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Human Papillomavirus (HPV) in Atypical Squamous Cervical Cytology: the Invader HPV Test as a New Screening Assay▽

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In surveillance for cervical neoplasia, a diagnosis of cytologically atypical squamous cells of undetermined significance (ASCUS) presents a significant clinical issue, often dependent on testing for high-risk (HR) human papillomavirus (HPV) for the triage of patients. HPV type 16 now appears to be a critical concern in the follow-up of patients with ASCUS. The Invader HPV (Inv2) test, by Third Wave Technologies, Inc., is a recently developed analyte-specific reagent assay that uses probe sets for the detection of 14 HR HPV subtypes. These probe sets are A5/A6 (HPV types 51, 56, and 66), A7 (HPV types 18, 39, 45, 59, and 68), and A9 (HPV types 16, 31, 33, 35, 52, and 58). This report describes the performance characteristics of the Inv2 test in the screening of ASCUS cervical cytology specimens and correlates the results of the Inv2 test with those of the Hybrid Capture II HPV (HC2) test by Digene. The linear array HPV genotyping test (Roche Molecular Systems) was used as a reference method for the testing of samples with discordant results. Ninety-four Pap smear samples with a cytological diagnosis of ASCUS and 39 samples with a negative diagnosis were tested. The results of the Inv2 test demonstrated a good (86.6%) concordance with those of the HC2 test, with an overall sensitivity and specificity of 96% for the Inv2 test. Additionally, the Inv2 assay, which offers high-throughput, semiautomated DNA extraction, allows the subgrouping of HPV types by differential probe sets, could provide a useful test for screening for HPV, and has the potential to provide an improved means of risk stratification and the selection of patients for further HPV subtyping.

The human papillomavirus (HPV) is responsible for 95 to 100% of the cases of cervical cancer, the second most prevalent malignant neoplasm among women worldwide (15, 18, 25). Approximately 50 million females undergo the Papanicolaou (Pap) test annually, with 7% of these individuals diagnosed with a pathological abnormality (10, 11). Atypical squamous cells of undetermined significance (ASCUS) remain a common cytological diagnosis and are detected in 4% of clinical cases. The poor cytological reproducibility of this diagnosis remains a problem, since 5 to 17% of ASCUS cases are subsequently diagnosed as cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or CIN3 on cervical biopsy (4–6, 13, 16, 20–24, 27), which is a significant clinical management issue.

HPV typing and colposcopy are ancillary tests for patients with an abnormal cervical cytology, while cervical biopsy for the demonstration of CIN3 remains the only diagnostic test for cervical neoplasia. Colposcopy is invasive and expensive and can miss one-third of CIN3 lesions (8). Although cervical cancer is induced by HPV, the different HPV types are not equally carcinogenic. Fifteen oncogenic or high-risk (HR) strains are implicated in cervical carcinogenesis. Of these HR HPV types, HPV type 16 (HPV 16) and HPV 18 are etiologically associated with CIN3 and invasive cervical carcinomas (CIN lesions of grade 3 or higher) in 60% and 10 to 20% of specimens, respectively (17). The ASCUS LSIL Triage Study Group reported that the 2-year cumulative absolute risk for CIN lesions of grade 3 or higher is 32.5% for HPV 16-positive ASCUS specimens (2), which raises a clinical urgency. Moreover, the persistence of HPV infection is most often associated with HPV 16 and 18 (9, 14, 24, 26), suggesting that the identification of these two HPV types may play an important role in the risk stratification of patients with ASCUS for appropriate monitoring and, possibly, heightened follow-up.

Approximately 50% of ASCUS specimens demonstrate HR HPV infections (7). Algorithmically, the need for clinical follow-up for ASCUS is considerably intensified by a positive screen for HR HPV. Most contemporary HPV tests that indicate positive results for HPV do not yet specifically identify HPV 16 and 18, nor do they indicate the level of inherent patient risk when the assay results are positive. As one approach to the recently recognized need for a level of specificity for HPV 16 and 18 in cervical cytology specimens (2, 12), we examined a new and yet undescribed HPV screening assay, the second-generation Invader HPV (Inv2) assay (Third Wave Technologies, Inc., Madison, WI). The Inv2 assay screens for HPV DNA and subgroups the HR HPV types with three separate HR HPV-specific probe sets.

