Clinical Performance of the BD Onclarity HPV Assay Using an Adjudicated Cohort of BD SurePath Liquid-Based Cytology Specimens

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Key Words: BD Onclarity HPV Assay; Hybrid Capture 2 assay; Cervical cancer; High-risk HPV

Am J Clin Pathol July 2014;142:43-50

DOI: 10.1309/AJCP53KMHNRDICBL

ABSTRACT

Objectives: *To compare the performance of the BD Onclarity HPV Assay (BD Diagnostics, Sparks, MD) in BD SurePath liquid-based cytology media with that of Hybrid Capture 2 (HC2, Qiagen, Germantown, MD) samples co-collected in specimen transport medium in an adjudicated patient cohort.*

Methods: *The performance of the BD Onclarity HPV Assay using BD SurePath media was compared with that of HC2 samples co-collected in specimen transport medium using 541 archived samples from a multicenter US clinical trial with histologically adjudicated cervical biopsy specimens.*

Results: *The sensitivity for cervical intraepithelial neoplasia (CIN) 2 positivity (n - 104) was 90.4% (95% confidence interval [CI], 83-95) and 93.3% (95% CI, 87-97) and specificity was 76.9% (95% CI, 73-81) and 77.8% (95% CI, 74-82) for the BD assay and HC2, respectively. Nine cases of CIN 2+ had results discordant with the high-risk HPV assay. All were found to have been correctly classified with the BD assay using a novel WAVE denaturing high-performance liquid chromatography double-stranded DNA sequencing method.*

Conclusions: *The clinical performance of The BD Onclarity HPV Assay with respect to histology end points was similar to HC2. Moreover, discordant analysis revealed improved performance of the BD assay with respect to ability to provide extended genotyping information and lack of cross-reactivity with low-risk HPV types associated with cellular abnormalities. The relative risks for CIN 3 disease for HPV 31 and HPV 33/58 (combined) were comparable to that of HPV 18 in this population, suggesting that these genotypes may warrant monitoring in future studies.*

The advancement of molecular technologies has led to the introduction of a number of tests that specifically detect high-risk human papillomaviruses (hrHPV). The Hybrid Capture 2 (HC2) HPV DNA test (HC2, Qiagen, Germantown, MD) was the first molecular test to be approved by the Food and Drug Administration (FDA) and is widely used both as an adjunct to cytology for cervical cancer screening and as a way to determine which women with minor cytologic abnormalities require colposcopy.[1] Most recent studies have clearly shown that hrHPV testing alone is significantly more sensitive than cytology for detecting cervical cancer and is only slightly less specific.[2] In addition, a growing body of evidence now shows that cervical cytology together with hrHPV testing offers little benefit over hrHPV testing alone. A number of countries are now moving to adopt hrHPV primary screening with a reflex to cytology or genotyping as a triage method for hrHPV-positive women.[3-5] Primary screening with hrHPV testing will require a highly accurate hrHPV test, because it alone will be used to determine which women need additional follow-up and which women can simply be rescreened at some interval. Moreover, although many hrHPV tests may exhibit acceptable clinical sensitivity, the specificity of hrHPV tests will also be important when hrHPV testing is used for primary screening. This is because even a small decrease in specificity will lead to increases in unnecessary referrals for follow-up, avoidable anxiety for patients, and a substantial increased cost to the health care system.

Once we begin to use hrHPV testing for primary screening, some form of triage will be required to reduce the number of HPV-positive women needing referral to colposcopy. A number of different triage strategies are being considered. Triage using cytology and/or genotyping for specific hrHPVs

most commonly found in association with cervical intraepithelial neoplasia (CIN) type 3 lesions and invasive cancers has been reported to have the best outcome in terms of avoiding unnecessary colposcopy referrals.[5,6] However, cytology has well-recognized limitations including both a false-positive rate (because of infection with low-risk HPV and non–HPV-associated cellular abnormalities) and a high false-negative rate (because of sampling and detection errors). Moreover, commonly used genotyping assays also have limitations. A large World Health Organization (WHO) global proficiency study of HPV genotyping tests reported a relatively low sensitivity for both HPV 16 and HPV 18 when they occurred in mixed HPV infections.[7] Therefore, it is important that the next generation of hrHPV testing methods have maximal *clinically valid* sensitivity without compromising on specificity and that they provide robust and accurate detection of the most important HPV genotypes when present in both single and mixed HPV infections.

