Comparison of the Third Wave Invader Human Papillomavirus (HPV) Assay and the Digene HPV Hybrid Capture 2 Assay for Detection of High-Risk HPV DNA[▽]

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This study compared the clinical performance of the Digene Hybrid Capture 2 (HC2) assay to that of a prototype Third Wave Invader human papillomavirus (HPV) (IHPV) analyte-specific reagent-based assay for the detection of oncogenic or “high-risk” (HR) HPV DNA using liquid-based cytology specimens. In total, 821 ThinPrep vials were tested using both assays. In accordance with the type-specific probes contained within each test, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the IHPV assay were 95.9%, 97.6%, 97.5%, and 96.1%, respectively, and those for the HC2 assay were 98.1%, 86.2%, 87.1%, and 97.9%. Overall, the sensitivity and NPV were comparable between the assays, but the IHPV assay demonstrated a better specificity and PPV, since the IHPV assay had fewer false-positive HR HPV results. The incorporation of an internal control to evaluate the cellularity of the test material is an important feature of the IHPV assay and should reduce the risk of false-negative results due to insufficient sample collection rather than the lack of HR HPV DNA. An additional benefit of the IHPV assay was the smaller sample volume required (1 ml versus 4 ml for the HC2 assay).

Cervical cancer is the second most common malignancy of women worldwide and a leading cause of death for middle-aged women (ages 35 to 55) in low-resource settings (10; GLOBOCAN 2000 software program). In the United States, the American Cancer Society estimated that ~10,370 new cases of cervical cancer would be diagnosed in 2005 and that ~3,710 women would die of the disease (<http://www/cancer.org>). Cervical cancer is a highly preventable disease when cytologic screening programs are employed that facilitate the detection and treatment of precancerous lesions (6). With early detection, the 5-year relative survival rate for the earliest stage of invasive cervical cancer is 92% and the overall (all stages combined) 5-year survival rate for cervical cancer is about 73% in the United States (<http://www/cancer.org>). Beginning in 2002, patient management guidelines have been published by various groups of health care professionals with recommendations on how women should be screened for cervical cancer according to age, other factors, and the presence of cytological abnormalities in a Pap test (4, 12, 17–21). These patient management guidelines all recommend testing for the presence of “high-risk” (HR) types of human papillomavirus (HPV) as an additional diagnostic tool for equivocal or ambiguous cytology results, including atypical squamous cells of undetermined significance, atypical squamous cells for which the possible presence of high-grade squamous intraepithelial lesions cannot be excluded, and low-grade squamous intraepithelial lesions.

Research worldwide has clearly shown that virtually all cervical cancer is caused by chronic infection with certain carci-

nogenic types of HPV (1, 2, 7, 8, 9, 15, 16). More than 100 types of HPV have been documented in the literature, approximately 40 of which are known to be sexually transmitted. In the United States, genital infection with HPV is the most common sexually transmitted viral infection, with an estimated 6.2 million new infections each year (<http://www/cancer.org>). Of the sexually transmitted types of HPV, approximately 15 types have been classified as oncogenic or HR in epidemiological studies (1, 7, 9, 16). Persistent infection with one or more of these HR types of HPV causes 95 to 100% of all cervical cancer (7, 9, 15, 16).

There are 13 types of HR HPV DNA for which clinical utility has been established and for which testing is recommended: HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (9, 12, 15–19). Recently the International Association for Research on Cancer reclassified HPV-66 as a high-risk, carcinogenic type of HPV and recommended that it be included as part of routine screening (2). Under current patient management guidelines, testing for HR HPV DNA is conducted in one of two settings: as a means of triage for patients with equivocal or ambiguous cytology results (atypical squamous cells of undetermined significance) to determine the need for referral to colposcopy or as an adjunct to cervical cytology analysis in women 30 years of age and older (4, 17–21). In either setting, the presence or absence of HR HPV DNA is considered in light of the patient’s cytology history and other risk factors in order to guide the patient’s management.