The Inv2 assay is available as analyte-specific reagents (ASRs) and provides a potentially semiautomated, high-throughput system for the detection of 14 HR HPV types. According to the manufacturer of the Inv2 assay ASRs, the Inv2 assay is similar to the first-generation Invader HPV HR assay but with the addition of a probe for HR HPV 66 (Table 1). The Inv2 assay HR HPV-specific probe sets include A5/A6 (HPV 51, 56, and 66), A7 (HPV 18, 39, 45, 59, and 68), and A9 (HPV 16, 31, 33, 35, 52, and 58). Positivity for probes A9 and A7 is suggestive of the presence of HPV 16 and 18, respectively (Table 1). The Inv2 assay features an internal positive control

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TABLE 1. HPV types detected by Inv2 and HC2 assay probes

HPV types detected by:				
Inv2 assay			HC2 assay	
Probe set A5/A6	Probe set A7	Probe set A9	Probe A	Probe B
51	18	16	6	16
56	39	31	11	18
66	45	33	42	31
	59	35	43	33
	68	52	44	35
		58		39
				45
				51
				52
				56
				58
				59
				68

through the detection of the human histone-2, or *H2be*, gene, ensuring the quality of the DNA and informative results for specimens with negative assay results.

We describe the performance characteristics of the Inv2 assay, with particular attention given to screening for HR HPV and the subgrouping of ASCUS cytology specimens. We compared the Inv2 assay to a commonly employed HR HPV screening test, the Hybrid Capture II HPV (HC2) test (Digene Corporation, Gaithersburg, MD). The HC2 assay (HC2 probe B) screens for 13 HR HPV types associated with cervical carcinoma (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). This evaluation of the Inv2 assay and HR HPV grouping is undertaken in the context of an increasing clinical focus on HPV 16 and 18 and the need to refine the risk stratification for patients with ASCUS cervical cytology results.

MATERIALS AND METHODS

Cytology and HC2 testing. Cervical cytology specimens were submitted in SurePath, an ethanol-based transport medium, from December 2006 to March 2007. This study included 39 specimens that were cytologically negative and 94 specimens with an ASCUS cytology. SurePath is a liquid medium used for routine Pap smears and is a fixative that excellently preserves squamous cells for accurate morphological interpretation and that extracts high-quality DNA for concurrent testing for HPV.

Specimens in SurePath medium were vortexed at 3,000 rpm for 20 s, subsequently enriched by dispensing approximately 8 ml of the specimen over 4 ml of PrepMate density reagent, and then centrifuged at 800 × *g* for 10 min. The supernatant was decanted, and the pellet was vortexed. Probe B of the HC2 test (Digene) was used for the postenrichment sample, with residual specimen stored at 4°C for a period up to 3 months prior to the Inv2 and amplification assays.

NucliSens easyMAG total nucleic acid extraction. Total nucleic acids were extracted from the specimens in SurePath medium by using the NucliSens easyMAG platform, a second-generation system for the automated isolation of nucleic acid from clinical samples that is based on the silica extraction technology (bioMérieux, Durham, NC). One to 2 ml of the sample in SurePath medium was centrifuged at 1,150 × *g* for 15 min. The supernatant was removed, and the cell pellet was resuspended in 400 μl of Genfind lysis buffer (Agencourt Bioscience Corporation, Beverly, MA). This mixture was incubated at 99°C for 10 min and then cooled to ambient (room) temperature. Nine microliters of proteinase K (Agencourt Bioscience Corporation) was added to each tube containing the reaction mixture, and the tubes were incubated at 37°C for 30 min on a thermomixer. Following incubation, the DNA was extracted by use of the NucliSens easyMAG platform and eluted to a final volume of 110 μl.

HPV typing by Inv2 assay. The extracted DNAs were tested for the presence of HR HPV DNAs in a laboratory-developed HPV assay with the Inv2 ASRs, including HPV Oligos (version 2.0; Third Wave Technologies, Inc.). Three Inv2 assay HPV reaction mixtures representing Inv2 assay oligonucleotides (probe sets) A5/A6, A7, and A9, respectively, were prepared and contained the specific oligonucleotides, the enzyme cleavase, $MgCl_2$, and oligonucleotides specific for the human *H2be* gene.

