JCM Journals.ASM.org

Comparison of Hybrid Capture II, Linear Array, and a Bead-Based Multiplex Genotyping Assay for Detection of Human Papillomavirus in Women with Negative Pap Test Results and Atypical Squamous Cells of Undetermined Significance

Manola Comar,[a,c] Michelle R. Iannacone,[b] Giorgia Casalicchio,[a] Sandrine McKay-Chopin,[b] Massimo Tommasino,[b] and Tarik Gheit[b]

Institute for Maternal and Child Health—IRCCS Burlo Garofolo, Trieste, Italy[a]; International Agency for Research on Cancer, Lyon, France[b]; and University of Trieste, Trieste, Italy[c]

Many methods with different levels of analytical sensitivity and clinical specificity have been developed to detect the presence of high-risk (HR) types of the human papillomavirus (HPV) in cervical samples. The Hybrid Capture II (HC-II) assay is broadly used for primary screening. In addition, several HPV genotyping assays, based on PCR methods, display higher sensitivity than the HC-II and are also used in screening programs. We evaluated the performance of three HPV DNA tests, namely, the HC-II, the Linear Array (LA) HPV genotyping assay, and an HPV type-specific E7 PCR bead-based multiplex genotyping assay (TS-MPG) that is a laboratory-developed method for the detection of HPV, in 94 women with atypical squamous cells of undetermined significance (ASC-US) and in cytological samples from 86 women with a negative Pap test. The HPV prevalence with the TS-MPG assay was increased compared to the prevalence with the LA and HC-II assays. The HPV DNA prevalence in women with ASC-US was greater with the TS-MPG assay (46.2%) than with the LA (36.3%) and HC-II (29.7%) assays. The HPV DNA prevalence in the control group was greater with the TS-MPG assay (32.1%) than with the LA assay (10.7%). Two women with ASC-US who were HPV DNA negative by the HC-II and positive by the TS-MPG or/and LA assays had lesions that progressed to low-grade squamous intraepithelial and high-grade squamous intraepithelial lesions. This study shows that the TS-MPG assay exhibited higher analytical sensitivity than the LA and HC-II assays for the detection of HPV DNA, which reduces the potential to incorrectly identify a woman's HPV infection status.

Infection by high-risk (HR) types of the human papillomavirus (HPV) has been demonstrated in almost all cervical carcinomas (3, 41). Women with persistent infections of HR-HPV types have a greater risk for developing premalignant lesions and therefore require additional screening (22, 36). Previous studies have shown that the risk of persistence and progression of infection differs by genotype (20, 31). Therefore, HPV genotyping has important implications in screening protocols, especially among women who have been diagnosed with premalignant lesions.

DNA detection of HR-HPV types is applicable in several clinical settings. HR-HPV DNA testing is recommended in combination with cytology in women 30 years or older and as a posttreatment follow-up test (37). In addition, HPV testing has proven to be useful for triage of the very problematic categories of equivocal cytological results that account for around half of abnormal results, such as atypical squamous cells of undetermined significance (ASC-US) (2).

The management of ASC-US, or a borderline Pap smear, has been problematic because the majority of women with this Pap result have no lesions, although approximately 5 to 11% have high-grade cervical intraepithelial neoplasia (CIN) and 1 per 1,000 have cervical cancer (10).

A large panel of methods with different levels of analytical sensitivity and clinical specificity has been developed to detect the presence of HR-HPV papillomavirus in cervical samples. The Hybrid Capture II (HC-II) assay (Digene, Gaithersburg, MD) is broadly used for primary screening in addition to cytology and for the triage of ASC-US (1). Despite the relatively low analytical sensitivity of the HC-II assay, which detects approximately 5,000 viral copies/ml of cervical sample suspension (4), it has demonstrated good clinical specificity to detect premalignant lesions (9).

In addition to HC-II, several HPV genotyping assays based on PCR methods have been developed (12, 27, 30, 34, 38–40). PCR amplification significantly increases the sensitivity of the HPV detection assays. However, the HPV DNA detection sensitivity is normally inversely correlated with the specificity in detecting cervical lesions. Therefore, the use of the highly sensitive HPV DNA detection methods in clinical settings is often questioned.

In this study, we evaluated the performance of three HPV DNA tests, namely, the HC II assay, the Linear Array (LA) HPV genotyping assay (Roche Molecular Systems, Alameda, CA), and an HPV type-specific E7 PCR bead-based multiplex genotyping assay (TS-MPG) that is a laboratory-developed method (14, 33) for the detection of HPV in women with ASC-US and in cytological samples from women with a negative Pap test.

