Experimental and Molecular Pathology 106 (2019) 149–156

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Analytical performance evaluation of the HPV OncoCheck assay for detection of high-risk HPV infection in liquid-based cervical samples

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ARTICLE INFO

Keywords:
Cervical cancer
Human papillomavirus
E6/E7 mRNA
Reverse transcriptase-quantitative PCR
Molecular diagnosis

ABSTRACT

Recent studies have demonstrated low specificity (false positive) of human papillomavirus (HPV) DNA testing for the screening and diagnosis of cervical samples. Therefore, we evaluated the performance of the HPV OncoCheck assay, which is an HPV E6/E7 mRNA-based assay, for the detection of 16 high-risk (HR)-HPVs including HPV 16 and HPV 18 genotypes in cervical samples using multiplex reverse transcriptase-quantitative PCR. In the present study, the analytical performance of the assay was evaluated using 16 HPV single strand DNAs. Clinical evaluation was performed using 319 Thinprep® liquid-based cytology samples obtained from women with cervical diseases, and the HPV OncoCheck assay results were compared with those of cytological diagnosis and sequence analysis.

All 16 types of HPVs were detected with a minimum detection sensitivity of 100 copies per reaction and high specificity was observed. The sensitivity and specificity of the HPV OncoCheck assay for detecting high-grade lesions were 94.1% (95% confidence interval (CI), 0.875–0.975; $p < .0001$) and 95.4% (95% CI, 0.868–0.989; $p < .0001$), and sequence analysis were 99.4% (95% CI, 0.965–0.999; $p < .0001$), and 98% (95% CI, 0.939–0.996; $p < .0001$), respectively. Moreover, the agreement between the HPV OncoCheck assay and sequence analysis for the detection of HR-HPV was 98.8% ($\kappa = 0.98$, 95% CI 0.967–0.996; $p < .0001$). The results of this study showed high agreement and specificity with cytological diagnoses and sequence analysis. Future studies with histological follow- up are needed to determine whether use of the HPV OncoCheck assay in cervical screening may aid detection of the most significant cervical disease while reducing false-positive results.

1. Introduction

Cervical cancer is the third most common cancer in females affecting 528,000 women each year and leading to 266,000 deaths worldwide (Ferlay et al., 2015). Persistent infection with at least one of the high-risk (HR) oncogenic human papillomavirus (HPV) genotypes is the primary risk factor for the development of cervical cancer (Guan et al., 2012). Therefore, testing for oncogenic HPV infection in cervical lesions may be an accurate means of identifying women who are at a risk of developing cervical cancer. > 100 types of HPV have been identified (Muñoz et al., 2003) and approximately 40 types of HPV infect the genital tract (Bosch et al., 2002). Of these, at least 13 HR-HPV genotypes—including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68—are detected in up to 99.7% of all cervical cancer cases and high-grade cervical lesions cases (Arbyn et al., 2014); particularly, HPV 16 and 18 are associated with a higher risk of cervical cancer progression worldwide, accounting for > 70% of the cases (Zeng et al., 2016).

In 2009, the American Society for Colposcopy and Cervical Pathology implemented clinical guidelines for individualized examinations that focused on the combination of an HPV DNA test and a Pap test for cervical cancer screening; HPV DNA testing has been considered as an adjunctive test for all cervical cytology samples in women over 30 years of age according to the current recommendations (Darragh et al., 2012). In 2014, the HPV DNA test was approved as a primary screening method by the Korea Ministry of Food and Drug Safety (Min et al., 2015). Accordingly, various assays, including probe hybridization, PCR, and DNA microarray, have been developed, and are now commercially available for the detection of HPV DNA to facilitate early cancer screening in clinical samples. The current HPV DNA test, mostly targeting the *L1* gene, has been reported to have the limitation of low specificity (false-positive results) and there is a lack of

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https://doi.org/10.1016/j.yexmp.2018.12.008
Received 8 October 2018; Received in revised form 2 December 2018; Accepted 29 December 2018
Available online 31 December 2018
0014-4800/

association with the prognosis of the patient (Schiffman and Solomon, 2009; Persson et al., 2012). Therefore, efficient biomarkers and new screening methods for diagnosing cervical lesions are required to improve the treatment of cervical cancer (Fan and Shen, 2018).

Due to the importance of HPV infection in the diagnosis of cervical cancer, the upregulation of HPV oncoproteins has been studied as a marker for increased risk of cervical cancer (Ratnam et al., 2010). Recently, commercialized diagnostic kits, such as APTIMA HPV assay, Hologic; HPV OncoTect 3Dx™, IncellDx; Nuclisens EasyQ HPV E6/E7 mRNA, Biomerieux, for cervical cancer screening based on HPV E6/E7 mRNA expression has been introduced (Coquillard et al., 2011; Binnicker et al., 2014; Munkhdelger et al., 2014) and has shown promise in increasing the specificity of HPV testing (Lie and Dristensen, 2008). Previous studies have reported that detection of these mRNA targets is more specific than detection of corresponding DNA targets and can predict women who would progress toward high-grade cervical cancer (Dockter et al., 2009). Expression of HPV E6/E7 mRNA is essential for the development and progression of cervical cancer. The E6 and E7 proteins stimulate the cell cycle by binding to and inactivating cellular p53 and pRb, leading to their degradation via the proteasomal pathway and, ultimately, to cervical cancer (McLaughlin-Drubin and Münger, 2009; Brimer and Vande Pol, 2014). These two oncogenes are uniformly retained and highly expressed in cervical cancer cells, and their continuous expression is required for maintaining the tumorigenic phenotype.