The BD Onclarity HPV Assay (BD Diagnostics, Sparks, MD) is a new real-time polymerase chain reaction (PCR)–based HPV screening test, which targets the E6 and E7 DNA regions of the HPV genome. These target regions are required during all stages of disease progression and the assay is designed to enable the detection of type-specific regions of the virus, as opposed to consensus amplification of conserved genomic regions detected with L1 primer sets. The assay can provide individual genotyping information for six HPV types, while simultaneously screening for all 14 high-risk virus types. The six genotypes identified individually with the assay include HPV 16, 18, 31, 45, 51, and 52. The performance of the BD assay has previously been reported to be equivalent to a number of FDA-approved and European conformity (CE)–marked HPV assays (including HC2) using cervical specimens collected in PreservCyt medium (Hologic, Marlborough, MA).[8-10] Here we evaluate the performance of the BD Onclarity HPV Assay using cervical specimens collected in BD SurePath medium and compare its performance to that of HC2 using cervical specimens collected at the same visit in specimen transport medium (STM).

Materials and Methods

Multicenter Clinical Trial Design

A total of 5,415 eligible women were enrolled with informed consent at 25 collection sites in the United States between September 2010 and February 2012. The selection criteria were as follows: age younger than 35 years; any age with high-risk status, defined as having been previously diagnosed with an abnormal Papanicolaou or positive HPV test; or not being screened in the previous 5 years. Two cervical samples were taken from each patient in accordance with the manufacturers' recommendations: a liquid-based cytology (LBC) sample was collected using the BD SurePath collection device and medium (BD Diagnostics, Burlington, NC) and a second sample was collected using the Digene HC2 DNA collection device and placed in STM (Qiagen). An LBC result was obtained from each patient and patients with either abnormal cytology or a positive HC2 result were referred to colposcopy, in which a four-quadrant biopsy specimen was taken via endocervical curettage. All testing was performed centrally in three independent laboratories. Abnormal cytology and all histology were adjudicated by an expert pathology panel and the consensus result was considered to be the final study result.

Retrospective Cohort Study Design

A total of 541 (10%) residual BD SurePath specimens were selected from the multicenter clinical trial samples for testing with the BD Onclarity HPV Assay. These included all samples with biopsy-confirmed CIN 2 (n = 104), 79 samples with abnormal cytology but lower than CIN 2 on cervical biopsy, and 358 samples that were negative for intraepithelial lesion or malignancy (NILM) on cytology and lower than CIN 2 on cervical biopsy. Residual samples were stored at room temperature and shipped on cold packs from the trial sites to BD within 60 days of collection where they were immediately stored at –20°C for 8 to 24 months before testing. All samples were tested in a blinded fashion using the BD HPV assay. The results were subsequently compared with the HC2 results obtained in the multicenter study from the fresh STM specimens.

HPV Testing

HC2 testing was performed at the time of collection in accordance with the manufacturer's recommended protocol[11] using the STM specimens collected in the multicenter trial. BD Onclarity HPV Assay was used on retrospective BD SurePath residual vial specimens using the fully automated Viper LT system (BD Diagnostics, Sparks, MD). The details of the BD Viper LT system will be reported elsewhere. Briefly, residual (pregradient) SurePath samples were brought to room temperature, vortexed briefly, and 0.5 mL of specimen was transferred to 1.7 mL of a proprietary BD HPV LBC diluent. The resultant mixture was heated at 120°C for approximately 30 minutes to lyse and homogenize the specimen and then cooled to room temperature. A volume of 0.8 mL of the resultant lysate was then extracted using BD Viper ferric oxide particle DNA binding and magnetic extraction and eluted in 400 μL of elution and neutralization buffer.[12] The BD Onclarity HPV Assay design selection criteria included the ability to detect 100 copies of one HPV type in the presence of one million copies of competing HPV targets, which

DOI: 10.1309/AJCP53KMHNRDICBL

ensures that mixed infections can be reliably detected.[13] It is a three-well, four-channel real-time PCR test that can provide individual genotyping information for six HPV types (HPV 16, 18, 31, 45, 51, and 52) while simultaneously screening for all 14 high-risk viruses. The rest of the 14 high-risk types are reported in groups of two or three viruses (HPV 33/58, HPV 56/59/66, and HPV 35/39/68). Each of three tubes contains fluorescent real-time PCR probes for four separate optical channels including one channel for human β-globin sequence, which acts as a sample adequacy and sample processing control **Table 1**. Aliquots (50 μL) of the DNA eluate were dispensed into each of three PCR tubes (G1, G2, and G3) to rehydrate the dried-down master mix and hot start Taq polymerase. PCR was performed using the following parameters: 95°C enzyme activation step for 15 minutes followed by 40 cycles at 95°C for 30 seconds and 55°C for 60 seconds. HPV-positive specimens were identified using a previously defined cycle threshold algorithm method that was derived using receiver operating characteristic curves.[8]

The BD Onclarity HPV Assay participated in the WHO HPV Laboratory Network (LabNet) HPV DNA Proficiency Study, 2013. The assay correctly identified each of the 46 blinded panel samples and was therefore found to be "proficient for detection of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a, and 68b" (personal communication, Joakim Dillner, MD, WHO HPV LabNet, International HPV Reference Center, Stockholm, Sweden).