MATERIALS AND METHODS

Study population and cervical tissue sample collection. In accordance with the diagnostic criteria for the Bethesda System 2001 (35), cytological

Received 7 August 2012 Returned for modification 7 September 2012
Accepted 26 September 2012
Published ahead of print 3 October 2012
Address correspondence to Tarik Gheit, icb@iarc.fr.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JCM.02105-12

TABLE 1 HPV DNA prevalences in women with ASC-US and controls by HC-II, TS-MPG, and LA assays

Variable	Samples positive for HPV DNA by:									
			LA				TS-MPG			
	HC-II in ASC-US patients ($n = 91$)		ASC-US patients ($n = 91$)		Controls ($n = 84$)		ASC-US patients ($n = 91$)		Controls ($n = 84$)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Any HPV type[a]	27	29.7	33	36.3	9	10.7	42	46.2	27	32.1
HPV types in both TS-MPG and LA[b]	27	29.7	30	33.0	9	10.7	40	44.0	23	27.4
HPV types in HC-II[c]	27	29.7	24	26.4	6	7.1	32	35.2	20	23.8

[a] Prevalence of DNA not restricted to HPV types included in the HC-II, TS-MPG, or LA assays.
[b] Prevalence of DNA for HPV types included in both TS-MPG and LA assays.
[c] Prevalence of DNA for the 13 HR-/pHR-HPV types included in the HC-II assay.

specimens from 94 women consecutively recruited and diagnosed with atypical squamous cells of undetermined significance (ASC-US) were included in the current analysis. According to the clinical protocol, follow-up cytological examination data were available for 47 women with ASC-US 1 year later. In addition, cytological samples from 86 women with negative Pap tests without history of precancerous lesions or cervical cancer were selected.

The cytological samples were collected at the Gynecological Service of the Children's Hospital Burlo Garofolo, a clinic setting for cervical cancer prevention. All study procedures have been approved by the IRCCS Burlo Garofolo Ethical Committee (C.I.B. 118/10 09/02/2010).

Cervical samples were obtained using the Cervex brush device (Rovers Medical Devices B.V., The Netherlands). Collected specimens were preserved in vials containing PreservCyt solution (Cytyc Corporation, Boxborough, MA) and transferred to the laboratory for HPV testing.

HPV detection methods. (i) Hybrid Capture II assay. Sample material collected in PreservCyt medium was made suitable for HC-II assay by using a sample conversion kit (Digene Corporation). HPV DNA testing by the HC-II assay method was performed automatically according to the manufacturer's protocol. HC-II is a sandwich capture molecular hybridization assay that utilizes chemiluminescent detection to provide a semiquantitative result. The assay is calibrated to detect approximately 5,000 genome equivalents per ml (or 1 pg/ml) of target HPV, represented by an RLU (relative light unit) measurement greater than or equal to the cutoff value calculated in each run by a series of standards. The samples were analyzed for the presence of 13 HPV types, including 12 HR types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and one pHR (probable high-risk) type (HPV 68). The positive and negative controls provided were included in each run.

(ii) Linear Array HPV genotyping test. The LA HPV genotyping test (Roche Diagnostics) uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 bp long. A pool of HPV primers is designed to amplify HPV DNA from 37 genotypes, including 20 HR-/pHR-HPV types (16, 26, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73 and 82), as defined in 2011 by the International Agency for Research on Cancer (IARC) classification (15), and for amplification of the β-globin human gene as a genomic DNA control. The LA assay was used manually as described by the manufacturer. At least one replicate of the Linear Array HPV-positive (HPV 16) and -negative controls was processed within each run.

(iii) HPV type-specific E7 PCR bead-based multiplex genotyping. The multiplex HPV type-specific E7 PCR utilizes HPV type-specific primers targeting the E7 region for the detection of 19 HR-/pHR-HPV types (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 68b, 70, 73, and 82) and 2 low-risk (LR)-HPV types (HPV 6 and 11), with detection limits ranging from 10 to 1,000 copies of the viral genome. The amplicon size varies between 210 and 258 bp. Two primers for amplification of the β-globin gene were also included to provide a positive control for the quality of the template DNA (14, 33). Following PCR amplification, 10 μl of each reaction mixture was analyzed by MPG using the Luminex technology (Luminex Corporation, Austin, TX) as described previously (32, 33).