As women infected with HPV 16 and/or HPV 18 are more likely to develop cervical intraepithelial neoplasia grade 2 or worse lesions than women infected with other HR-HPV genotypes (Bulk et al., 2007), the HPV OncoCheck—based on HPV E6/E7 mRNA expression—is an assay that can distinguish between the HPV 16 and HPV 18 genotypes and can detect 16 HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69) added in 12 HR-HPV types classified as IARC carcinogenicity group 1 (Bouvard et al., 2009; IARC, 2012). Furthermore, the HPV OncoCheck assay includes an internal control (IC) in each well to determine whether samples contain PCR inhibitors. In the present study, we assessed the analytical and clinical performance of the HPV OncoCheck assay (Optipharm, Osong, Republic of Korea) as a potential cervical cancer screening method using multiplex reverse transcriptase-quantitative PCR (RT-qPCR).

2. Materials and methods

2.1. HPV single strand DNA control

HPV single strand (ss) DNA was prepared as the positive control to determine the analytical sensitivity and specificity of the HPV OncoCheck assay. It was synthesized by Integrated DNA Technologies (IDT; Coralville, IA) with each of the 16 HPV genotypes at E6 and E7 regions using GenBank BLAST and was adjusted to 1×10^{12} copies with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

2.2. Cell lines

SiHa, HeLa, and C33A cell lines were purchased from American Type Culture Collection (ATCC; Manassas, USA) and Korean Cell Line Bank (Seoul, Republic of Korea). SiHa and HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. C33A cells were cultured in MEM with Earle's BSS and 10% FBS. Cells were counted using the T20™ Automated cell counter (Bio-Rad, Hercules, USA) according to the manufacturer's instructions, and each cell was measured as 10^6 cells/mL.

2.3. Clinical samples and cytological diagnosis

Liquid-based cytology samples from 319 women were obtained over a 1-year period (2014–2015) at Yonsei University Wonju Severance Christian Hospital, Wonju, Republic of Korea. This study was approved by the Institutional Ethics Committee at Yonsei University Wonju College of Medicine, and all patients provided written informed consent. All clinical samples were collected using ThinPrep® (Hologic, Inc., Bedford, MA, USA) Pap materials containing PreservCyt solution. For liquid-based cytology slides (Pap smears) were carried out according to the manufacturer's recommendations. After preparing the Pap slide, 1 mL of an exfoliated cervical cell sample was transferred to each of the two 1.5-mL microcentrifuge tubes and stored at −70 °C until use. The clinical diagnosis was performed by cytopathologists using the 2001 Bethesda System terminology (Solomon et al., 2002). Cytological cases of squamous cell carcinoma (SCC), high-grade squamous intraepithelial lesion (H-SIL), low-grade SIL, typical squamous cells of undetermined significance (ASC-US), and normal (includes reactive change due to inflammation, fungal infection, and atrophy) were included. The remaining fluid samples were stored at 4 °C after cytology slide preparation, but before RNA extraction.

2.4. Total RNA isolation

After cytological slide preparation, a 3 ml of remaining fluid sample was used for RNA isolation. Total cellular RNA was isolated using the High Pure RNA Isolation Kit no. 11828665001 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 400 μL of lysis/binding buffer was added to the cell pellet. Cells were lysed by vortexing or repeated pipetting and allowed to stand at 25 °C for 15 s. The mixture was transferred to a High Pure filter tube and centrifuged at 8,000 × *g* for 15 s. Add 500 μL of Wash buffer I and II to the upper reservoir of the filter tube assembly and was centrifuged at 8,000 × *g* for 15 s, respectively. Finally, 200 μL of Wash buffer II was added to the upper reservoir of the filter tube, centrifuge 13,000 × *g* for 2 min to remove any residual Wash buffer, discard the collection tube, and was transferred to a new tube. The RNA pellet was dried and eluted in 50 μL of elution buffer to the upper reservoir. The purity and concentration of total RNA were determined by measuring the absorbance at 260 and 280 nm using an Infinite 200® spectrophotometer (Tecan, Vienna, Austria). All steps in the preparation and handling of total RNA were conducted in a laminar flow hood under RNase-free conditions. The isolated total RNA was stored at −70 °C until use.

2.5. cDNA synthesis

cDNA was synthesized using the PrimeScript™ RT Master Mix (Perfect Real Time; TAKARA Bio, Shiga, Japan), which was designed to perform reverse transcription (RT) optimized for two-step RT-qPCR, according to the manufacturer's recommendations. Briefly, 16 μL of total RNA was added to the master mix comprising 4 μL of the 5× premixed reagent containing all components needed for RT-qPCR (PrimeScript RTase, RNase inhibitor, random 6-mers, oligo dT primer, dNTP mixture, and reaction buffer) in PCR tubes. The cDNA synthesis reaction was performed at 37 °C for 15 min (reverse transctiption) and 85 °C for 5 s (for heat inactivation of RT).