Currently, the Digene Hybrid Capture 2 (HC2) assay (Digene, Gaithersburg, MD) is the only method cleared by the FDA for the detection of HPV DNA from both endocervical samples and liquid-based cytology specimens (package insert; Digene Corporation, Gaithersburg, MD). Several other methods, including the PCR-based Roche Amplicor and Roche Linear Array HPV tests (14), the GenProbe Aptima HPV test

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for the detection E6/E7 mRNA, and the Third Wave Invader HPV assay (Third Wave Technologies, Madison, WI) (13, 22) are currently under investigation as alternative technologies for HPV detection. This study evaluated the use of the Invader chemistry as an alternative method for detecting HR HPV DNA in samples submitted for routine HPV DNA testing (13, 22). Similar to all Invader assays, the prototype Invader HPV (IHPV) detection method (for research use only) uses isothermal signal amplification and requires no specialized instrumentation to perform (3). The prototype IHPV method utilizes sequence-specific Invader DNA probes, a structure-specific Cleavase enzyme, and a universal fluorescent resonance energy transfer system combined with interpretive software and a multiwell fluorometer to semiquantitatively detect 15 HR types of HPV DNA in cervical epithelium cell specimens (3). The reagents are designed to query for the presence of known sequence polymorphisms and to identify specific nucleic acid sequences through the analysis of structure-specific cleavage events driven by the presence of any 1 of 15 types of HPV and single-copy human DNA sequences, respectively. The HPV types detected by the IHPV prototype assay are the HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and non-oncogenic types 67 and 70. The IHPV reagents were also designed to be able to detect mixed infections of certain combinations of HR HPV. The bplex format of the IHPV reagents enables simultaneous detection of two DNA sequences, HPV DNA if present and a nonvarying segment of the human alpha actin 1 (ACTA1) gene in a single well. The detection of the ACTA1 DNA serves as an internal control both for assay performance and for determining if sufficient cellular material is present in the reaction. IHPV results were compared to those obtained using the current laboratory assay, the Digene HC2 HR HPV test.

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MATERIALS AND METHODS

Sample acquisition. Endocervical specimens ($n = 821$) of unknown cytological profile were submitted in ThinPrep vials (Cytoc, Marlborough, MA) for routine Pap smear analysis and HR HPV DNA testing using the HC2 test. After completion of the HC2 testing, the residual samples were deidentified and used for IHPV testing under an institutional review board-approved protocol.

HC2 HPV DNA test. HC2 tests were performed and analyzed as per the manufacturer's instructions using the HR HPV probe cocktail (package insert; Digene Corporation, Gaithersburg, MD). The HR probe cocktail is capable of detecting HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Based upon in-house validation studies, the established laboratory criteria for the interpretation of results were as follows. Samples were scored as negative for HR HPV DNA when samples yielded relative light unit (RLU) values of ≤ 0.8 , scored indeterminate with RLU values of ≥ 0.8 to < 5.0 , and scored positive for HR HPV DNA with RLU values of ≥ 5.0 . When a sufficient volume was present, samples with values in the indeterminate range were retested with the HC2 assay. If the repeat HC2 test was < 1.0 , the sample was considered negative. For a sample to be reported as positive, both RLU values had to be ≥ 2.0 . If only one result was ≥ 2.0 , then the result would remain indeterminate. If insufficient sample was available for retesting, the result would remain indeterminate.

DNA extraction for Invader HPV assay. DNA was extracted from 1 ml of the residual ThinPrep solution using a modified PureGene (Gentra, Minneapolis, MN) protocol. Briefly, cells were lysed using the PureGene cell lysis solution with the assistance of heating to 55°C for 15 min. Protein was precipitated using the PureGene protein precipitation solution. Next, 20 mg/ml glycogen was used to create a DNA pellet. Finally, the DNA was washed, dried, and rehydrated in 60 μl using nuclease-free water.