Statistical analysis. For the TS-MPG and LA assays, the independent HPV type-specific DNA prevalence was estimated as the proportion of patients who tested positive for DNA of a given HPV type. For each of the three assays (i.e., HC-II, LA, and TS-MPG), DNA prevalence was also calculated as the proportion of patients who tested positive for DNA for at least one tested HPV type included in that particular assay. Comparisons between the TS-MPG and LA assays for the number of HPV types testing DNA positive were made using Fisher's exact test. Agreement between genotyping assays was estimated using the unadjusted kappa coefficient. To compare the overall DNA prevalence found by the HC-II assay independently with that found by the TS-MPG assay and by the LA assay, 2 different approaches were used to estimate the agreement rate. First, the agreement rate was estimated regardless of the specific HPV types included in the different assays. Second, the agreement rates were estimated by restricting the prevalence estimates to include only those types common across the HC-II, TS-MPG, and LA assays.

RESULTS

Analyses were restricted to cervical specimens that tested positive for the β-globin gene, corresponding to 91 women diagnosed with ASC-US and 84 women with a negative Pap test serving as a control group. The median age of the women was 34.5 years for both groups. Among the 91 women diagnosed with ASC-US, 29.7% were identified as HPV DNA positive by the HC-II assay (Table 1). In comparison, DNA prevalence among ASC-US cases was 26.4% and 35.2% by the LA and TS-MPG assay, respectively, for HPV types included in the HC-II assay (Table 1).

Additionally, 84 women with no history of precancerous lesions or cervical cancer were also tested by the LA and TS-MPG assays. The HR-/pHR-HPV DNA prevalence among the controls was lower than that observed among the ASC-US cases, with estimated prevalences of 7.1% and 23.8% by the LA and TS-MPG assays, respectively (Table 1).

The increase of HPV prevalence in the ASC-US group in comparison to the prevalence in the control group was detected independently of the HPV genotyping assay (Table 1).

Table 2 presents the number of ASC-US cases that tested HPV DNA positive either by the HC-II and TS-MPG assays or by the HC-II and LA assays, as well as the independent agreement (unadjusted kappa test) between the HC-II assay and the TS-MPG

TABLE 2 ASC-US HPV DNA positivity by HC-II compared to that in TS-MPG and LA assays

Variable	HC-II and TS-MPG		HC-II and LA	
	No. of HPV DNA-positive samples	Kappa coefficient	No. of HPV DNA-positive samples	Kappa coefficient
Any HPV type[a]	23	0.70	21	0.77
HPV types in HC-II[b]	18	0.71	15	0.74

[a] DNA positivity not restricted to HPV types included in the HC-II, TS-MPG, or LA assay.
[b] DNA positivity restricted to the 13 HR-/pHR-HPV types included in the HC-II assay.

TABLE 4 HPV DNA prevalence for multiple infections in women with ASC-US and controls across TS-MPG and LA assays

No. of infections[a]	HPV DNA prevalence									
	ASC-US patients (n = 91)					Controls (n = 84)				
	TS-MPG		LA			TS-MPG		LA		
	No.	%	No.	%	P^b	No.	%	No.	%	P^b
0	49	53.9	58	63.7		57	67.9	75	89.3	
1	19	20.9	16	17.6		16	19.1	6	7.1	
2	20	22.0	14	15.4		7	8.3	3	3.6	
≥3	3	3.3	3	3.3	<.0001	4	4.8	0	0.0	<.0001

[a] Prevalence of DNA positivity for HPV types included in both TS-MPG and LA assays.
[b] Fisher's exact P value.

assay and the LA assay. Despite variations in the HPV types included in each assay, the number of ASC-US cases that tested HPV DNA positive was greater using the TS-MPG assay (Table 2). The agreement between the HC-II and TS-MPG assays (kappa statistic = 0.70) and between the HC-II and LA assays (kappa statistic = 0.77) was substantial. Furthermore, restricting analyses to the HPV types included in the HC-II assay did not significantly alter the agreement rate of DNA detection between the TS-MPG and HC-II assays (kappa statistic = 0.71) or the LA and HC-II assays (kappa statistic = 0.74) (Table 2).