Ten microliters of each sample or control was added to three separate wells, which each contained one of the targets for the three HPV-specific probe sets, in a 96-well reaction plate. Twenty microliters of Chill-Out liquid wax (Bio-Rad, Hercules, CA) was added to each well. After incubation at 95°C for 5 min and cooling to 63°C, 10 μl of each Inv2 assay reaction mixture was added to the appropriate specimens and controls. The reaction plate was incubated at 63°C for 4 h and then read in a GENios reader (Tecan Systems, Inc., San Jose, CA) with the following settings: for the 6-carboxyfluorescein (FAM) signal, excitation at 485 nm and emission at 535 nm; for the red signal, excitation at 560 nm and emission at 612 nm. Data analysis and HR HPV detection were performed with a Microsoft Excel software-based HPV (version 2.0) Invader data analysis worksheet (Third Wave Technologies, Inc.).

Analytic criteria for Inv2 assay results and cutoff values. The fluorescent signals of all specimens were expressed as the FAM (HPV specific) and red (*H2be*-specific) signals. The levels of the red and FAM signals over zero (red fold over zero [Red FOZ] and FAM fold over zero [FAM FOZ]) were calculated for each of the three probe sets as follows: FAM FOZ = FAM signal of sample/FAM signal of no-DNA target solution and Red FOZ = red signal of sample/red signal of no-DNA target solution.

The FAM FOZ ratio was subsequently calculated with the FAM FOZ values from each of the three probe sets for a given sample (see below). When the lowest FAM FOZ value was <1, the value was set equal to 1.0 for the calculation of the FAM FOZ ratio: FAM FOZ ratio = highest FAM FOZ value/lowest

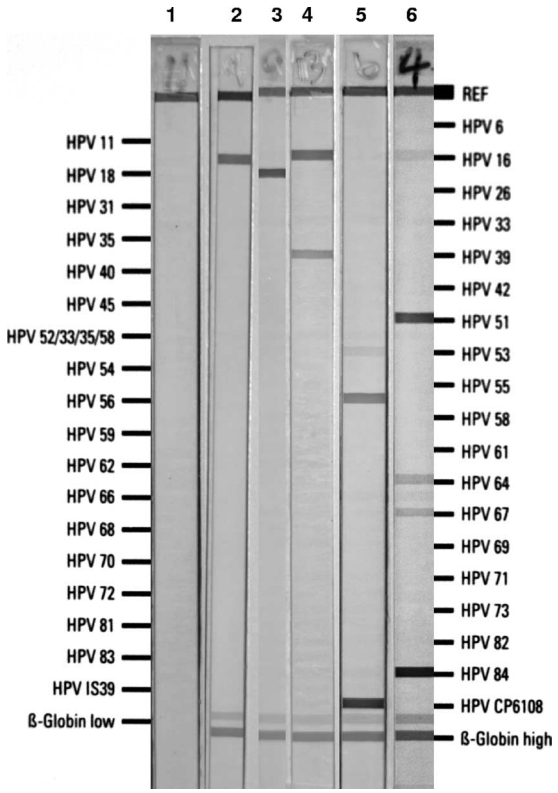

FIG. 1. Examples of PCR-LA results. The PCR-LA result for each sample was determined by comparing the band pattern to the PCR-LA reference guide. Lane 1, PCR-LA HPV-negative control with no visible bands; lane 2, PCR-LA HPV-positive control with HPV 16 band; lane 3, HeLa cell DNA positive for HPV 18; lane 4, specimen Sp133 positive for HPV 16 and 39; lane 5, specimen N167 positive for HPV 53, 56, and CP6108; lane 6, specimen Sp79 positive for HPV 16, 51, 64, 67, and 84. In addition, except for the negative control, all specimens were positive for β-globin high and β-globin low molecular weight (see Materials and Methods).

TABLE 2. Correlation of results obtained by Inv2 and HC2 assays with specimens with a cytological diagnosis of ASCUS

HC2 assay result (no. of specimens)	No. of specimens with the following Inv2 assay results:					
	Negative	Positive				Indeterminate[a]
		Probe set A5/A6	Probe set A7	Probe set A9	Mixed probe sets	
Negative (48)	40	2	1	0	2[b]	3
Positive (46)	7	7	7	18	7[c]	0
Total (94)	47	9	8	18	9	3

[a] Indeterminate indicates specimens that showed 3FS and for which the results were diagnostically indeterminate.
[b] The two specimens were positive for probe sets A5/A6 and A9.
[c] Two specimens were positive for probe sets A5/A6 and A7, three specimens were positive for probe sets A7 and A9, and two specimens were positive for probes sets A5/A6 and A9.