Denaturing High-Performance Liquid Chromatography (DHPLC) Double-Stranded Sequencing Discordant hrHPV Test Method

Proprietary HPV type-specific primers (Transgenomic Inc, Omaha, NE) were designed to detect 14 high-risk strains (HPV 16, 18, 31, 33, 35, 39 45, 51, 52, 56, 58, 59, 66, and 68) and 11 low-risk genotypes (HPV 6, 11, 26, 30, 53, 67, 69, 70, 82, 85, and 97) in the L1 region of the virus. Prototype designs were validated empirically using human and HPV type-specific DNA. Residual SurePath samples (blinded to the original HPV results) were extracted using a commercially available extraction kit[14] and the purified DNA was used for PCR amplification using gene-specific primer sets. Each PCR reaction also contained a primer pair specific for the human *KRAS* gene that acted as an internal (sample adequacy/processing) control. Each experimental run was performed using a positive control containing 8,000 copies of the relevant plasmid DNA from each of the 25 HPV genotypes in the assay and 60 ng of human genomic DNA. Negative run controls were also included to ensure system integrity. Analysis of the strain-specific amplifications was performed on the WAVE HS System (Transgenomic Inc) **Figure 1**. Each amplification reaction was routinely run on a triethylammonium acetate/acetonitrile gradient under nondenaturing conditions and compared using plasmid control amplifications. Any amplification that showed a peak at the expected retention time for a given genotype was bidirectionally sequenced using standard Sanger sequencing. Approximately 100 bases of the resulting sequence for any predicted strain was then confirmed using nucleotide sequencing alignment and the nucleotide database

Table 1
Design of the BD Onclarity HPV Assay[a]

G1	G2	G3
HPV 16	HPV 33/58	HPV 51
HPV 18	HPV 31	HPV 52
HPV 45	HPV 56/59/66	HPV 35/39/68
IC	IC	IC

G, genotyping wells; HPV, human papillomavirus; IC, internal control.
[a] Each of the three genotyping wells contains four distinct probes detected in separate dye channels, one of which is dedicated to the β-globin IC. The other three channels are used to detect HPV types (either singly or in groups of two or three viruses) as shown.

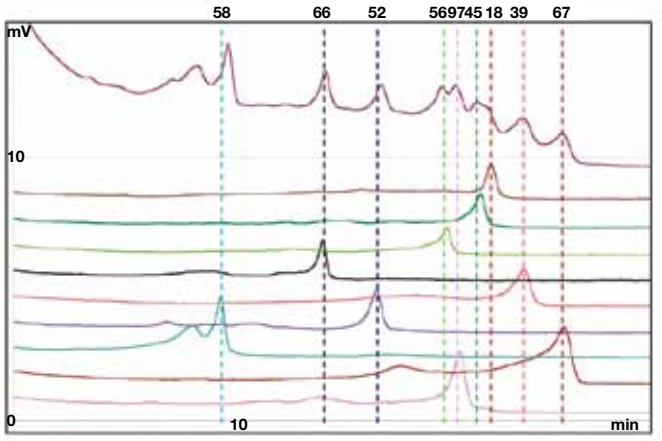

Figure 1 Ability of the denaturing high-performance liquid chromatography (DHPLC) technique to resolve mixed human papillomavirus (HPV) infections. The WAVE HS System DHPLC chromatogram shows the analysis of the HPV type-specific L1 polymerase chain reaction amplifications. Each amplification reaction contains HPV-specific primers and human *KRAS* (internal control) primers. The top trace shows the results of a UV detector scan of eluted DNA fragments from a mixture of nine different HPV types (indicated on top of figure) eluted from the DHPLC column and illustrates how different mixed HPV genotypes are separated. Each individual peak corresponds to the designated HPV type. The lower traces show the results from the corresponding HPV types run individually on the same column. The dotted vertical lines demonstrate that the genotype-specific peaks had the same retention time on the column whether present as a mixture or individually. In patients with mixed infections, the different genotypes are separated on the column, eluted as distinct fractions, and then sequenced individually using double-stranded Sanger sequencing chemistry.