The DNA prevalence for any HPV type included in both assays was greater for ASC-US patients (46.2%) and controls (32.1%) with the TS-MPG assay than for ASC-US patients (36.3%) and controls (10.7%) with the LA assay (Table 3). Additionally, compared to the results for the LA assay, the TS-MPG assay detected a statistically significantly higher proportion of multiple infections among both ASC-US patients and controls ($P = <0.0001$) (Table 4). Specifically, 23 ASC-US patients were HPV positive for more than one HPV type by TS-MPG, while 17 women were positive by LA (Table 4). Among controls, 11 women were HPV positive for more than one HPV type by TS-MPG, while only 3 women were positive by LA (Table 4).

Nineteen women with ASC-US were HPV DNA positive for a single HPV type by TS-MPG and 16 by LA. In the controls, 16 women were positive for a single HPV type by TS-MPG and 6 by LA (Table 4).

The prevalence of specific HPV types established by highly sensitive assay demonstrated that HPV 53 was highly prevalent in the women with ASC-US using the LA assay (8.8%) and the TS-MPG assay (14.3%) (Table 3). Among ASC-US cases, a greater prevalence of DNA of HPV types 16, 33, 35, 39, 45, 51, 53, 56, 59, and 66 was detected with the TS-MPG than with the LA assay (Table 3). By the LA assay, HPV 16 was the predominant type (9.9%) present in women with ASC-US, followed by HPV 53 (8.8%), while in

TABLE 3 HPV DNA prevalence and agreement rate across TS-MPG and LA in women with ASC-US and controls

HPV type	HPV DNA prevalence									
	ASC-US patients (n = 91)					Controls (n = 84)				
	TS-MPG		LA		Kappa coefficient (%)	TS-MPG		LA		Kappa coefficient (%)
	No.	%	No.	%		No.	%	No.	%	
Any[a]	42	46.2	33	36.3	0.87	27	32.1	9	10.7	0.75
6	3	3.3	3	3.3	1.00	1	1.2	0	0.0	0.91
11	0	0.0	0	0.0	1.00	0	0.0	0	0.0	0.92
16	11	12.1	9	9.9	0.98	7	8.3	2	2.4	0.86
18	0	0.0	0	0.0	1.00	1	1.2	0	0.0	0.91
26	0	0.0	0	0.0	1.00	0	0.0	0	0.0	0.92
31	5	5.5	5	5.5	1.00	5	6.0	1	1.2	0.87
33	6	6.6	3	3.3	0.96	0	0.0	0	0.0	0.92
35	2	2.2	1	1.0	0.99	0	0.0	0	0.0	0.92
39	7	7.7	4	4.4	0.96	1	1.2	1	1.2	0.92
45	2	2.2	1	1.1	0.99	2	2.4	0	0.0	0.89
51	3	3.3	1	1.1	0.98	1	1.2	0	0.0	0.91
52	0	0.0	0	0.0	1.00	1	1.2	0	0.0	0.91
53	13	14.3	8	8.8	0.94	3	3.6	4	4.8	0.91
56	1	1.1	0	0.0	0.99	3	3.6	0	0.0	0.88
58	1	1.1	1	1.1	1.00	4	4.8	1	1.2	0.88
59	6	6.6	2	2.2	0.95	3	3.6	1	1.2	0.47
66	3	3.3	0	0.0	0.00	2	2.4	1	1.2	0.91
68	0	0.0	0	0.0	1.00	0	0.0	0	0.0	0.92
82	0	0.0	1	1.1	0.99	0	0.0	0	0.0	0.92

[a] Prevalence of DNA positivity for HPV types included in both TS-MPG and LA assays.

women with ASC-US tested by TS-MPG, the predominant type was HPV 53 (14.3%), followed by HPV 16 (12.1%). The rank of other HPV types differed according to the HPV DNA test used. Among ASC-US cases, with the exception of HPV 66 (kappa statistic = 0.00), the unadjusted kappa statistic test demonstrated almost perfect agreement between the TS-MPG and LA assays (kappa statistic ranging from 0.87 to 1.00) (Table 3). Among controls, a higher DNA prevalence was detected for HPV types 6, 16, 18, 31, 45, 51, 52, 56, 58, 59, and 66 with the TS-MPG assay than with the LA assay, respectively (Table 3). HPV 16 was the predominant type (8.3%), followed by HPV 31 (6.0%), when the samples were tested by TS-MPG, while in women tested by LA, the predominant type was HPV 53 (4.8%), followed by HPV 16 (2.4%). Additionally, among controls, the agreement between the genotyping assays was substantial for any HPV type (kappa statistic = 0.75), moderate for HPV 59 (kappa statistic = 0.47), and almost perfect for all other types (kappa statistic ranging from 0.86 to 0.92) (Table 3).