FAM FOZ value and FAM FOZ ratio = middle FAM FOZ value/lowest FAM FOZ value.

Specimens with FAM FOZ ratios ≥1.4 were considered positive for HR HPV. When the FAM FOZ ratio was <1.4 but the specimen demonstrated a FAM FOZ ratio of ≥1.5 with all three HPV-specific probe sets, the specimen was called multiple positive, or the presence of "three fluorescence signals" (3FS).

An average Red FOZ value was calculated for each specimen and served as an indicator for genomic DNA levels. A negative reaction for HPV for specimens with average Red FOZ values <1.5 was considered insufficient for a result.

PCR-LA. The linear array HPV genotyping assay (PCR-LA; Roche Molecular Systems, Inc., Branchburg, NJ) employs the amplification of target DNA by PCR and nucleic acid hybridization and has been designed to detect 37 cervical HPV DNA genotypes, including 13 HR types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). PCR-LA is for research use only (Fig. 1).

PCR-LA was performed with extracted DNAs, according to the manufacturer's instructions. Briefly, DNAs were amplified in a final reaction volume of 100 μl with a working master mixture containing Tris buffer, KCl, dATP, dCTP, dUTP, dGTP, dTTP, AmpliTaq Gold DNA polymerase, uracil-*N*-glycosylase, $MgCl_2$, and biotinylated HPV-specific and β-globin-specific primers. The mixture was incubated in a GeneAmp PCR system 9700 instrument for 2 min at 50°C and 9 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C, with a final extension at 72°C for 5 min. Positive and negative controls were included in all runs to assess the performance of the assay. Following amplification, the biotinylated HPV and human β-globin amplicons were denatured by adding 100 μl of denaturation solution to each PCR tube. The denatured amplicons were hybridized to an immobilized probe array strip. HPV detection and genotyping were achieved by the protocol provided by the manufacturer. The hybridization strips were manually interpreted by using the linear array HPV reference guide.

RESULTS

Semiautomated extraction of cytology specimens in SurePath medium by Inv2 assay. High-quality DNA was successfully extracted from all 133 specimens by semiautomated techniques, as demonstrated by a Red FOZ value of >1.5 (see Materials and Methods).

For determination of the reproducibility of the Inv2 assay, the coefficients of variation were established through repeat testing for high to low viral "loads" in dilution assays, through replicate testing in separate runs, on different days. CaSki and HeLa cell DNAs, which were the source materials for HPV 16 and HPV 18, respectively, were employed for known viral "copy" numbers (Advanced Biotechnology, Inc., Columbia, MD). The reaction mixtures contained HPV 16 (1.2×10^3 to 10×10^3 copies in four dilutions) or HPV 18 (5×10^3 to 10×10^3 copies in two dilutions). For HPV 16 and 18, the coefficients of variation for the FAM FOZ ratios ranged from 6.63 to 2.53% and from 9.33 to 4.72%, respectively (range from low to high viral loads). The FAM FOZ ratios were reproduced with good precision.

All specimens were screened for the presence of HPV by the Inv2 and HC2 assays, and the results were considered within the context of ASCUS or negative cytology results (Tables 2 to 5).

Specimens with ASCUS cytology and HPV typing. A total of 94 extracted specimens previously identified cytologically as ASCUS were screened for HPV by the Inv2 and HC2 assays and were stratified into four groups (Table 2): 40 Inv2 assay-negative and HC2 assay-negative specimens, 39 Inv2 assay-positive and HC2 assay-positive specimens, 7 Inv2 assay-negative and HC2 assay-positive specimens, and 5 Inv2 assay-positive and HC2 assay-negative specimens. Three samples demonstrated multiple fluorescence signals (3FS) when they were tested by the Inv2 assay, according to the parameters outlined in the Materials and Methods section. These specimens were all retested and had the same results (3FS) on retesting. Of these specimens, two were negative and one was positive for HPV 59 by PCR-LA. Accordingly, results of 3FS were considered diagnostically uninformative, or indeterminate (Table 2). Otherwise, samples positive by both HPV typing methodologies (the Inv2 and HC2 assays) variously included 7 samples positive for Inv2 assay probe set A5/A6, 7 samples positive for Inv2 assay probe set A7, 18 samples positive for Inv2 assay probe set A9, 2 samples mixed positive for Inv2 assay probe sets A5/A6 and A7, 2 samples mixed positive for Inv2