2.6. HR-HPV E6/E7 mRNA assay

Detection of HPV E6/E7 mRNA in cervical samples was performed with HPV OncoCheck (Optipharm), a quantitative reverse transcriptase-PCR (RT-qPCR)-based assay, using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) for thermocycling and fluorescence detection. The assay consisted three different sets of HR-HPVs and detected 16 HR-HPV genotypes in three tubes [Group I: HPV16 (FAM), HPV 31, 33, 35, 52, and 58 (HEX), and internal control (IC) (Cy5); Group II: HPV 18 (FAM), HPV 39, 45, 51, and 68 (HEX), and IC (Cy5); and Group III: HPV 53, 56, 59, 66, and 69 (HEX), and IC (Cy5)] by incorporating specific TaqMan probes labeled with different

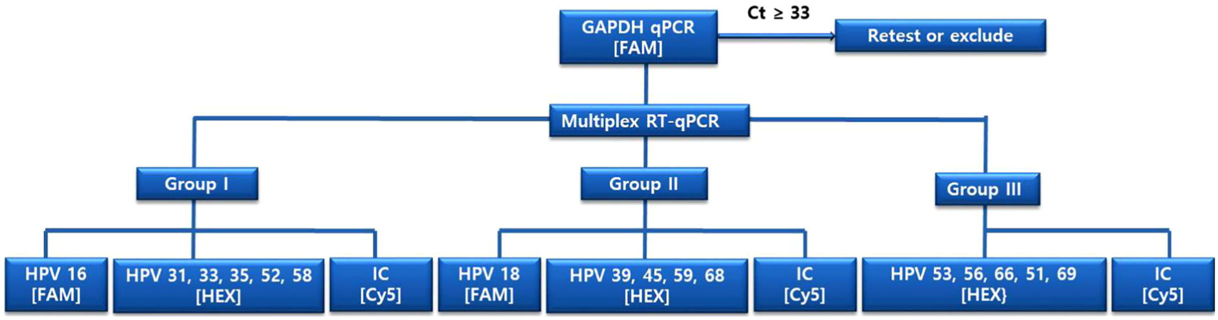

Fig. 1. Scheme of the HPV OncoCheck assay.

fluorophores (Fig. 1). The results can be also visualized separately for each group. Each real-time PCR kit contained IC DNA, which was used to indicate successful nucleic acid extraction, the quality of the sample, and to check for the presence of PCR inhibitors in the reaction. IC DNA was designed to have minimal sequence similarity to the target gene and served to detect false-negatives. Therefore, it did not directly compete with the amplification of the species-specific target in multiplex real-time PCR. When no target was detected, positive detection of IC DNA indicated the absence of PCR inhibition and that the concentration of target in the sample was below the detection limit. The IC signal showed a constant value under the following conditions: successful nucleic acid extraction, satisfactory sample quality, absence of PCR inhibition. Real-time PCR amplification of HPV E6/E7 mRNA was performed in a reaction mixture containing 10 µL of 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 µL of primer and TaqMan probe mixture, 2 µL of template cDNA, and distilled water (DW) to a final volume of 20 µL for each sample. Positive and negative controls were included throughout the procedure. No-template controls containing sterile DW rather than template DNA were incorporated in each run. Each sample was tested in duplicate, and all PCR runs were performed in duplicate. PCR cycling conditions were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 3 s, and 55 °C for 30 s. The mRNA expression level was quantified by determining the cycle threshold (C_T), which is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than that of background fluorescence. To avoid false-negatives because of mRNA degradation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control, and C_T values of ≥33 were excluded. A positive result for Group I, II, and III was indicated when the C_T value was < 35 after observing signal formation of wavelength from each channel. Target gene mRNA expression levels relative to GAPDH were automatically calculated using the comparative quantification cycle values (Cq) method using the CFX Manager Software v3.0 (Bio-Rad).

2.7. Sequence analysis

To confirm HR-HPVs identified by the HPV OncoCheck assay, HPV E6/E7 gene regions were sequenced. The primer sets used to amplify the target HPV E6/E7 gene were 60F-5′-CCGAAAMCGGTKVRTATAA-AAGCA-3′ and 970R-5′-GTACCTKCWGGATCAGCCAT-3′. Amplified PCR products were sequenced using the ABI Prism BigDye Terminator and ABI 3730 automated DNA sequencer (Cosmo Genetech, Seoul, Republic of Korea). The obtained sequences were compared with sequences of the National Center for Biotechnology Information GenBank database for species assignment.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 5.02 (GraphPad, Inc., La Jolla, CA, USA) and SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). For the HPV OncoCheck assay, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and 95% confidence interval (CI) of predictive ability were estimated. The Agreement between the HPV OncoCheck assay and sequence analysis results was calculated using Cohen's kappa coefficients (k), which were classified as follows: 0.00–0.19, poor; 0.20–0.39, fair; 0.40–0.59, moderate; 0.60–0.79, strong; 0.80–1.00, excellent (Estrade and Sahli, 2014). Significance between proportions was assessed with McNemar's test. *p*-values < .05 were statistically significant.

3. Results

3.1. Analytical sensitivity of the HPV OncoCheck assay

To determine the analytical sensitivity of the assay, cervical cancer cell lines—HPV 16-infected SiHa, HPV 18-infected HeLa, and HPV-negative C33A—were serially diluted 10-fold from 1×10^4 to 1×10^0 cells/mL. The analytical sensitivity was estimated as the last serial linear concentration that yielded positive results in all five replicates, and the corresponding Ct value was selected as the analytical cutoff. The Cq values for GAPDH with SiHa and Hela cell concentrates (10^4–10^0 cells/mL) ranged from 23.4 ± 0.3 (95%, CI 23–23.8) to 30.2 ± 0.6 (95%, CI 29.5–30.9) and 21.9 ± 0.1 (95% CI, 21.7–22) to 37.4 ± 0.04 (95% CI, 37.3–37.5), respectively. The detection limit of the assay for SiHa and HeLa cell lines was 10^2 cells/mL and 10^1 cells/mL, respectively (Table 1). For the C33A cell line, all tests of each group of the HPV OncoCheck assay showed negative results. In addition, the analytical sensitivity of the assay for detecting HPV E6/E7 mRNA was determined using 10-fold serially diluted (10^3 copies to 1 copy) control HPV ssDNA obtained from all 16 HR-HPV genotypes. The analytical sensitivity was estimated as the last serial linear concentration that yielded positive results in all 20 replicates, and the corresponding Ct value was selected as the analytical cutoff. HPV 51, 53, and 68 were detected at a concentration of 1 copy per reaction; HPV 56 and 58, at 100 copies per reaction; and other HPV-type ssDNA, at 10 copies per reaction. The CV of this assay was below 3%, and the mean Cq value ranged from 25 ± 0.4 (95% CI, 24.8–25.2) to 34.6 ± 0.5 (95% CI, 34.4–34.8) (Table 2).