Of the 91 ASC-US patients, 19 (20.9%) tested DNA positive for any HPV type included in the TS-MPG and/or LA assays but HPV DNA negative by the HC-II assay (data not shown). Among the 19 samples, 12 were HPV positive by the LA and TS-MPG assays, and 7 were HPV positive by the TS-MPG assay only. Six of 19 samples (31.5%) were infected with HPV types 6, 53, 66, and 68a, which are not included in the HC-II assay (data not shown).

Without restricting the analysis to the 13 HPV types included in the HC-II assay, we found that 7 women were HPV positive by HC-II and HPV negative by the TS-MPG and/or LA assays. Specifically, 6 women were HPV negative by LA and 4 by TS-MPG.

In addition, follow-up cytological examination data were available for only 47 out of 91 women with ASC-US 1 year later. At the initial visit, 15 of 47 women with ASC-US (31.9%) were HPV positive by HC-II and by TS-MPG and/or LA. Twenty-one of 47 women with ASC-US (44.6%) were HPV negative by the HC-II, TS-MPG, and LA assays. Ten of 47 (21.2%) were HPV DNA negative as determined by the HC-II assay, while they tested positive by the TS-MPG and/or LA assays. One ASC-US patient of 47 (2.1%) was HPV DNA positive as determined by the HC-II assay only.

The data showed that 5 of 47 patients (10.6%) were diagnosed with higher-grade cervical lesions in the second visit. Specifically, three patients had a cytological diagnosis of low-grade squamous intraepithelial lesion (LGSIL), and two had a high-grade squamous intraepithelial lesion (HGSIL). One HGSIL and one LGSIL patient were found to be positive for HPV types 33, 51, and 70 by the TS-MPG and/or LA assays and negative by HC-II. The three other patients (2 LGSIL and 1 HGSIL) were HPV positive by HC-II and TS-MPG and/or LA. None of the ASC-US patients that were concomitantly HPV negative by the HC-II, TS-MPG, and LA assays had developed higher-grade cervical lesions 1 year later.

Among patients with negative cytological data in the second visit ($n = 41$; 87.2%), 6 women who were HPV negative by HC-II were found to be HPV positive at the first visit by using highly sensitive HPV DNA detection assays. Of these, 4 out of 6 were found to be HPV positive by the TS-MPG and LA assays, and 2 out of 6 were found to be HPV positive by TS-MPG only.

DISCUSSION

New technologies for rapid, specific, sensitive, and cost-effective methods to enable HPV detection and genotyping in a clinical setting are being actively investigated. Although the performance indicators of the adopted test depend on HPV prevalence in the target population, HPV testing has been widely adopted for the triage of patients with ASC-US (1, 42) and as an adjunct to cervical cytology analysis as a primary screening tool in women older than 30 years (26). Biopsy specimens revealed that 22.2% of patients with a cytological diagnosis of ASC-US had concurrent cervical intraepithelial neoplasia (CIN). CIN grades 1 and 2/3 were identified in 16.9% and 5.3% of women with ASC-US, respectively (19).

In Italy, the frequency of ASC-US diagnosis is extremely variable and is underestimated with a median value of 3% (range, 0 to 7%) (25). Currently, the HC-II assay is widely used for the triage of ASC-US cases (1).

In this study, two highly sensitive HPV DNA detection methods were evaluated by testing HPV DNA in a series of ASC-US and normal cytological samples. The results from the TS-MPG and the LA assays were then compared with those of the HC-II HPV assay. We found substantial agreements between the assays (HC-II versus TS-MPG and HC-II versus LA), with kappa values ranging from 0.70 to 0.77. The agreement was almost perfect (kappa statistic = 0.87) when the TS-MPG and LA assays were compared for HPV types included in both assays.

However, the TS-MPG assay increased the rate of detection of HPV DNA compared to the rates of detection of the LA and HC-II assays. For ASC-US samples, the DNA prevalence for any HPV type included in each assay was greater with the TS-MPG assay (46.2%) than with the LA (36.3%) and HC-II (29.7%) assays. When we restricted the analysis to the 13 HPV types included in the HC-II assay, the TS-MPG remained the most sensitive assay, with an HPV DNA prevalence of 35.2%, compared to 26.4% for the LA assay and 29.7% for the HC-II assay. The same trend was observed in the controls, where the TS-MPG assay appeared to be more sensitive than the LA assay. As expected, the HR-/pHR-HPV DNA prevalence among controls was lower than that observed among the ASC-US cases, with estimated prevalences of 7.1% and 23.8% by the LA and TS-MPG assays, respectively.