TABLE 1. User-defined criteria for Invader data analysis using Excel software

Result ^a	Criterion
Positive	FFOZ ratio ≥ 1.500
Negative	FFOZ ratio < 1.500
Valid avg	RFOZ > 1.30
Valid % CV (RFOZ)	$< 25\%$
HPV flag (A5/A6 pool)	FFOZ > 3.00
HPV flag (A7 pool)	FFOZ > 3.00
HPV flag (A9 pool)	FFOZ > 3.00

^a FFOZ, 6-carboxyfluorescein fold-over-zero value; RFOZ, RED fold-over-zero value; CV, coefficient of variation. FFOZ ratio values represent the highest FFOZ value obtained using three probe pools for a given sample divided by lowest FFOZ value obtained for the three probe pools. The term "flag" is used to indicate that in cases where all three probe pools for a sample exhibit elevated FFOZ values but the FFOZ ratio is < 1.5 , an additional call criterion is triggered to determine the HPV result. When each pool gives an FFOZ value ≥ 3.00 but an FFOZ value of < 1.5 , the sample is called positive for HPV.

Invader HPV reactions. The IHPV reagents were composed of three pools of HR HPV type-specific probes and fluorescent resonance energy transfer cassettes, a probe set specific for the ACTA1 gene, Cleavase enzyme, and Invader buffer (Third Wave Technologies). IHPV reactions were carried out in triplicate using 10 μl of the nucleic acid extract and three separate oligonucleotide pools for each sample. Pool A5/A6 contained the oligonucleotides required for the detection of HPV-51 and -56, pool A7 the oligonucleotides for the detection of HPV-18, -39, -45, -59, -68, and -70, and pool A9 the oligonucleotides for the detection of HPV-16, -31, -33, -35, -52, -58, and -67. Reactions were allowed to proceed for 4 h at 63°C before the signal was read using a multiwell Genios FL fluorometer (Tecan, Durham, NC). The HPV signal generated using each of the three oligonucleotide pools produced a 6-carboxyfluorescein signal, while a Redmond Red [RED; 3-(3-oxo-7-pivaloylphenoxazin-2-yl)-propanamino-1-(2-*O*-dimethoxytrityloxymethyl)-pyrrolidin-4-yl-*O*-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] signal was produced by the internal control, ACTA1, contained within each reaction.

Invader data analysis. Raw data from the multiwell fluorometer was exported and analyzed using Microsoft Excel 2000. "Fold-over-zero" (FOZ) values were calculated for standards and unknown samples by dividing the raw signal obtained for each by the raw signal of the standard no-target control. For HPV qualitative detection, the FOZ values indicative of the presence of HR HPV and for a positive internal control are shown in Table 1. Invader reactions would be repeated when indeterminate results were generated due to failure of either assay or if the sample was determined to have an insufficient amount of cellular DNA based upon a RED FOZ (RFOZ) result of < 1.2 . If not enough sample remained for repeat testing, the result would be scored as indeterminate.

Discordant analysis. Discrepant results between the HC2 and the IHPV methods were resolved using consensus PCR with sequencing utilizing the GP5+/GP6+ and L1C1/L1C2 primer sets as previously described (5, 23). PCRs were done in a volume of 25 μl with 1 μl of template DNA. The PCR mixture consisted of 1 $\times$ PCR buffer without MgCl_2 (Roche Molecular Systems, Pleasanton, CA), 0.4 μl of a 1:1 mixture of Amplitaq polymerase (Roche Molecular Systems) and TaqStart antibody (BD Biosciences, San Jose, CA), 200 μM (each) deoxynucleoside triphosphates, 4 mM MgCl_2 , and 1 μM of each of two primers (GP5+ and GP6+ or L1C1 and L1C2). Reactions were run in a Hybaid thermocycler (Thermo Fischer Scientific, Inc., Waltham, MA) for one cycle of 5 min at 95°C and then eight cycles of 94°C for 30 s, 40 to 47°C for 30 s (starting at 40°C and then increasing by 1°C per cycle), and 72°C for 1 min. This was followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min. The total volume of each PCR product was run on a 2% agarose in 1 $\times$ Tris-acetate-EDTA gel with ethidium bromide. The approximately 150-bp band from the GP5+/GP6+ amplifications and the approximately 250-bp band(s) from the L1C1/L1C2 amplifications were excised and transferred to individual 1.7-ml tubes. The DNA was extracted with the QiaexII gel extraction system (Qiagen, Valencia, CA) and eluted with 20 μl Tris-EDTA buffer. The products were ligated into the pGEM-T Easy vector (Promega, Madison, WI), transformed into chemically competent JM109 cells, and then plated on two LB-plus-ampicillin plates containing isopropyl- β -D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. After overnight growth, two white colonies were picked from each plate (total of four per sample) and grown overnight for DNA extraction (using