3.2. Analytical specificity of the HPV OncoCheck assay

To assess the potential cross-reactivity with other bacteria and viruses, which include normal flora or sexually-transmitted infectious agents present in the female genitalia, analytical specificity was performed with 23 bacteria and 27 viruses exhibiting 16 HR-HPV genotypes. At medically significant concentrations, 10^6 colony forming units (CFU)/mL for bacteria and 10^5 plaque forming units (PFU)/mL for virus were mixed with HPV C33A cell line at 10^6 cells/mL. Specificity test results of the assay showed that groups I, II, and III, which allowed HPV-specific probes in three tubes, were detected accurately without cross-reactivity (Table 3).

Table 1
Analytical sensitivity of the HPV OncoCheck assay using cervical cell lines.

Cell lines		Cells/mL*, Cq indicates (95% CI)				
		10^4 cells	10^3 cells	10^2 cells	10^1 cells	1 cell
SiHa	GAPDH	23.4 (23.0–23.8)	25.8 (25.6–26.4)	27.8 (27.5–28.1)	30.2 (29.5–30.9)	N/A
(n = 10)	CV (%)	1.4	0.8	0.8	2	
	HPV 16	27.9 (27.2–28.7)	29.9 (29.3–30.5)	32.4 (31.7–33.0)	38.1 (37.5–38.7)	N/A
	CV (%)	2.8	2.1	2.2	1.6	
HeLa	GAPDH	21.9 (21.7–22.0)	25 (24.9–25.1)	28.7 (28.2–29.2)	30.4 (30.0–31.4)	37.4 (37.3–37.5)
(n = 10)	CV (%)	0.6	0.3	1.5	1.5	0.1
	HPV 18	22.5 (22.2–22.9)	25.5 (25.3–25.7)	28.5 (28.3–28.7)	33.4 (32.9–33.8)	37.2 (37–37.4)
	CV (%)	1.4	0.7	0.5	1.2	0.5

Abbreviations: *, assumes that each SiHa and HeLa cell contains approximately 1–2 copies and 10–50 copies, respectively, of integrated HPV 16 and 18 DNA [19]; Cq, mean of the quantification cycle in five repeated tests; 95% CI, 95% confidence interval; CV, coefficient of variation; N/A, not amplified.

3.3. Results of the HPV OncoCheck assay with cervical samples

The age of the women who participated in the study ranged from 21 to 87 years with a mean age of 44 ± 12.5 years (95% CI, 42.5–45.5). To evaluate the HPV OncoCheck assay performance, 319 cervical samples were used, and the results were compared with those of cytological diagnosis. Of the 319 cytologically diagnosed cases, the HR-HPV positive rates determined by the HPV OncoCheck assay were 94.7% (36 of 38) in SCC cases, 93.8% (60 of 64) in HSIL cases, 61.4% (35 of 57) in LSIL cases, 38.9% (37 of 95) in ASC-US cases, and 4.6% (3 of 65) in normal cases. Nine cases of multiple infections were detected among the positive samples, with eight LSIL cases and one ASCUS case (Table 4). All 319 ThinPrep® Pap samples were positive for GAPDH mRNA expression, and the Cq values ranged from 19.4 to 31.7 (24.8 ± 2.5, 95% CI, 24.5–25.1). The positive Cq values for each group detected by the HPV OncoCheck assay ranged from 19.1 to 34.1 (28.5 ± 4.4, 95% CI, 27.4–29.6) for HPV 16, 17.6 to 33.5 (26.9 ± 4.4, 95% CI, 24.4–29.4) for HPV 18, 17.9 to 33.8 (28.6 ± 4.5, 95% CI, 27.4–29.8) for groups I, 19.8 to 31.5 (25.9 ± 3.3, 95% CI, 23.9–27.9) for group II, 13.6 to 30.2 (22.4 ± 5.2, 95% CI, 20.5–24.3) for group III, and 15.0 to 32.0 (23.3 ± 5.0, 95% CI, 20.9–25.6) for multiple infection, respectively (Table 4).

3.4. Comparison of the results between the HPV OncoCheck assay and sequence analysis for HR-HPV genotype identification of the cervical samples

In order to confirm the results obtained from the HPV OncoCheck

Table 2
Detection limits analysis for the 16 high-risk HPV types.