TABLE 3. Correlation of HPV results obtained by Inv2 and HC2 assays with cytologically negative specimens

HC2 assay result (no. of specimens)	No. of specimens with the following Inv2 assay results:					
	Negative	Positive				Indeterminate[a]
		Probe set A5/A6	Probe set A7	Probe set A9	Mixed probe sets[b]	
Negative (37)	30	1	1	2	0	3
Positive (2)	1	0	0	1	0	0
Total (39)	31	1	1	3	0	3

[a] Specimens that showed 3FS and that were thus diagnostically indeterminate (and not included in the analysis).
[b] Specimens positive for two probe sets.

TABLE 4. Summary of results for discordant specimens

Cytologic diagnosis	HPV result by[a]:		No. of specimens	
	Inv2 assay	HC2 assay	Total	Positive for HR HPV by PCR-LA
ASCUS	Pos	Neg	5	3
ASCUS	Neg	Pos	7	2
Negative	Pos	Neg	4[b]	1
Negative	Neg	Pos	1	ND[c]

[a] Pos, positive for HR HPV; Neg, negative for HR HPV.
[b] Only two of four samples could be tested by PCR-LA.
[c] ND, not done.

assay probe sets A5/A6 and A9, and 3 samples mixed positive for Inv2 assay probe sets A7 and A9. Five samples that were Inv2 assay positive and HC2 assay negative were HPV subtyped into two specimens positive for probe set A5/A6, one specimen positive for probe set A7, and two specimens mixed positive for probe sets A5/A6 and A9. A total of 38.5% (35/91) of ASCUS specimens were positive for Inv2 assay probe sets A9 and A7, representative of the possible presence of HPV 16 and/or HPV 18.

Twelve ASCUS specimens had discordant results and comprised seven Inv2 assay-negative and HC2 assay-positive specimens and five Inv2 assay-positive and HC2 assay-negative specimens, all of which underwent PCR-LA testing (Tables 4 and 5). Of the seven Inv2 assay-negative and HC2 assay-positive samples, two specimens were found to contain HR HPV types, including a sample with HPV 35 and 56 and a second sample with HPV 16 and 39. Among the five specimens that were Inv2 assay positive and HC2 assay negative, three samples had HR HPV types, including a specimen with both HPV 16 and HPV 51, a specimen with HPV 39, and a specimen with HPV 66. Of all the discordant specimens, PCR-LA detected HPV 16 in 2 of the 12 samples: 1 specimen that was Inv2 assay positive and HC2 assay negative and 1 specimen that was Inv2 assay negative and HC2 assay positive, with the Inv2 and HC2 assays each failing to detect a sample in which HPV 16 was detected by PCR-LA (Fig. 1). For samples with an ASCUS cytology, the concordance between the Inv2 and HC2 tests was 86.8% (79/91 samples).

Specimens with negative cytology results and HPV typing. Thirty-nine specimens with negative cytology results underwent testing for HPV (Table 3). The specimens were grouped into four categories: Inv2 assay positive and HC2 assay positive (1 specimen), Inv2 assay positive and HC2 assay negative (4 specimens), Inv2 assay negative and HC2 assay positive (1 specimen), and Inv2 assay negative and HC2 assay negative (30 specimens). Three specimens were identified (including by repeat assays) as having 3FS and were thus considered indeterminate and excluded from analysis. A total of five specimens demonstrated HPV by the Inv2 assay, with one specimen positive for probe set A5/A6, one specimen positive for probe set A7, and three specimens positive for probe set A9. The Inv2 assay detected the potential presence of HPV 16 and/or HPV 18 (probe sets A9 and A7) in 11.0% (4/36) of specimens with negative cytology results.

For the specimens with negative cytology results, 5 discordant specimens were identified among 36 informative specimens (3 of 39 specimens had 3FS and, thus, indeterminate).