The DNA prevalence for any HPV type included in both assays was greater for ASC-US patients and controls with the TS-MPG assay than with the LA assay. In controls, the prevalence of HPV 16 was 8.3% for the samples tested by TS-MPG, while the HPV 16 prevalence dropped to 2.4% with the LA assay, underlining the higher sensitivity of the TS-MPG assay than of the LA assay for the detection of this HPV type. This trend is confirmed by the fact that most of the types included in both assays (HPV 6, 16, 18, 31, 45, 51, 52, 56, 58, 59, and 66) were preferentially detected by the TS-MPG assay. Similarly, in women with ASC-US, a greater DNA prevalence for most of the HPV types included in both assays (HPV 16, 33, 35, 39, 45, 51, 53, 56, 59, and 66) was detected with the TS-MPG assay than with the LA assay, which confirms the ultrasensitivity of the TS-MPG assay (33).

The higher sensitivity of the TS-MPG assay than of the LA assay for the detection of specific HPV types can be explained by the different amplicon lengths which are generated by the two assays. The size of amplicons generated by the TS-MPG assay varies between 210 and 258 bp, while the amplicons generated by LA are twice as long (450 bp).

Interestingly, HPV 53 was highly prevalent in the ASC-US lesions with the LA (8.8%) and the TS-MPG (14.3%) assays, followed by HPV 16 (9.9% and 12.1%, respectively). HPV 53 was

originally isolated from a woman without cytological abnormality (13) and subsequently classified as a pHR-HPV type, although its association with the development of neoplastic cervical disease has not been established. Nevertheless, specific mutations in the *L1* gene of HPV 53 were recently found to be associated with LGSIL lesions, although they cannot presently be considered predictive of a further worsening of the cervical pathology (7).

Additionally, TS-MPG was able to detect the presence of coinfections and demonstrate that the prevalence of low-copy-number HPV infections in an ASC-US lesion may be underestimated by LA. The TS-MPG assay detected a statistically significantly greater proportion of multiple infections among both ASC-US patients and controls ($P = <0.0001$) than the LA assay (25.3% versus 18.7% and 13.1% versus 3.6%, respectively). The increased sensitivity of the TS-MPG assay for the detection of multiple HPV infections can be explained by the use of HPV type-specific primers rather than only one pair of consensus primers as for the LA assays (14, 33). In the case of multiple infections, different HPV types will not compete for the same primer set, which is in contrast to the case for the LA assay.

To date, 20 to 40% of HPV-positive women have been reported to be infected with multiple HPV genotypes (17). These multiple infections are found more frequently among younger women (5, 18) and in patients with cytological abnormalities of different grades. In this series of ASC-US samples, the high sensitivity of the TS-MPG assay confirmed the widespread presence of multiple HPV types with a low viral load. Coinfections with multiple HPV types could play a role in the induction and progression of the low-grade cervical lesion (8, 11, 16, 21, 28).

We have shown that among the 91 ASC-US patients, 7 women with ASC-US were HPV positive by the HC-II and HPV negative by the TS-MPG and/or LA assays. Specifically, 6 women were HPV negative by LA and 4 by TS-MPG. The possibility of false-negative results could be due to mutations, natural variations, or deletions leading to an HPV DNA-negative result by the LA or TS-MPG assays, Alternatively, we can hypothesize that known or unknown HPV types which are not included in the TS-MPG and LA assays cross-reacted with the HC-II probe cocktail (6, 23, 24, 29).

Of the 91 ASC-US patients, 19 tested DNA positive for any HPV type included in the TS-MPG and/or LA assays but HPV DNA negative by the HC-II assay. However, 6 of these 19 women were infected with HPV types not included in the HC-II assay. Ten of these 19 women with ASC-US had a follow-up sample available. Interestingly, 2 of them who tested DNA positive for the HR-HPV types 33 and 51 had lesions that progressed 1 year later to HGSIL and LGSIL. However, 8 ASC-US patients regressed to a negative Pap test ($n = 7$) or remained ASC-US ($n = 1$), showing a limited benefit of the use of highly sensitive HPV DNA detection methods in the triage of ASC-US patients.

Among patients with a negative Pap test in the second visit, 6 women who were HPV negative by HC-II were found to be HPV positive by using highly sensitive HPV DNA detection assays 1 year earlier. Although the positive predictive value may increase with increased analytical sensitivity, this is clearly in detriment to the negative predictive value and clinical specificity of the HPV assay in the triage of women with ASC-US.