TABLE 2. Initial test results from HC2 and Invader HPV assays

HC2 assay result	No. of samples with Invader HPV assay result			
	Indeterminate	Negative	Positive	Total
Indeterminate	2	15	3	20
Negative	21	330	16	367
Positive	18	73	343	434
Total	41	418	362	821

Qiaprep Spin; Qiagen) and sequencing (with the ABI Big Dye Terminator v3.1 cycle sequencing kit and an ABI 377 sequencer; Applied Biosystems, Foster City, CA). The primer used for sequencing was J5 (5'-GACGTCGCATGCTCCC-3'). The sequences were trimmed to exclude the amplification primers and vector sequence. The resulting sequences were subjected to BLAST analysis versus GenBank and the top hit for each sequence listed as the genotype.

Statistical analysis. Assay sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using standard formulas.

RESULTS

Of the 821 samples tested (Table 2), initial results were concordant for 673 samples (81.97%) and discordant for 148 samples (18.03%). Discordant results included 16 IHPV-positive/HC2-negative samples (1.95%), 73 IHPV-negative/HC2-positive samples (8.89%), 20 indeterminate HC2 results (2.44%), and 41 indeterminate IHPV results (4.99%), of which 2 samples were indeterminate by both methods. Twenty-six (23) of the 41 IHPV samples were scored as indeterminate due to insufficient cellular material in the samples, as denoted by a RFOZ value of <1.2.

PCR and sequencing were performed on 84/89 positive/negative discordant samples to determine if HR HPV DNA was in fact present in each sample. The remaining five samples (IHPV negative/HC2 positive) did not have sufficient quantity remaining for sequencing studies (Table 3). In addition, PCR and sequencing were performed on 1 of the 2 samples indeterminate by both HC2 and IHPV and 10 and 11 samples indeterminate by HC2 and IHPV (Table 4). Of the 84 positive/negative discordant samples sequenced, 60 (71.43%) resolved in favor of the IHPV assay (Table 3) when the detection probes present within the IHPV assay were considered. The HC2 assay gave false-positive results for 38 samples, including 13 samples with no HPV DNA detected by PCR and 25 samples with low-risk (LR) HPV types, shown in Table 3. The HC2 assay detected 15 more positive samples than the IHPV assay for HR types listed in Table 3. The HC2 assay also detected 15 samples with HR HPV type 66, although a specific probe for type 66 is not present in their HR HPV probe cocktail. This

version of the IHPV assay does not contain a probe for type 66, and therefore, these samples were negative with the IHPV assay. The IHPV assay gave false-positive results for eight samples, including three samples with no HPV DNA detected by PCR and five samples with LR HPV LR types (Table 3). The IHPV assay detected HR HPV types in seven additional samples negative with the HC2 assay.

Repeat testing of all the indeterminate HC2 and IHPV results with the appropriate assay was not performed due to having an insufficient residual specimen. However, sufficient sample was available to perform PCR and sequencing for 22 indeterminate samples, and the results are shown in Table 4. For the HC2-indeterminate/IHPV-negative samples, four had no HPV DNA detected by PCR, four had an LR HPV strain, and two were positive for HR HPV (types 16 and 31). The two HR-positive samples were positive by the IHPV assay. For the samples with IHPV-indeterminate/HC2-negative results, three had no HPV DNA detected by PCR and three had an LR HPV strain. For the IHPV-indeterminate/HC2-positive samples, two contained an LR HPV strain and three HR HPV (types 51 and 66). As noted above, the IHPV assay does not contain a probe for type 66. Two of the three samples with HR HPV types would have been reported as indeterminate due to insufficient cellular material.