HPV type		Copy number of HPV ssDNA per reaction			
		10^3 copies	10^2 copies	10 copies	1 copy
HPV 16	Cq avg. (95% CI)	25 (24.8–25.2)	28.4 (28.3–28.5)	32 (31.9–32.1)	35.5 (35.3–35.7)
	CV (%)	1.4	0.7	0.7	1
HPV 18	Cq avg. (95% CI)	25.9 (25.8–25.9)	30.5 (30.2–30.8)	34.1 (33.9–34.3)	38 (37.7–38.3)
	CV (%)	0.0	2	1.3	1.8
HPV 31	Cq avg. (95% CI)	27.7 (27.5–27.9)	31.4 (31.3–31.5)	34.3 (34.2–34.4)	37.6 (37.4–37.8)
	CV (%)	1.7	0.7	0.9	1.3
HPV 33	Cq avg. (95% CI)	26.2 (26.1–26.3)	30.6 (30.5–30.7)	34 (33.9–34.1)	38.7 (38.5–38.8)
	CV (%)	0.9	0.8	0.7	1.0
HPV 35	Cq avg. (95% CI)	25.8 (25.7–25.9)	29.3 (29.2–29.4)	32.7 (32.6–32.8)	38.3 (37.7–38.9)
	CV (%)	1	0.9	0.6	3.7
HPV 39	Cq avg. (95% CI)	26.8 (26.7–26.9)	31.1 (30.9–31.3)	34.6 (34.4–34.8)	N/A
	CV (%)	1.2	1.5	1.4	
HPV 45	Cq avg. (95% CI)	26.1 (25.8–26.3)	28.8 (28.4–29.2)	33.9 (33.7–34.1)	36.4 (36.2–36.6)
	CV (%)	0.8	2.6	1.5	1.4
HPV 51	Cq avg. (95% CI)	25.5 (24.7–26.4)	27.9 (26.3–27.6)	30.2 (29.9–30.5)	33.4 (33.1–33.8)
	CV (%)	2.3	1.8	2.1	1.4
HPV 52	Cq avg. (95% CI)	27.4 (27.3–27.5)	30.5(30.4–30.5)	33.7 (33.6–33.8)	37.1 (36.9–37.3)
	CV (%)	0.7	0.5	0.8	1.1
HPV 53	Cq avg. (95% CI)	24.7 (24.4–24.9)	27.9 (27.6–28.2)	31.3 (31.2–31.4)	34.8 (34.6–35.0)
	CV (%)	0.8	2.1	1	0.0
HPV 56	Cq avg. (95% CI)	30.5 (30.4–30.6)	33.9 (33.8–34.0)	37.5 (37.2–37.8)	N/A
	CV (%)	0.8	0.7	1.5	
HPV 58	Cq avg. (95% CI)	28.7 (28.6–28.8)	32.1 (32.0–32.2)	36.1 (35.9–36.3)	35.4 (33.5–37.2)
	CV (%)	0.7	0.6	1	2.7
HPV 59	Cq avg. (95% CI)	24.4 (24.3–24.4)	26.8(26.5–27.0)	30.1 (30.0–30.2)	36.0 (35.8–36.2)
	CV (%)	0.1	0.7	0.1	1.3
HPV 66	Cq avg. (95% CI)	25.2 (25.1–25.3)	28.6 (28.5–28.7)	32 (31.9–32.1)	36.1 (35.8–36.4)
	CV (%)	1.2	1.2	0.6	1.8
HPV 68	Cq avg. (95% CI)	26.8 (26.3–27.3)	27.9 (27.7–28.2)	29.3 (29.3–29.4)	33.2 (32.7–33.6)
	CV (%)	1.4	0.7	0.1	1.0
HPV 69	Cq avg. (95% CI)	24.7 (24.6–24.8)	27.0 (26.4–27.7)	31.4 (31.4–31.6)	35.4 (35.2–35.6)
	CV (%)	0.3	1.7	0.3	1.2

Abbreviations: HPV ssDNA, HPV single strand DNA; Cq avg., average of the quantification cycle in 20 repeated tests; 95% CI, 95% confidence interval; CV, coefficient of variation; N/A, not amplified.

Table 3
Analytical specificity of the HPV OncoCheck assay.