DOI: 10.1309/AJCP53KMHNRDICBL

of the National Library of Medicine (Basic Local Alignment Search Tool). Primer designs were optimized to maximize the distance between individual genotype peaks so that even mixed infections would be resolved. Mixed infections that resulted in fully or partially overlapping peaks were not typically observed. However, the DHPLC method can be adjusted to resolve these samples by rerunning the DNA mixture under partially denaturing conditions on the column. The peak-to-peak separation is then increased, allowing the different DNA species to be separated before they are collected in individual fractions for sequencing.

Statistical Analysis

Test performance estimates were calculated with exact 95% confidence intervals (CIs) using the Pearson-Klopper method in the binom R package.[15] McNemar exact test comparisons were calculated using the exact 2 × 2 R package.[16] Differences between the HC2 and BD HPV assays were calculated using Fisher exact test in Minitab 16 statistical software (Minitab, State College, PA). The risk for disease among patients with a given genotype relative to HPV-negative patients was calculated by unconditional maximum likelihood estimation using the epitools R package.[17]

Results

The multicenter US cohort samples were collected from predominantly younger women (median age of patients whose samples were tested was 28 years) or women at high risk for cervical disease (those with a history of disease or who had not been screened in the previous 5 years). This resulted in a much higher HPV positivity rate among women than that typically observed in US screening populations **Table 2**.[18] The positivity rates were comparable for both assays across all histology categories, indicating that this was a population effect and reflected the high-risk group enrolled in the study. This is also evident from the high yield of CIN 2–positive disease detected (63 women diagnosed with CIN 2 [12%] and 41 with CIN 3 disease [8%]).

The hrHPV positivity rate of the BD Onclarity HPV Assay in women with NILM cervical cytology was 22.7% (95% CI, 18.7-27.1) and the HPV positivity rate of the HC2 assay in the NILM group was 20.7% (95% CI, 16.8-25.0) **Table 3**. The difference between the two assays was not statistically significant ($P = .488$). The sensitivity for CIN 2–positive disease for all patients (n = 104) was 90.4% for the BD HPV assay and 93.3% for HC2 ($P = .614$). The specificity for CIN 2 positivity was 76.9% and 77.8% with the BD assay and HC2, respectively ($P = .808$). The assays also showed no difference in performance in the entire cohort as well as the clinically important subset of women with NILM cervical cytology (Table 3).

Discordant hrHPV results for the two assays are shown **Table 4** for all patients as well as for women with CIN 2 positivity. Among all 541 women, 20 were hrHPV positive on HC2 testing but negative on the BD Onclarity HPV Assay, and 21 cases were hrHPV positive with the BD assay but negative with HC2. The difference between the two assay results was not significant using the McNemar exact test (odds ratio, 0.95; $P = 1.0$). Among the 104 women with biopsy-confirmed CIN 2 positivity, nine cases were discordant. Six cases were hrHPV positive with HC2 but negative with the BD assay and three cases were hrHPV positive with the BD assay but negative with HC2. The difference between the two assay results in women with CIN 2 positivity was not significant using McNemar exact test (odds ratio, 2.00; $P = .508$). The nine discordant samples from women with biopsy-confirmed CIN 2+ were blindly adjudicated using type-specific L1 PCR, DHPLC, and sequencing **Table 5**. In all nine cases, the BD assay using BD SurePath media had provided the correct hrHPV result and the HC2 test using STM had provided an erroneous result. Five of the cases should be classified as HC2 false-positive results, because only low-risk HPV types were identified using DHPLC and sequencing. Three cases were false-negative HC2 results: in one case, HC2 gave a negative result but the BD assay detected hrHPV, two cases had HPV 18, and one had HPV 31, which was confirmed using DHPLC and sequencing. The remaining case was HC2-positive and BD-negative, and

Table 2
Positivity of BD Onclarity HPV and Hybrid Capture 2 Assays vs Histologic Classification

	Consensus Histology				
	WNL	**CIN 1**	**CIN 2**	**CIN 3**	**Total (%)**
Total No. of patients	407	30	63	41	541
BD HPV test (–)	329	7	6	4	346 (63)
BD HPV test (+)	78	23	57	37	195 (36)
HC2 (–)	335	5	6	1	347 (64)
HC2 (+)	72	25	57	40	194 (35)

CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HPV, human papillomavirus; WNL, within normal limits; +, positive; –, negative.