For the final overall analysis, there were in total 757 samples with valid HC2 and IHPV results, and when indicated for discordant resolution, the comparison to PCR and sequencing analysis was done (Table 5). Results for each method are presented in two manners: (i) based upon the detection only of the HR HPV types with the type-specific probes present in the HR probe mixes (Table 5, rows 1 and 3) and (ii) detection of all HR HPV types, including HR type 66, which was not present in either the IHPV or HC2 probe mixes (Table 5, rows 2 and 4). Overall, there were 380 HR HPV-positive samples (including 15 positive for type 66) and 377 HR HPV-negative samples. Based upon resolution with PCR and sequencing, overall the HC2 test was slightly more sensitive for the detection of HR HPV DNA than the IHPV assay (98.08% and 98.16% versus 95.89% and 92.11%, respectively) and therefore had a higher NPV (97.94% and 97.94% versus 96.08% and 91.85%, respectively) (Table 5). The increased sensitivity of the HC2 assay was primarily related to the detection of HR HPV type 66, for which a type-specific probe is not present in the HC2 probe mix or in the IHPV probe pools. The HC2-positive results may be due to the presence of other HR HPV types for which probes are present in the HC2 probe mix but which were not detected by PCR and sequencing. Alternatively, type 66 may have been detected due to cross-reactivity

TABLE 3. Resolution by PCR/sequencing for discordant positive/negative samples

Assay	True HR HPV-positive results		False HR HPV-positive results			HPV-negative results	
	No. of HR DNA-positive results (types identified)	No. of samples showing type 66 cross-reactivity with HR probes	No. of HPV DNA-negative results	No. of samples showing HR probe cross-reactivity with LR types (types identified)	Total no. of false-positive results	No. of true-negative results	No. of false-negative results (type)
Invader HPV	7 (16, 45, 56, 59, 66)	0	3	6 (6, 67, 73, 84, 91)	8	38	30 (66)
HC2	15 (16, 31, 35, 52, 53, 56, 58, 59, 68)	15	13	25 (6, 30, 42, 43, 53, 61, 72, 73, 84, 87, 91)	38	7	9

TABLE 4. Resolution of indeterminate HC2 and IHPV results

Results ^a	No. of samples with PCR/sequencing result (type[s] identified)			
	No HPV DNA detected	LR HPV detected	HR HPV detected	HR HPV cross-reactive ^b
HC2 Ind, Invader Neg	4	4 (6, 41, 42, 81, 90)	0	0
HC2 Ind, Invader Pos	0	0	2 (16, 31)	0
HC2 Ind, Invader Ind	0	0	1 (67)	0
HC2 Neg, Invader Ind	3	3 (6, 91)	0	0
HC2 Pos, Invader Ind	0	2 (53, 90)	1 (51)	2 (66)

^a Ind, indeterminate; Neg, negative; Pos, positive.^b HR HPV detected due to cross-reactivity with probes in HC2 HR probe mix.

with other HC2 probes present in the probe mix. Analysis of the discordant results demonstrated that the HC2 method, in comparison to the IHPV assay, has a lower specificity (86.16% and 89.67% versus 97.61% and 97.41%, respectively), a lower PPV (87.10% and 90.75% versus 97.49% and 97.49%, respectively), and a higher overall rate of false positives (7.0% and 5.02% versus 1.19%, respectively). This rate of HC2 false positives is a result of samples being found to be HPV HR positive while PCR/sequencing determined that either no HPV DNA was present or there was a substantial degree of cross-reactivity with HPV types not included in the HC2 HR probe set or for which the test has label claims of being able to detect with the HC2 HR probe pool. The HC2 method demonstrated cross-reactivity with the LR HPV types 6, 30, 42, 43, 53, 61, 72, 73, 84, 87, and 91, while the IHPV method demonstrated cross-reactivity with LR types 6, 73, 84, and 91. In addition to false negatives that were PCR/sequencing positive, the HC2 method does not contain probes capable of detecting “possible HR” HPV type 66 yet demonstrated significant cross-reactivity for this type. However, one must use caution when interpreting these rates of false positives and false negatives. The methods employed here will detect only false positives/negatives that occur in only one method but not the other, since only discrepant samples were sequenced. To determine the true rates of false-positive and false-negative results for each method, all samples would have to be sequenced. In addition, low levels of HR HPV may have been present in some samples without being detected by the PCR method used in this study.