Bacteria (10^6 CFU/mL)	ATCC no.	Positive % (n/total)			Viruses (10^5 PFU/mL)	ATCC no.	Positive % (n/total)		
		Group I	Group II	Group III			Group I	Group II	Group III
Chlamydia trachomatis	VR-573	100 (0/15)	100 (0/15)	100 (0/15)	Herpes simplex virus 1	VR-1493	100 (0/15)	100 (0/15)	100 (0/15)
Gardnerella vaginalis	14018	100 (0/15)	100 (0/15)	100 (0/15)	Herpes simplex virus 2	VR-734	100 (0/15)	100 (0/15)	100 (0/15)
Ureaplasma urealyticum	27618	100 (0/15)	100 (0/15)	100 (0/15)	Cytomegalovirus	VR-677	100 (0/15)	100 (0/15)	100 (0/15)
Neisseria gonorrhoeae	49226	100 (0/15)	100 (0/15)	100 (0/15)	Adenovirus 2	VR-846	100 (0/15)	100 (0/15)	100 (0/15)
Mycoplasma hominis	23114	100 (0/15)	100 (0/15)	100 (0/15)	Adenovirus 5	VR-1516	100 (0/15)	100 (0/15)	100 (0/15)
Bacteroides fragilis	25285	100 (0/15)	100 (0/15)	100 (0/15)	HPV 16	45113D	0 (15/15)	100 (0/15)	100 (0/15)
Bifidobacterium breve	15700	100 (0/15)	100 (0/15)	100 (0/15)	HPV 31	65446	0 (15/15)	100 (0/15)	100 (0/15)
Fusobacterium mortiferum	25557	100 (0/15)	100 (0/15)	100 (0/15)	HPV 33	Clinical isolate	0 (15/15)	100 (0/15)	100 (0/15)
Staphylococcus aureus	29213	100 (0/15)	100 (0/15)	100 (0/15)	HPV 35	40330	0 (15/15)	100 (0/15)	100 (0/15)
Staphylococcus epidermidis	12228	100 (0/15)	100 (0/15)	100 (0/15)	HPV 52	Clinical isolate	0 (15/15)	100 (0/15)	100 (0/15)
Streptococcus faecalis	29221	100 (0/15)	100 (0/15)	100 (0/15)	HPV 58	Clinical isolate	0 (15/15)	100 (0/15)	100 (0/15)
Streptococcus pyogenes	12344	100 (0/15)	100 (0/15)	100 (0/15)	HPV 18	Clinical isolate	100 (0/15)	0 (15/15)	100 (0/15)
Streptococcus agalactiae	13813	100 (0/15)	100 (0/15)	100 (0/15)	HPV 39	Clinical isolate	100 (0/15)	0 (15/15)	100 (0/15)
Lactobacillus acidophilus	4356	100 (0/15)	100 (0/15)	100 (0/15)	HPV 45	Clinical isolate	100 (0/15)	0 (15/15)	100 (0/15)
Corynebacterium diphtheriae	11913	100 (0/15)	100 (0/15)	100 (0/15)	HPV 59	Clinical isolate	100 (0/15)	0 (15/15)	100 (0/15)
Enterococcus faecalis	29212	100 (0/15)	100 (0/15)	100 (0/15)	HPV 68	Clinical isolate	100 (0/15)	0 (15/15)	100 (0/15)
Peptostreptococcus anaerobius	27337	100 (0/15)	100 (0/15)	100 (0/15)	HPV 51	Clinical isolate	100 (0/15)	100 (0/15)	0 (15/15)
Escherichia coli	25922	100 (0/15)	100 (0/15)	100 (0/15)	HPV 53	Clinical isolate	100 (0/15)	100 (0/15)	0 (15/15)
Enterobacter cloaceae	13047	100 (0/15)	100 (0/15)	100 (0/15)	HPV 56	40549	100 (0/15)	100 (0/15)	0 (15/15)
Pseudomonas aeruginosa	27853	100 (0/15)	100 (0/15)	100 (0/15)	HPV 66	Clinical isolate	100 (0/15)	100 (0/15)	0 (15/15)
Klebsiella oxytoca	700324	100 (0/15)	100 (0/15)	100 (0/15)	HPV 69	Clinical isolate	100 (0/15)	100 (0/15)	0 (15/15)
Proteus vulgaris	49132	100 (0/15)	100 (0/15)	100 (0/15)	HPV 6	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)
Candida albicans	36802	100 (0/15)	100 (0/15)	100 (0/15)	HPV 11	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)
					HPV 40	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)
					HPV 54	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)
					HPV 84	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)
					HPV 87	Clinical isolate	100 (0/15)	100 (0/15)	100 (0/15)

Abbreviations: CFU, Colony forming units; ATCC, American type culture collection; PFU, Plaque forming units.

assay, sequence analysis was performed using the same cervical samples (Table 5). The results of the HPV OncoCheck and sequence analysis methods were all concordant for all but four cases. Of the four discrepant cases, three cases (group I, II, and III) detected by the HPV OncoCheck assay were not identified by sequencing, and the case that was not detected by the HPV OncoCheck assay was identified as HPV 16 by sequence analysis (Table 5).

4. Discussion

The purpose of this study was to evaluate the analytical and clinical performance of the HPV OncoCheck assay for the detection of HPV 16 and 18 genotypes as well as 14 HR-HPVs (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69) in bulks in cervical cancer screening and describe HPV E6/E7 mRNA as an early biomarker with cytological diagnosis. To assess the performance and accuracy of the HPV OncoCheck assay, a synthesized HPV ssDNA was used as the reference standard and Cq values were determined in 20 replicate measurements. As all of the 16 HR-HPV genotypes were detected at 10–100 copies per reaction, the detection limit of the assay was determined to be 100 copies per reaction. These results are similar to those obtained for the cervical cell lines (10–100 cells) as SiHa and HeLa cell lines contain 1–2 and 10–50 copies, respectively, of HPV 16 and 18 DNA/cell (Mincheva et al., 1987). The assay was tested using bacterial and viral specimens in combination with HPV C33A cell lines. Consequently, no cross-reaction was observed between the specimens, demonstrating that the presence of other samples in HPV-infected endocervical specimens did not affect the performance of the assay. In addition to sensitivity and specificity, we also conducted analytical performance tests of interference and reproducibility: i) For interfering reactions, we used the following 11 substances—PreservCyt Solution, five vaginal tablets (Nowon Vaginal Suppositories, Ovestin Vaginal Suppositories, Gynoflor Vaginal Tab, Gynobetadine Vaginal Suppositories, and Crinone vag gel), two antifungal agents (Canesten Cream and Polygynax), a lubricant (Surgytube; Clear Transparent Jelly), blood, and EDTA that could be included in the cervical cytology fluid. In nine substances, except for blood and EDTA, no interference was observed at the highest tested concentration (10%), but interference was observed at 10% with blood and 20 mM EDTA. (ii) Repeatability and reproducibility of this assay was performed with a total of 144 intra-laboratory test (12 days × 2 runs/day × 2 replicates × 3 lots). These results showed that the intra-laboratory reproducibility over time was 95.1% ($\kappa = 0.9$, 95% CI, 0.832–0.973), and CV of the assay was below 3% (data not shown). These results suggested that the assay might yield stable results.