DOI: 10.1309/AJCP53KMHNRDICBL

Table 3
Clinical Performance of the BD Onclarity HPV Assay Using BD SurePath Media and HC2 Assay Using STM

Performance Measure	BD HPV Test (95% CI)	HC2 (95% CI)
All women (n = 541)		
hrHPV positivity rate, %	36.0 (32-40)	35.9 (32-40)
Sensitivity for CIN 2+ (n = 104), %	90.4 (83-95)	93.3 (87-97)
Specificity for CIN 2+, %	76.9 (73-81)	77.8 (74-82)
Positive predictive value, %	48.2 (41-56)	50.0 (43-57)
Negative predictive value, %	97.1 (95-99)	98.0 (96-99)
Women with NILM (n = 392)		
hrHPV positivity rate, %	22.7 (19-27)	20.7 (17-25)
Sensitivity for CIN 2+ (n = 34), %	91.2 (76-98)	91.2 (76-98)
Specificity for CIN 2+, %	83.8 (80-88)	86.0 (82-90)
Positive predictive value, %	34.8 (25-46)	38.3 (28-50)
Negative predictive value, %	99.0 (97-100)	99.0 (97-100)

CI, confidence interval; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HPV, human papillomavirus; hrHPV, high-risk HPV; NILM, negative for intraepithelial lesion or malignancy; STM, specimen transport medium.

DHPLC and sequencing failed to identify any of the 25 HPV types that could be identified by the type-specific PCR and DHPLC assay.

The samples used in this study came from a relatively young population of women at elevated risk for cervical disease, especially cases having biopsy-confirmed CIN 2 (19.2% of all samples). We thought it would be useful to evaluate the relative risk for CIN 2 and higher and CIN 3 disease based on specific HPV type **Table 6**. HPV 16 has the highest relative risk for both CIN 2 and higher and CIN 3 disease, taking into consideration both single and multiple hrHPV infections. This is in agreement with a number of published studies. Interestingly, the relative risk for HPV 31 is slightly higher than that for HPV 18 for both CIN 2 and higher and CIN 3 disease. The risk for the paired HPV 33/58 types shows a similar pattern. However, none of the differences in relative risk observed between specific HPV types are significant because of the number of CIN 2 (n = 63) and CIN 3 (n = 41) lesions. The prevalence of mixed infections in this population was high, with 44.1% of patients harboring two or more viral types (range, 2-7 HPV types). This prompted us to investigate the impact of multiple infections by comparing the relative risks for all infections (including patients with multiple HPV genotypes) with that of single HPV infections. HPV types 16 and 33/58 showed a marked increase in relative risk for CIN 3 disease compared with CIN 2 or higher disease in all as well as single infections.

Table 4
Comparison of BD Onclarity HPV and HC2 Assays

	HC2 (–)	HC2 (+)	Odds Ratio[a] (*P*)
All women			
BD (–)	326	20	0.95 (1.0)
BD (+)	21	174	
Women with CIN2+ disease			
BD (–)	4	6	2.00 (.508)
BD (+)	3	91	

CIN, cervical intraepithelial neoplasia; HC2, Hybrid capture 2; HPV, human papillomavirus; +, positive; –, negative.
[a] McNemar exact test.

Table 5
Patients With CIN 2+ and Discordant BD Onclarity HPV Assay and HC2 Test Results[a]

Adjudicated Histology Result	Sample No.	Laboratory Cytology	HC2 Result	BD HPV Assay Result	DHPLC and Sequencing Result
CIN 2	1	LSIL	Positive	Negative	53 (LR)
	2	ASCUS	Negative	31	31
	3	NILM	Negative	18	18
	4	NILM	Positive	Negative	82 (LR)
CIN 3	5	ASCUS	Positive	Negative	Negative for HR
	6	LSIL	Positive	Negative	53 (LR)
	7	NILM	Negative	18	18
	8	NILM	Positive	Negative	67 (LR)
	9	ASCUS	Positive	Negative	82 (LR)

ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; DHPLC, denaturing high-performance liquid chromatography; HC2, Hybrid capture 2; HPV, human papillomavirus; HR, high-risk HPV type; LR, low-risk HPV type; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; +, positive.
[a] Discordance was resolved using DHPLC and sequencing.

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Table 6
Relative Risk by HPV Type for CIN 2 or Higher Disease and CIN 3 Disease