In addition to lower rates of false positives, the IHPV method is also capable of detecting the presence of multiple types of HR HPV DNA present within a single sample. The use of three separate oligonucleotide pools enables the IHPV

method to detect these mixed infections, something the HC2 method is unable to do. The IHPV method was able to detect at least 47 positive samples that contained mixed HR HPV infections (data not shown).

DISCUSSION

Overall, the HC2 and IHPV assays demonstrated comparable sensitivities for the detection of HR HPV when the type-specific probes present in each assay are considered. When taking into account all positive results, the slightly increased sensitivity of the HC2 assay was mainly based on the ability of HC2 to detect HR HPV type 66 due to potential probe cross-reactivity. Recently the International Association for Research on Cancer reclassified HPV-66 as a high-risk, carcinogenic type of HPV and recommended that it be included as part of routine screening (2). While the IHPV probe pools used in this study did not contain probes for type 66, the manufacturer has announced its inclusion in all newer versions of the reagents (22). In this study and in previous studies, the HC2 HR probe pool, which does not contain a type 66-specific probe, has been shown to detect type 66 due to a high degree of cross-reactivity between type 66 and other HR probes present in the HC2 HR probe pool (11). Although the cross-reactivity resulted in more HR HPV-positive samples, the HC2 product was not FDA approved with label claims for reliably and reproducibly being able to detect type 66 and its demonstrated cross-reactivity with other nontargeted HPV types. Therefore, the ability of the HC2 assay to detect type 66 should not be relied upon.

When combined with a negative Pap smear, HR HPV DNA testing has been shown to have an NPV for disease progression to cervical cancer of greater than 98% (16, 17). However, other

TABLE 5. Summary of results after discordant analysis^a

Assay	No. of samples with result					% Sens	% Spec	PPV (%)	NPV (%)
	Pos/Neg (total) ^b	True Pos ^b	False Pos ^c	True Neg ^b	False Neg ^c				
IHPV (w/o 66)	365/392	350	9	368	15	95.89	97.61	97.49	96.08
IHPV (with 66)	380/377	350	9	338	30	92.11	97.41	97.49	91.85
HC2 (w/o 66)	365/392	358	53	330	7	98.08	86.16	87.10	97.94
HC2 (with 66)	380/377	373	38	330	7	98.16	89.67	90.75	97.94

^a “w/o 66” (rows 1 and 3) indicates analysis without including data for type 66. Data are presented based upon detection of HR HPV types in accordance with type-specific probes present in each assay. “with 66” (rows 2 and 4) indicates that data are presented including detection of HR HPV type 66 due to HC2 probe cross-reactivity, since samples were scored as positive with the HC2 assay and confirmed by PCR/sequencing to contain type 66. Pos, positive; Neg, negative; Sens, sensitivity; Spec, specificity.

^b Total true-positive and true-negative samples were determined based upon concordant HC2/IHPV testing and discordant analysis.