This study evaluated the positive rates of the cervical lesions that were cytologically diagnosed based on the Bethesda system using the HPV OncoCheck assay; the overall positive rates were the highest for high-grade lesions (SCC and HSIL), followed by low-grade lesion (LSIL, ASCUS, and normal). During comparative analysis of HR-HPV, a good agreement (94.6%, $\kappa = 0.89$, 95% CI, 0.816–0.959) was observed between the results of the HPV OncoCheck assay and high-grade cytology diagnosis. The clinical sensitivity, specificity, PPV, and NPV of the HPV OncoCheck assay for detecting high-grade lesions were 94.1% (95% CI, 0.875–0.975, $p < .0001$), 95.4% (95% CI, 0.868–0.989, $p < .0001$), 97% (95% CI, 0.911–0.993, $p < .0001$), and 91.2% (95% CI, 0.817–962, $p < .0001$), respectively. These results are similar to a previously reported sensitivity of 91.9–92.6% and specificity of 98.5–98.6% with the APTIMA HPV assay (Dockter et al., 2009) and sensitivity of 84–91% and specificity of 85–96% with the InCellDx Oncotect E6/E7 mRNA assay (Coquillard et al., 2011). However, the presently reported sensitivity and specificity were higher than the previously reported sensitivity of 91.8–92.1% and specificity of 39–45.6% with the Luminex XMAP (Zhao et al., 2017). Previous studies have reported an upregulation in E6 and E7 mRNA levels with lesion severity; therefore, the detection of HPV E6/E7 mRNA may be of higher prognostic value and may improve specificity and PPV compared with those by cytological analysis and HPV DNA testing, which are used in traditional cervical cancer screening (Castle et al., 2007; Benevolo et al., 2011; Duvlis et al., 2015). Our results also showed that the HPV

Table 4
HPV OncoCheck assay results for the discrimination of the 16 HR-HPV genotypes, including HPV 16 and 18, in 172 cytologically diagnosed samples.

HPV OncoCheck assay	Range of IC Cq range (mean ± SD)	SCC (*n* = 36)		HSIL (*n* = 60)		LSIL (*n* = 35)	ASCUS (*n* = 37)		Normal (*n* = 3)			Total (*n* = 172)
		n (%)	Cq range (mean ± SD)	n (%)	Cq range (mean ± SD)	n (%)	Cq range (mean ± SD)	n (%)	Cq range (mean ± SD)	n (%)	Cq range	
HPV 16	21.3–31.7 (25.4 ± 2.9)	22 (61.1)	24.3–34.1 (28.7 ± 3.3)	22 (36.7)	19.1–33.9 (28.7 ± 4.1)	3 (8.6)	22.9–31.6 (28.1 ± 4.6)	9 (24.3)	20.2–28.5 (27.3 ± 6.7)	1 (33.3)	27.73	57 (33.1)
HPV 18	22.0–27.9 (26.1 ± 2.1)	3 (8.3)	26.1–29.2 (27.3 ± 1.7)	3 (5.0)	21.8–33.5 (29.6 ± 6.2)	2 (5.7)	17.6–31.5 (24.5 ± 9.8)	3 (8.1)	27.2–31.8 (28.9 ± 2.4)	1 (33.3)	25.02	12 97.0)
Group I	19.4–30.3 (25.3 ± 2.5)	11 (30.6)	25.7–33.2 (29.8 ± 2.1)	32 (53.3)	17.9–33.8 (29.5 ± 4.5)	5 (14.3)	16.4–27.7 (22.6 ± 4.5)	5 (13.5)	23.1–32.6 (27.6 ± 3.9)			53 (30.8)
Group II	21.2–25.7 (22.9 ± 1.6)					4 (11.4)	19.8–31.5 (25.2 ± 4.9)	7 (18.9)	23.5–30.0 (26.3 ± 2.3)			11 (6.4)
Group III	21.1–26.4(24.1 ± 2.4)			3 (5.0)	26.3–30.2 (28.9 ± 2.2)	13 (37.1)	13.6–27.1 (20.7 ± 4.6)	13 (35.1)	13.2–27.1 (22.3 ± 5.5)	1 (33.3)	27.46	30 (17.4)
Multiple infection (*n* = 9)	21.7–25.5 (23.2 ± 1.3)											9 (5.2)
HPV 16 + group III	21.7–24.9 (23.6 ± 1.6)					3 (8.6)	21.3–32.0 (25.8 ± 3.9)					3 (1.7)
HPV 18 + group III	21.9–25.5(23.1 ± 1.6)					4 (11.4)	15.0–31.3 (21.7 ± 5.6)					4 (2.3)
Group I + group III	22.7–23.3 (22.9 ± 0.4)					1 (2.9)	16.4–22.5 (19.4 ± 4.3)	1 (12.5)	26.6–26.9 (26.8 ± 0.2)			1 (1.2)

Abbreviations: HR-HPV, high-risk human papillomavirus; SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance; Cq, quantification cycles; The assay consisted three different sets of HR-HPVs [Group I: HPV16 (FAM), HPV 31, 33, 35, 52, and 58 (HEX), and internal control (IC) (Cy5); Group II: HPV 18 (FAM), HPV 39, 45, 51, and 68 (HEX), and IC (Cy5); and Group III: HPV 53, 56, 59, 66, and 69 (HEX), and IC (Cy5)] by incorporating specific TaqMan probes labeled with different fluorophores.

Table 5
Comparison of the HPV OncoCheck assay and sequence analysis for the detection of the HR-HPV genotypes in 319 cervical samples.