All Infections						Single Infections					
				Relative Risk (95% CI)						Relative Risk (95% CI)	
HPV Type	≥ CIN 2	CIN 3	Frequency[a]	≥ CIN 2	CIN 3	HPV Type	≥ CIN 2	CIN 3	Frequency[a]	≥ CIN 2	CIN 3
16	46	22	64	24.9 (13-47)	29.7 (11-83)	16	22	11	35	21.7 (11-42)	27.2 (9-81)
18	11	3	19	20 (10-41)	13.7 (3-57)	18	3	0	8	13.0 (4-38)	0
31	17	5	28	21 (11-41)	15.4 (4-54)	31	4	1	8	17.3 (7-44)	10.8 (1-86)
33/58	12	7	24	17.3 (8-36)	25.2 (8-80)	33/58	8	6	11	25.2 (12-51)	47.8 (16-143)
39/68/35	23	7	43	18.5 (9-36)	14.1 (4-46)	39/68/35	4	0	13	10.7 (4-30)	0
45	9	3	17	18.3 (9-39)	15.3 (4-63)	45	1	0	4	8.7 (1-53)	0
51	4	0	14	9.9 (4-28)	NA	51	0	0	1	0	0
52	13	2	28	16.1 (8-33)	6.2 (1-32)	52	2	1	10	6.92 (2-28)	8.65 (1-71)
59/56/66	15	3	46	11.3 (5-24)	5.6 (1-24)	59/56/66	3	0	19	5.5 (2-18)	0
HR negative	10	4	346	1 (0-2)	1 (0-4)						
HR positive	94	37	195	16.7 (9-31)	16.4 (6-45)						

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HR, high-risk HPV type.
[a] Frequency refers to the number of observed infections with this HPV type (including < CIN2).

This suggests that HPV types 16 and 33/58 are more likely to cause progressive disease, even when found together with other hrHPV types. Conversely, HPV 18, 31, and all other high-risk types showed a decrease in relative risk in the CIN 3 disease category.

Discussion

The performance of the BD Onclarity HPV Assay using frozen retrospective SurePath LBC specimens is equivalent to that of HC2-tested fresh STM specimens analyzed in the original multicenter trial. This is in agreement with our previous study, which showed both BD SurePath and PreservCyt specimens to be stable and providing accurate genotyping information after being in storage for up to 2.5 years at 4°C.[19] It is also noteworthy that most of the BD SurePath samples used in this study had been stored at room temperature for up to 60 days before being stored at –20°C. This provides further evidence that BD SurePath media stabilizes both human and viral genomic DNA over extended storage times and temperatures.

The women selected for this study did not comprise a normal screening population. The high positivity rates and incidence of CIN 2 can be explained by the age and high-risk status of the population. The results therefore provide a more reliable estimate of the sensitivity, rather than the specificity, of the BD assay. However, the specificity and positive predictive value of the BD assay performed using SurePath media compared with those of HC2 using FDA-cleared STM suggests that performance of the assays were equivalent with regard to CIN2+ end points. This is in agreement with a previous screening population study in which the BD HPV assay showed comparable performance to four clinically validated tests, including HC2.[20] The type-specific L1 PCR combined with DHPLC discordant sequencing method used in this study is a significant advance over PCR sequencing technology because it enables separation of two or more HPV types in the same sample on the basis of their sequence and/or size using the DHPLC column. Traditional PCR amplification and discordant sequencing methods can be difficult to perform and frequently do not amplify all of the genotypes present, or they coamplify two or more types, which results in poor or no sequencing results.

Using the new DHPLC method, one can, in principle, resolve all mixed infections. This may have important implications for future studies for tracking the predominant HPV type. The method also has the added advantage of targeting a different part of the HPV genome (L1) than that used by the BD HPV assay (targets E6/E7), which avoids potential experimental bias in the discordant resolution method. It has been known for some time that the HC2 assay, which was designed to identify hrHPV types, can cross-react with low-risk HPV types, particularly HPV 53 and 82.[21] This was evident in the current study, in which five of the 104 CIN 2–positive lesions were found to be HC2 positive but BD assay negative because of the cross-reaction of HC2 with low-risk HPV types. Even though three of these five lesions were histologically found to be CIN 3, given the low prevalence of HPV 53 and 82 in invasive cervical cancers (<1%), it is unlikely that these lesions represent precursors to invasive cervical cancer (ICC).[22] Conversely, the BD assay correctly identified hrHPV type positive CIN 2–positive lesions, including three cases missed with HC2. Four additional cases in the study were discordant

DOI: 10.1309/AJCP53KMHNRDICBL

with histology, all of which were classified as CIN 2, with each case having a negative result on both the HC2 and the BD assays. Three of these patients were characterized on cytology as having atypical squamous cells of undetermined significance (ASCUS) and the fourth was NILM. The double-negative HPV result and the cytologic finding of ASCUS or lower suggest that these patients were unlikely to harbor a progressive HPV infection.

