samples from women who visited the hospital for a health checkup, and the diagnostic results were compared with the results obtained via the cytological assay for the same population. With a detection rate of 19.91%, the HPV DNA multiplex real-time PCR assay showed higher sensitivity and efficacy for HPV detection than the cytological assay, which had a detection rate of 2.52%. Moreover, the HPV DNA assay detected 305 positive cases among the 1660 samples classified as normal via the cytological assay.

Hence, the addition of an HPV DNA multiplex real-time PCR assay to the current cytological diagnostic assay enables more precise HPV diagnosis; contributes to the prevention, treatment, and long-term management of cervical cancer; and is essential to public health-care programs.

Acknowledgments

The authors acknowledge Dankook University, which provided funding for this research in 2015.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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