HPV OncoCheck assay	No (%) of samples	Sequence analysis, no. (%)				Sensitivity (%)	95% CI	Specificity (%)	95% CI
		HPV genotypes	Consistent results	Not detected	Discrepant result				
HR-HPV positive	**172 (53.9)**		**169 (98.3)**	**3 (1.7)**		98.3	0.948–0.996	100	0.978–1.000
Single infection	**163 (94.8)**		**160 (98.2)**	**3 (1.8)**		98.3	0.945–0.996	100	0.979–1.000
HPV 16	57 (35.0)	HPV 16	57 (35.0)			100	0.946–1.000	100	0.970–1.000
HPV 18	12 (7.4)	HPV 18	12 (7.4)			100	0.784–1.000	100	0.979–1.000
Group I	53 (32.5)		52 (98.1)	1 (1.9)*		98.1	0.891–1.000	100	0.971–1.000
		HPV 33	20 (37.7)						
		HPV 58	13 (24.5)						
		HPV 52	9 (17.0)						
		HPV 35	6 (11.3)						
		HPV 31	4 (7.5)						
Group II	12 (7.4)		11 (91.7)	1 (8.3)*		91.7	0.625–0.999	100	0.979–1.000
		HPV 68	8 (66.7)						
		HPV 39	3 (25.0)						
Group III	29 (17.8)		28 (99.6)	1 (3.4)*		99.6	0.814–0.999	100	0.976–1.000
		HPV 53	12 (41.4)						
		HPV 51	7 (24.1)						
		HPV 56	7 (24.1)						
		HPV 69	2 (6.9)						
Multiple infection	**9 (5.2)**					100	0.731–1.000	100	0.979–1.000
HPV 16 + group III	3 (33.3)	HPV 16, 53	2 (66.7)						
		HPV 16, 66	1 (33.3)						
HPV 18 + group III	4 (44.4)	HPV 18, 53	3 (75.0)						
		HPV 18, 66	1 (25.0)						
Group I + group III	2 (22.2)	HPV 35, 53	1 (50.0)						
		HPV 52, 53	1 (50.0)						
HR-HPV negative	**147 (46.1)**	HPV 16	146 (99.3)		1 (0.7)†	99.3	0.959–0.999	99.3	0.959–0.999
Total	319 (100)		315 (98.8)	3 (0.9)	1 (0.3)	98.8	0.967–0.996	99.7	0.981–0.999

Abbreviations: HR-HPV, high-risk human papillomavirus; 95% CI, 95% confidence interval; *, three cases detected by the HPV OncoCheck assay were not identified by sequencing; †, the one case not detected by the HPV OncoCheck assay was identified as HPV 16 by sequence analysis; The assay consisted three different sets of HR-HPVs [Group I: HPV16 (FAM), HPV 31, 33, 35, 52, and 58 (HEX), and internal control (IC) (Cy5); Group II: HPV 18 (FAM), HPV 39, 45, 51, and 68 (HEX), and IC (Cy5); and Group III: HPV 53, 56, 59, 66, and 69 (HEX), and IC (Cy5)] by incorporating specific TaqMan probes labeled with different fluorophores.

OncoCheck assay yielded a significantly lower rate of positive results (4.6% for HPV OncoCheck vs. 27.7% for HPV microarray or 10.8% for REBA HPV-ID; $p < .05$) in patients with normal cytology than those with the HPV DNA test (data not shown). These results suggest that the selective targeting used in the HPV OncoCheck assay can reduce the positive detection rate of HPV infections in women with normal or low-grade cytology lesions. Because HPV OncoCheck is an assay based on E6/E7 mRNA, the accuracy of the assay was compared with sequence analysis. Sensitivity and specificity between the two assays were 99.4% (95% CI, 0.965–0.999, $p < .0001$) and 98% (95% CI, 0.939–0.996, $p < .0001$), respectively. We also found that the agreement between the HPV OncoCheck assay and sequence analysis for the detection of HR-HPV was 98.8% ($\kappa = 0.98$, 95% CI, 0.967–0.996, $p < .0001$). Of the four discrepant cases, three were not detected by sequencing results because of low yields of the PCR product, and lack of matching nucleotide sequences. Additionally, group I (HPV 31, 33, 35, 52, and 58) including HPV 16 genotype was detected in approximately 36% of the total samples, whereas HPV 18 was detected in approximately 5% of the total samples. The results of the HPV OncoCheck assay were confirmed by sequence analysis. The HPV OncoCheck assay may offer accurate HR-HPV detection and would be useful for prediction of cervical cancer in patients with undetermined cytological diagnosis; however, future studies with histological follow-up are needed and it requires additional comparative testing with several commercialized products according to the guidelines. The quantitative real-time PCR (qPCR) assay is conducive for screening multiple samples simultaneously and has the potential to become a standard procedure in terms of its performance, speed, accuracy, sensitivity, broad range, and high-throughput capacity. Furthermore, fluorescent PCR monitoring offers advantages over existing PCR methods for quantification (Ntoulia et al., 2006). The result of the present study showed that multiplex qPCR is a rapid method with a turnaround time that usually requires 3 h, including 1.5 h for RNA preparation and cDNA synthesis and 1.5 h for target amplification. It allowed for the rapid identification of 16 HR-HPV genotypes, including HPV 16 and 18, without post-PCR processing.

The potential limitations of our study were the relatively small sample size and no information about the histological diagnosis of the samples. Therefore, it will be necessary to determine whether the HPV OncoCheck assay shows similar analytical performance and efficacy in detecting HPV with a larger number of samples confirmed by histological diagnosis.

In conclusion, the HPV OncoCheck assay generally showed high agreement and specificity with cytological diagnosis and sequence analysis. When similar results are obtained by histological diagnosis, use of this newly developed molecular diagnostic assay in cervical screening may help to detect the most significant cervical disease while reducing false-positive results and has the potential to serve as a sensitive and specific tool for the early detection and diagnosis of cervical cancer.

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