^c Samples were scored as false positive or false negative if results were not concordant between IHPV, HC2, and sequencing.

studies have also shown that the significant degree of cross-reactivity exhibited by the HC2 HR HPV probe pool does not have the same effect on the PPV of HPV DNA testing using that assay, since the types detected are not always HR HPV. Previous investigation has shown that the HC2 HR probe pool cross-reacts with at least 15 types that its probes were not designed to detect, many of them LR HPV types (11). The IHPV probes in the three probe pools used in this study were designed to identify the presence of type-specific single nucleotide polymorphisms and by taking this approach were able to significantly decrease the cross-reactivity with LR HPV types and the potential for false-positive results being generated. This factor accounts for the better specificity and PPV of the IHPV assay than of the HC2. Recent studies by Schutzbank et al. that compared the IHPV assay and the HC2 assay also demonstrated a higher specificity of the IHPV assay than the HC2 assay due to cross-reactivity of the HC2 assay with HPV LR types (13). A high specificity and PPV are desired to reduce the number of unnecessary colposcopies that might be conducted based upon a false-positive HR HPV result. One limitation of this study was that only those samples with discordant results were sequenced, thereby not identifying all samples within the total data set that may have been called HR HPV positive but contained only LR types. In addition, although four clones per sample were sequenced, a low-level HR HPV type may have actually been present in the sample but not detected. Conversely, samples negative by both assays may have contained HR HPV. PCR and sequence analysis of all 821 samples was not feasible.

The manufacturer of the IHPV reagents has also improved the designs of several of the probes in the A5/A6 and A9 probe pools to further enhance the ability of laboratories to detect specific HPV types and has removed the probes specific for the nononcogenic HPV types 67 and 70. Studies by Wong et al. using this second-generation IHPV assay demonstrated good concordance with the HC2 assay (86.6%) and an overall sensitivity and specificity of 96% (22). In addition, since both the IHPV testing and HC2 assay utilize signal amplification instead of target amplification, both tests allow for the detection of clinically significant levels of HPV DNA, avoiding issues associated with target amplification technologies that often detect low levels of HPV DNA that may lack clinical significance in disease progression to cervical cancer.

Another feature incorporated into the IHPV assay was the use of ACTA1 probes as an internal control for the degree of cellularity in the ThinPrep samples tested. Since the ACTA1 gene is found in two copies per human cell, a positive ACTA1 signal assures that the sample contains sufficient DNA to allow the detection of HR HPV DNA should it be present in the sample. The lack of such an internal control in the HC2 test results in the possibility that a negative HR HPV result may actually be due to insufficient cellularity of the sample instead of the actual lack of HR HPV DNA. Low cellularity was found in 26/41 (63.4%) of the indeterminate IHPV samples, which included 2 samples that contained HR HPV type 51 or 66. Without the internal control, the 26 samples would have been reported as potentially false negative for HR HPV DNA. Low cellularity in a ThinPrep sample may also result in an insufficient volume of residual sample being available after cytology testing for HPV testing using HC2, since HC2 requires 4 ml of

sample. The requirement of less than 2 ml of sample for the Invader-based test minimizes the occurrence of samples with insufficient volume for HPV DNA testing.

Recent studies have suggested that the risk of progression toward cervical cancer can be as high for simultaneous infections with multiple HPV types as it is for infection with just one of the highest-risk HPV types, such as type 16 (16). This study has demonstrated that the IHPV testing is capable of identifying mixed infections, with multiple HR HPV types in 47 of 821 samples (5.7%) identified in this sample population alone. The use of three separate probe pools, one each for phylogenetically related HR HPV types, allows the IHPV test to identify samples that contain such mixed HPV infections, something unable to be done using the HC2 test.

The results of this study demonstrated the improved specificity of the IHPV reagents, the importance of the incorporation of an internal control to assure that negative results are due to the absence of HR HPV DNA instead of a lack of sample cellularity, and the assay's ability to detect mixed HR HPV infection within a single sample. Due to the manual extraction process, the IHPV assay required approximately 1 h more hands-on time than did the HC2 assay. However, studies have shown that the extraction process can be adapted to automated platforms, such as the Qiagen BioRobot M48 (Qiagen, Inc., Valencia, CA) and the bioMérieux NucliSENS easyMAG (bioMérieux, Durham, NC) (13, 22). Overall, these findings suggest that the IHPV method for the detection of HR HPV DNA is an easy-to-use and robust technology that may offer several advantages over current methods for the detection of HPV DNA.

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