It is now widely recognized that the 14 hrHPV types differ enormously in their relative oncogenic potential. Therefore, genotyping assays are being increasingly used to provide additional risk stratification above that which can be achieved using pooled hrHPV testing. Current US guidelines recommend genotyping for only HPV 16 and 18, and detection of either of these two HPV types is sufficient to warrant referral to colposcopy.[23] However, another finding in the current study is that hrHPV types other than HPV 16 and HPV 18 were frequently associated with adjudicated histologically confirmed CIN 2. When women without multiple HPV infections were analyzed, the association between CIN 2 positivity or CIN 3 and infection with HPV 33/58 was just as strong as the association found for HPV 16. Eight (72.7%) of 11 women with HPV 33/58 but no other hrHPV type had CIN 2 positivity, which is similar to the finding that 22 (62.9%) of 35 women with HPV 16 but no other hrHPV type had CIN 2 positivity. It should be cautioned, however, that the number of cases of CIN 2 positivity associated with specific genotypes was relatively limited in this study and that women without CIN 2 positivity were at high risk for having HPV infections given their age or clinical history. Therefore, the current study lacks statistical power to be definitive on this point. However, our findings are consistent with those of other studies that also suggested that it may be clinically useful to identify HPV types other than HPV 16 and 18. For example, a recent European study reported that HPV 31 infection has a higher disease risk for both CIN 2 positivity and CIN 3 than does HPV 18.[24] Similarly, large long-term longitudinal follow-up studies have found that HPV types 31 and 33, as well as 35, 52, and 58 in one Japanese study, are quite important in predicting which women will progress to CIN 3 positivity.[25,26] Finally, a large study of 47,617 women in New Mexico found that the age-adjusted odds ratio for cytologic high-grade disease compared with negative cytology in women with single infections was highest for HPV 33, followed by HPV types 16, 18, 31, and 58.[27]

A recent meta-analysis of the prevalence of different HPV types in the progression of disease from normal cytology through CIN 3 to ICC in 115,789 HPV-positive women demonstrated important differences in HPV type distribution between CIN 3 lesions and ICC. The most virulent types (HPV 16, 18, and 45) were overrepresented in cancers and HPV types 31, 33, and 58 were enriched when normal cytology was compared with CIN 3; this was shown in other studies as well.[28,29] However, the relative importance of HPV types 31, 33, and 58 dropped from CIN 3 to ICC, which suggests that these types are less carcinogenic than HPV 16, 18, and 45.[30] From a clinical risk stratification perspective, large genotyping studies are needed to be conducted in intended use populations that are statistically powered to accurately determine the prevalence of specific hrHPV types in women both with and without cervical disease. This will allow guideline makers to balance the benefits of rapidly detecting cervical disease by genotyping for a specific HPV type against the health care burden of working up women without disease who harbor that specific HPV type.

In summary, the current data indicate that the BD Onclarity HPV Assay using frozen retrospective BD SurePath LBC specimens has a sensitivity that is equivalent to that of HC2-tested fresh STM specimens analyzed during the original multicenter trial. The BD assay has the ability to provide accurate genotyping information both in single and mixed HPV infections and this will allow accurate estimates of the contribution of individual HPV types to end point disease. Based on our discordant analysis, the BD assay also shows excellent specificity and does not react with low-risk HPV types. Worldwide prevalence data demonstrate that HPV low-risk type 6, 11, 70, 73, and 82 account for 13.4% and 6.8% of low- and high-grade cytology, respectively.[31] The recent WHO global proficiency testing of HPV genotyping assays suggests that there is considerable room for improvement in the area of genotyping. Of the 132 panels that were tested with 24 different genotyping assays in 98 different laboratories, only 54% were proficient in detecting more than one HPV type.[7] By contrast, the BD Onclarity test was found to be 100% proficient in the 2013 LabNet Study. Therefore the BD Onclarity HPV Assay offers the prospect of improved HPV type detection. The assay's ability to predict CIN 2–positive outcomes is currently being assessed in large-scale trials.

This work was supported by Becton Dickinson and Company. The BD Onclarity HPV Assay and the Viper LT instrument are not available for sale in all countries.

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References

1. Arbyn M, Sasieni P, Meijer CJ, et al. Chapter 9: clinical applications of HPV testing: a summary of meta-analyses. *Vaccine*. 2006;24(suppl 3):78-89.
2. Cuzick J, Clavel C, Petry KU, et al. Overview of the European and North American studies on HPV testing in primary cervical cancer screening. *Int J Cancer*. 2006;119:1095-1101.