Given our results, although the highly sensitive HPV DNA tests appear to perform better at the analytical level than the HC-II assay, the clinical benefit remains limited to a widespread use of such tests in the triage of ASC-US patients, which could contribute to unnecessary procedures and expenses. However, the number of follow-up cytological examination data available ($n = 47$) is too small to determine whether highly sensitive HPV DNA tests should be used instead of the HC-II assay in the triage of women with ASC-US. Additional and larger follow-up studies including ASC-US patients are needed to draw conclusions about the use of highly sensitive HPV DNA tests in the triage of women with ASC-US.

In conclusion, the TS-MPG assay appears to be highly sensitive and suited for research purposes, to analyze the significance of multiple infections in the development of cervical lesions, to study the natural history and latency of HPV infection, and to monitor the impact of HPV vaccination in the targeted population.

ACKNOWLEDGMENTS

We thank Annick Rivoire for her help in the preparation of the manuscript.

We declare no conflicts of interest.

All study procedures have been approved by the IRCCS Burlo Garofolo Ethical Committee (C.I.B. 118/10 09/02/2010).

The study was partially supported by the European Commission, grant HPV-AHEAD (FP7-HEALTH-2011-282562), and by grant RC 11/11 from the Italian Health Ministry.

REFERENCES

1. **Arbyn M, et al.** 2004. Virologic versus cytologic triage of women with equivocal Pap smears: a meta-analysis of the accuracy to detect high-grade intraepithelial neoplasia. J. Natl. Cancer Inst. **96**:280–293.
2. **Arbyn M, et al.** 2009. Triage of women with equivocal or low-grade cervical cytology results: a meta-analysis of the HPV test positivity rate. J. Cell. Mol. Med. **13**:648–659.
3. **Bosch FX, et al.** 2008. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine **26**(Suppl 10):K1–K16.
4. **Carozzi FM, et al.** 2005. Reproducibility of HPV DNA testing by Hybrid Capture 2 in a screening setting. Am. J. Clin. Pathol. **124**:716–721.
5. **Castellsague X, et al.** 2001. Human papillomavirus genotypes in rural Mozambique. Lancet **358**:1429–1430.
6. **Castle PE, Gravitt PE, Solomon D, Wheeler CM, Schiffman M.** 2008. Comparison of linear array and line blot assay for detection of human papillomavirus and diagnosis of cervical precancer and cancer in the Atypical Squamous Cell of Undetermined Significance and Low-Grade Squamous Intraepithelial Lesion Triage Study. J. Clin. Microbiol. **46**:109–117.
7. **Cento V, et al.** 2012. Human papillomaviruses 53 and 66: clinical aspects and genetic analysis. Virus Res. **163**:212–222.
8. **Chaturvedi AK, et al.** 2011. Human papillomavirus infection with multiple types: pattern of coinfection and risk of cervical disease. J. Infect. Dis. **203**:910–920.
9. **Clavel C, et al.** 2000. Human papillomavirus detection by the hybrid capture II assay: a reliable test to select women with normal cervical smears at risk for developing cervical lesions. Diagn. Mol. Pathol. **9**:145–150.
10. **Cox JT.** 2001. Management of atypical squamous cells of undetermined significance and low-grade squamous intra-epithelial lesion by human papillomavirus testing. Best Pract. Res. Clin. Obstet. Gynaecol. **15**:715–741.
11. **Cuschieri KS, et al.** 2004. Multiple high risk HPV infections are common in cervical neoplasia and young women in a cervical screening population. J. Clin. Pathol. **57**:68–72.
12. **Fujinaga Y, et al.** 1991. Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction. J. Gen. Virol. **72**(Pt 5):1039–1044.
13. **Gallahan D, et al.** 1989. Human papillomavirus type 53. J. Virol. **63**:4911–4912.
14. **Gheit T, et al.** 2006. Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. J. Clin. Microbiol. **44**: 2025–2031.