TABLE 5. Roche PCR-LA HPV types for specimens with discordant results by HC2 and Inv2 assays

Specimen no.	SID[a]	Cytology result	Result[b]		PCR-LA type(s)[c]
			HC2 assay, probe B	Inv2 assay	
1	Sp61	ASCUS	+	−	54, 61, GTIS39
2	Sp67	ASCUS	+	−	61
3	Sp84	ASCUS	+	−	33, **35**, 52, **56**, 58
4	Sp108	ASCUS	+	−	82
5	Sp124	ASCUS	+	−	53, CP6108
6	Sp133[d]	ASCUS	+	−	**16, 39**
7	Sp134	ASCUS	+	−	53, 82
8	Sp78	ASCUS	−	+	84
9	Sp79[e]	ASCUS	−	+	**16**, **51**, 64, 67, 84
10	Sp81	ASCUS	−	+	**39**
11	Sp122	ASCUS	−	+	
12	Sp142	ASCUS	−	+	**66**, 84
13	N56	Negative	+	−	
14	N167	Negative	−	+	53, **56**, CP6108
15	N178	Negative	−	+	

[a] SID, specimen identification numbers of 12 discordant ASCUS specimens and 3 discordant cytologically negative specimens.
[b] +, positive for HR HPV; −, negative for HR HPV.
[c] HR HPV types are indicated in boldface.
[d] Specimen with HPV 16 not detected by Inv2 assay.
[e] Specimen with HPV 16 not detected by HC2 assay.

These included four specimens positive by the Inv2 assay and one specimen negative by the Inv2 assay but positive by the HC2 assay (Tables 4 and 5). PCR-LA was performed with three of the five discordant specimens with a negative cytology result, with the detection of HR HPV 56 in a single specimen which was Inv2 assay positive (Table 5). The concordance rate between the Inv2 and HC2 assays for the specimens with a negative cytology was 86.1% (31/36).

Overall, the results of the Inv2 assay demonstrated a concordance of 86.6% compared to the results of the HC2 assay. By use of PCR-LA as the reference assay for discordant specimens, the Inv2 assay had an overall sensitivity of 95.6% and a specificity of 96.1%, a positive predictive value of 93.6%, and a negative predictive value of 97.4%.

All of the specimens with an ASCUS result and all specimens with a negative cytology result were reevaluated in the context of the Inv2 assay results. No cytomorphological differences were seen among the specimens, whether they were positive, negative, or indeterminate for HPV (Fig. 2 and 3).

DISCUSSION

The use of the Pap test for the prevention of invasive cervical epithelial disease represents a medical triumph (7). There is now an increasingly important role for the clinical characterization of the etiologic agents of this disease, HR HPV types. In a recent study, Castle et al. (2) were able to stratify the absolute risk for progression to CIN of grade 3 or higher in women with an ASCUS cytology, based upon the type of HR HPV detected at the baseline screening. Women with an ASCUS cytology positive for HPV 16 had the highest risk for progression, and this risk was fivefold greater than the collective risk attributable to infections with other HR HPV types. In