DOI: 10.1309/AJCP53KMHNRDICBL

3. Ronco G, Biggeri A, Confortini M, et al. [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. *Epidemiol Prev.* 2012;36(suppl 1):e1-e72.
4. Murphy J, Kennedy EB, Dunn S, et al. HPV testing in primary cervical screening: a systematic review and meta-analysis. *J Obstet Gynaecol.* 2012;34:443-452.
5. Rijkaart DC, Berkhof J, van Kemenade FJ, et al. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. *Int J Cancer.* 2012;130:602-610.
6. Cox JT, Castle PE, Behrens CM, et al. Comparison of cervical cancer screening strategies incorporating different combinations of cytology, HPV testing, and genotyping for HPV 16/18: results from the ATHENA HPV study. *Am J Obstet Gynecol.* 2013;208:e1-e11.
7. Eklund C, Zhou T, Dillner J. Global proficiency study of human papillomavirus genotyping. *J Clin Microbiol.* 2010;48:4147-4155.
8. Szarewski A, Mesher D, Cadman L, et al. Comparison of seven tests for high-grade cervical intraepithelial neoplasia in women with abnormal smears: the Predictors 2 study. *J Clin Microbiol.* 2012;50:1867-1873.
9. Cuzick J, Cadman L, Mesher D, et al. Comparing the performance of six human papillomavirus tests in a screening population. *Br J Cancer.* 2013;108:908-913.
10. Mesher D, Szarewski A, Cadman L, et al. Comparison of human papillomavirus testing strategies for triage of women referred with low-grade cytological abnormalities. *Eur J Cancer.* 2013;49:2179-2186.
11. Qiagen. *Hybrid Capture 2 High-Risk HPV DNA Test Package Insert: L00665 Rev 2.* 2007.
12. Felder RA, Foster ML, Lizzi MJ, et al. Process evaluation of a fully automated molecular diagnostics system. *J Assoc Lab Automation.* 2009;14:262-268.
13. Vaughan L, Chen C, Gutierrez E, et al. Design and development of a novel molecular HPV assay. Abstract presented at the Twenty-Sixth International Papillomavirus Conference and Clinical Workshop; July 5, 2010; Montréal, Canada. Abstract P474.
14. Clontech. *NucleoSpin Tissue Kits User Manual.* Mountain View, CA: Clontech Laboratories Inc; 2004.PT3629-1 (PR41616).
15. Sundar D-R. Binomial confidence intervals for several parameterizations. binom2009. Version 1.0-5. Available at: http://CRAN.R-project.org/package=binom. Accessed October 2013.
16. Fay M. Two-sided exact tests and matching confidence intervals for discrete data. *R J.* 2010;2:53-58.
17. JT. Epidemiology tools. R package version 0.5-7. 2012. Available at: http://CRAN.R-project.org/package=epitools. Accessed October 2013.
18. Wright TC Jr, Stoler MH, Behrens CM, et al. The ATHENA human papillomavirus study: design, methods, and baseline results. *Am J Obstet Gynecol.* 2012;206:46 e1-e11.
19. Agreda PM, Beitman GH, Gutierrez EC, et al. Long-term stability of human genomic and human papillomavirus DNA stored in BD SurePath and Hologic PreservCyt liquid-based cytology media. *J Clin Microbiol.* 2013;51:2702-2706.
20. Cuzick J, Cadman L, Mesher D, et al. Comparing the performance of six human papillomavirus tests in a screening population. *Br J Cancer.* 2013;108:98-113.
21. Castle PE, Solomon D, Wheeler CM, et al. Human papillomavirus genotype specificity of hybrid capture 2. *J Clin Microbiol.* 2008;46:2595-2604.
22. de Sanjose S, Quint WG, Alemany L, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol.* 2010;11:1048-1056.
23. Saslow D, Solomon D, Lawson HW, et al. American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. *Am J Clin Pathol.* 2012;137:516-542.
24. Halfon P, Lindemann ML, Raimondo A, et al. HPV genotype distribution according to severity of cervical neoplasia using the Digene HPV genotyping LQ test. *Arch Virol.* 2013;158:1143-1149.
25. Kjaer SK, Frederiksen K, Munk C, et al. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. *J Natl Cancer Inst.* 2010;102:1478-1488.
26. Matsumoto K, Oki A, Furuta R, et al. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. *Int J Cancer.* 2011;128:2898-2910.
27. Wheeler CM, Hunt WC, Cuzick J, et al. A population-based study of human papillomavirus genotype prevalence in the United States: baseline measures prior to mass human papillomavirus vaccination. *Int J Cancer.* 2013;132:198-207.
28. Smith JS, Lindsay L, Hoots B, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer.* 2007;121:621-632.
29. Clifford GM, Smith JS, Aguado T, et al. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. *Br J Cancer.* 2003;89:101-105.
30. Guan P, Howell-Jones R, Li N, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. *Int J Cancer.* 2012;131:2349-2359.
31. Information Centre on Human Papilloma Virus (HPV) and Cancer. Low-risk HPV type prevalence in low and high grade cytology. Available at http://www.hpvcentre.net/dataquery.php. Accessed October 2013.

DOI: 10.1309/AJCP53KMHNRDICBL