15. **IARC.** 2011. A review of human carcinogens part B: biological agents. Human papillomaviruses. IARC Monogr. Eval. Carcinog. Risks Hum. **100B**:1–475.
16. **Mejlhede N, Bonde J, Fomsgaard A.** 2009. High frequency of multiple HPV types in cervical specimens from Danish women. APMIS **117**:108–114.
17. **Mendez F, et al.** 2005. Cervical coinfection with human papillomavirus (HPV) types and possible implications for the prevention of cervical cancer by HPV vaccines. J. Infect. Dis. **192**:1158–1165.
18. **Molano M, et al.** 2002. Low grade squamous intra-epithelial lesions and human papillomavirus infection in Colombian women. Br. J. Cancer **87**: 1417–1421.
19. **Morin C, et al.** 2000. Cytologic predictors of cervical intraepithelial neoplasia in women with an ASCUS Pap smear. Acta Cytol. **44**:576–586.
20. **Munoz N, et al.** 2004. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int. J. Cancer **111**: 278–285.
21. **Pista A, Oliveira A, Verdasca N, Ribeiro F.** 2011. Single and multiple human papillomavirus infections in cervical abnormalities in Portuguese women. Clin. Microbiol. Infect. **17**:941–946.
22. **Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM.** 2007. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. J. Infect. Dis. **195**:1582–1589.
23. **Poljak M, Brencic A, Seme K, Vince A, Marin IJ.** 1999. Comparative evaluation of first- and second-generation Digene Hybrid Capture assays for detection of human papillomaviruses associated with high or intermediate risk for cervical cancer. J. Clin. Microbiol. **37**:796–797.
24. **Poljak M, et al.** 2011. Comparison of clinical and analytical performance of the Abbott RealTime high risk HPV test to the performance of Hybrid Capture 2 in population-based cervical cancer screening. J. Clin. Microbiol. **49**:1721–1729.
25. **Rassu M, et al.** 2005. HPV genotype prevalence in cervical specimens with abnormal cytology: a report from north-east Italy. Scand. J. Infect. Dis. **37**:476–481.
26. **Rebolj M, Njor SH, Lynge E.** 2012. Restriction of human papillomavirus DNA testing in primary cervical screening to women above age 30: systematic review. Eur. J. Cancer Prev. **21**:73–81.
27. **Resnick RM, et al.** 1990. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. J. Natl. Cancer Inst. **82**:1477–1484.
28. **Rousseau MC, et al.** 2003. Predictors of cervical coinfection with multiple human papillomavirus types. Cancer Epidemiol. Biomarkers Prev. **12**:1029–1037.
29. **Safaeian M, et al.** 2007. Comparison of the SPF10-LiPA system to the hybrid capture 2 assay for detection of carcinogenic human papillomavirus genotypes among 5,683 young women in Guanacaste, Costa Rica. J. Clin. Microbiol. **45**:1447–1454.
30. **Sasagawa T, et al.** 2000. A new PCR-based assay amplifies the E6-E7 genes of most mucosal human papillomaviruses (HPV). Virus Res. **67**:127–139.
31. **Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S.** 2007. Human papillomavirus and cervical cancer. Lancet **370**:890–907.
32. **Schmitt M, et al.** 2006. Bead-based multiplex genotyping of human papillomaviruses. J. Clin. Microbiol. **44**:504–512.
33. **Schmitt M, et al.** 2010. Abundance of multiple high-risk human papillomavirus (HPV) infections found in cervical cells analyzed by use of an ultrasensitive HPV genotyping assay. J. Clin. Microbiol. **48**:143–149.
34. **Snijders PJ, et al.** 1990. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J. Gen. Virol. **71**:173–181.
35. **Solomon D, et al.** 2002. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA **287**:2114–2119.
36. **Syrjanen K.** 2008. New concepts on risk factors of HPV and novel screening strategies for cervical cancer precursors. Eur. J. Gynaecol. Oncol. **29**: 205–221.
37. **Thrall MJ, et al.** 2010. High-risk HPV testing in women 30 years or older with negative Papanicolaou tests: initial clinical experience with 18-month follow-up. Am. J. Clin. Pathol. **133**:894–898.
38. **Tieben LM, et al.** 1993. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. J. Virol. Methods **42**: 265–279.
39. **van den Brule AJ, Meijer CJ, Bakels V, Kenemans P, Walboomers JM.** 1990. Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type-specific polymerase chain reaction. J. Clin. Microbiol. **28**:2739–2743.
40. **van den Brule AJ, et al.** 2002. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J. Clin. Microbiol. **40**:779–787.
41. **Walboomers JM, et al.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. **189**:12–19.
42. **Wright TC, Jr, Cox JT, Massad LS, Twiggs LB, Wilkinson EJ.** 2002. 2001 Consensus Guidelines for the management of women with cervical cytological abnormalities. JAMA **287**:2120–2129.