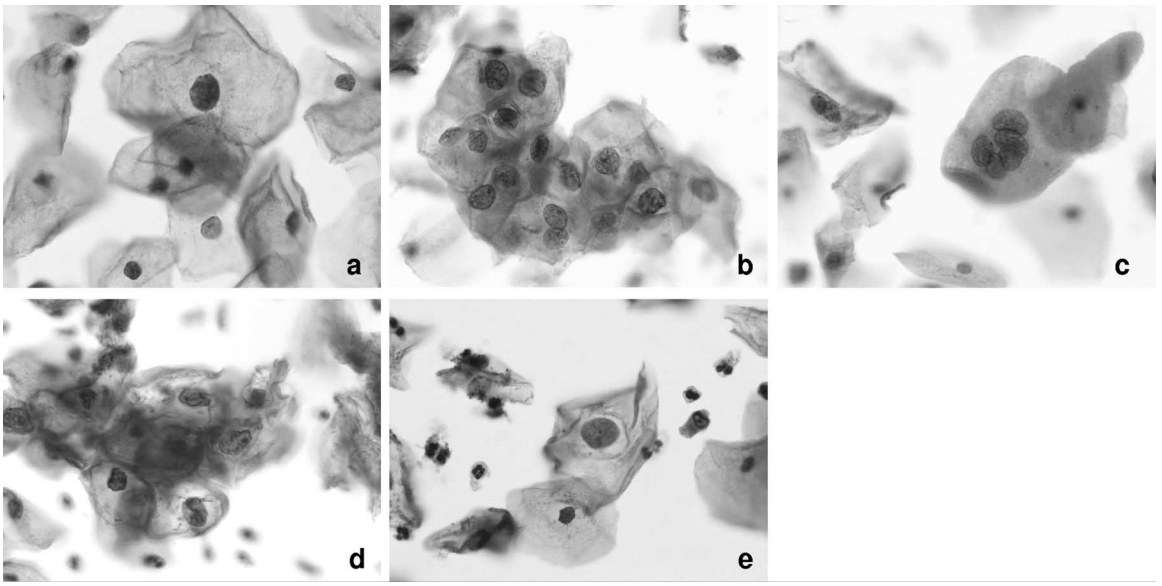

FIG. 2. Examples of specimens cytologically classified as ASCUS. (a) Negative for HPV by both the Inv2 and the HC2 assays; (b) positive for HPV by both the Inv2 and the HC2 assays; (c) negative for HPV by the Inv2 assay but positive by the HC2 assay; (d) positive for HPV by the Inv2 assays but negative by the HC2 assay; (e) indeterminate for analysis, as the sample demonstrated multiple fluorescence signals (3FS).

the future, tests for HPV must anticipate such priorities—the clinical implications of infection with HPV 16 and 18—and screening methods commensurate with the urgency of these two HR HPV subtypes must be augmented.

This study describes the performance characteristics of a new HPV test, the second-generation Inv2 assay. Clinically, the Inv2 test detects 14 oncogenic HPV types and segregates the viruses into three groups according to the results obtained with separate probe sets, thus allowing the selection of women for whom further subtyping is needed. In this study, cervical samples were submitted in SurePath ethanol-based transport medium, with DNA extractions for Inv2 testing obtained by semiautomated methods. SurePath medium has been increasingly popular as a liquid fixative for gynecologic cytology; moreover, screening for HPV from a liquid fixative is rapidly growing as an important testing modality for cancer prevention. For the Inv2 assay, *H2be* serves as an internal control and ensures that informative DNA is extracted from the specimen. In this study, the extractions for the Inv2 assay were "clean"; that is, the semiautomated extraction resulted in a low level of extraneous background DNA and provided quality specimen DNA.

In examining the performance characteristics of the Inv2 assay with specimens with an ASCUS cytology, the results obtained by the Inv2 assay were compared to those obtained by the HC2 assay. The discordances between the two tests were examined by a third method, PCR-LA. For ASCUS specimens, the results obtained by the Inv2 assay demonstrated a good correlation (86.8%) with those obtained by screening by the HC2 assay. The Inv2 test demonstrated that 48.4% (44/91) of ASCUS specimens were positive for HR HPV, with 38.4% (35/91) representing potential HPV types 16 and 18. This incidence of HR HPV correlates with that of 45.5 to 59% published previously for females with an ASCUS cytology with HR HPV (7).

Otherwise, 12 ASCUS specimens were discordant for HPV typing by the Inv2 assay and the HC2 assay. With PCR-LA used as a reference, for specimens with an ASCUS cytology, the rates of false-positive results for HR HPV were 4.6%

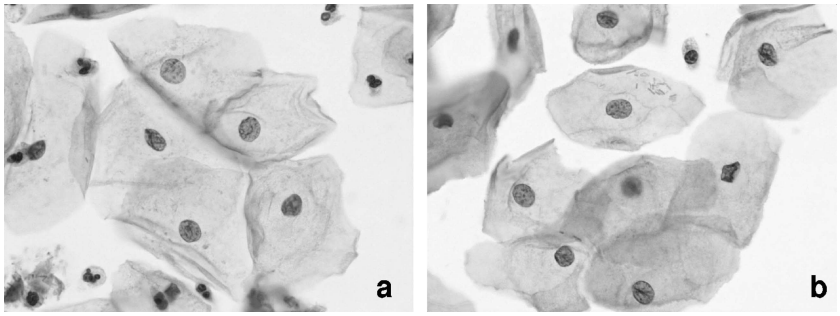

FIG. 3. Examples of specimens cytologically diagnosed as negative. (a) Positive for HPV by both the Inv2 and the HC2 assays; (b) negative for HPV by both the Inv2 and the HC2 assays.

(2/44) for the Inv2 assay and 10.9% (5/46) for the HC2 assay. The rates of false-negative results were 4.3% (2/47) for the Inv2 assay and 6.7% (3/45) HC2 assay. The results of the Inv2 assay were falsely negative for HR HPV 16, 35, 39, and 56 in two specimens, and the results of the HC2 assay were falsely negative for HPV 16, 39, 51, and 66 in three specimens. Two ASCUS cases screened negative for HPV, one each by the Inv2 assay and the HC2 assay. This finding is notable, since each specimen was positive for HPV 16 by PCR-LA.

HPV typing of 39 specimens with negative cytology results demonstrated HR HPV by the Inv2 assay (with probe set A9 or A7) in 13.9% (5/36) of specimens, and 4 (11.1%) of theses specimens potentially involved HPV 16 and/or 18. These findings correlate with the previously published findings that 17% of patients with a negative cytology were infected with HR HPV (7). Specimens with a negative cytology screened as discordant for HPV by the Inv2 and HC2 assays were also examined by PCR-LA. Of the three specimens tested, PCR-LA detected an HR HPV type (type 56) in a single specimen that was Inv2 assay positive.

Overall, the results of the Inv2 assay demonstrated a concordance of 86.6% compared to the results of the HC2 assay. When PCR-LA was used as a reference for discordant specimens, the Inv2 assay had an overall sensitivity and specificity of 96%, a positive predictive value of 94%, and a negative predictive value of 97%.

Six specimens (three specimens with an ASCUS cytology and three specimens with a negative cytology result) demonstrated 3FS, which was classified as an indeterminate result in this study. All of these specimens were negative by the HC2 assay. All specimens had FAM FOZ values $\leq$4.0. PCR-LA was performed with the three specimens with an ASCUS cytology and an indeterminate result and demonstrated HPV 59 in one specimen. The significance of indeterminate results is not clear at present. Additional specimens with indeterminate results will need to be studied. It is possible that the cutoff values for the Inv2 test will need to be further optimized in the future. Alternatively, for the SurePath fixative, the specimen DNA concentration and/or the duration of fixation may require standardization to prevent indeterminate results. Importantly, no differences in cytological morphology were seen among the specimens that were positive, negative or indeterminate for HPV.

The specificity of assays for HPV subtypes is now a major concern, namely, because patients may be subjected to unnecessary stress and procedures secondary to false-positive results for HR types. The HC2 HPV screening assay detects at least 15 HPV genotypes not included in its HR probe set (19). It may be argued that it is important to detect HPV with sensitivity, irrespective of specificity; however, this may not attend to the caveats of risk and the economic cost associated with a false-positive test result. Specificity for HR HPV and the associated clinical implications—the need for close follow-up, including colposcopy—are relevant issues which suggest that a high sensitivity and a high specificity are paramount to clinical acumen. The Inv2 and HC2 tests appear to demonstrate similar sensitivities, although the Inv2 test may offer an improvement in specificity (19). From the results of the present study, the Inv2 test offers a reliable means of grouping of HR HPV types, which is potentially of advantage in triaging of patients for follow-up for possible HPV 16 and 18 infections. We have semiautomated the extraction phase for Inv2 testing for high throughput. Otherwise, the LA-PCR allows the specific recognition of individual types within both low-risk and HR HPV groups, although the test remains unavailable for clinical use at this time. In its present format, the application of PCR-LA would be problematic (because of strip hybridization) for high throughput.

In characterizing the Inv2 assay, the assay appears to be simple to perform, is amenable to use with the SurePath fixative, may be semiautomated for DNA extraction, and shows a good sensitivity and a good specificity for ASCUS cytology cases. For routine surveillance for HPV, the Inv2 test appears to be potentially useful as a first-line test for screening for HPV. The Inv2 test demonstrates few false-positive results without compromising the sensitivity of detection of critical HPV types, including HPV 16 and 18. Patients positive by the Inv2 test with the A9 and/or A7 probe set may warrant further close follow-up and/or testing for possible HPV 16 and 18 infection.

In the future, new modalities for testing for HPV should address HPV types 16 and 18. However, on the basis of even a limited association with invasive cervical disease (28), because of the obvious clinical stakes, it will remain prudent to continue to screen for multiple HR HPV types, such as for ASCUS cytology cases, as well as for additional triage for HPV types 16 and 18.

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