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Performance of Human Papillomavirus DNA and mRNA Testing Strategies for Women with and without Cervical Neoplasia▽

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In the present study we investigated the cross-sectional positivity for DNA and E6/E7 mRNA from high-risk human papillomavirus (HPV) types in 643 women with high-grade cervical neoplasia (135 cases of cervical intraepithelial neoplasia grade 2 [CIN2], 495 cases of CIN3/adenocarcinoma in situ [ACIS], and 13 cases of invasive carcinoma) and in 736 women with normal cytology by using the Amplicor and PreTect HPV-Proofer assays. In addition, genotyping was performed using Linear Array for women with normal cytology and a positive HPV test and in all women with histologically confirmed CIN2+. In women with normal cytology, 8.3% (61/736) were Amplicor positive and 3.3% (24/736) were PreTect HPV-Proofer positive ($P < 0.001$). Concordant results between the Amplicor and PreTect HPV-Proofer tests were present in 90.3% (665/736). In women with CIN2+ lesions 96.4% (620/643) were positive by Amplicor, 98.4% (633/643) by linear array, and 64.1% (412/643) by PreTect HPV-Proofer. Concordant results for the three HPV assays were present in 63.8%. The genotype profile detected by linear array and PreTect HPV-Proofer showed substantial agreement for HPV types 16, 18, 33, and 45. HPV type 16 and/or 18 was detected in 58.8% (378/643) of the women with high-grade neoplasia. Detection of E6/E7 mRNA by PreTect HPV-Proofer increased with severity of the cervical lesion. Detection of HPV DNA, however, was not associated with histology grade. In conclusion, the detection of HPV varied according to the assay used, and the concordance between the tests was poor. Our results indicate that mRNA testing may be a biomarker for progression of cervical neoplasia, but the optimal genotype mix remains to be determined.

Infection with human papillomavirus (HPV) is considered the cause of the vast majority of premalignant and malignant epithelial lesions of the cervix (8, 21, 29, 31). However, most HPV infections are asymptomatic and transient, and more than 90% of new infections will resolve within 2 years (19). Progression to carcinoma is associated with a persistent infection with high-risk (HR) HPV types, integration of the HPV genome into the host chromosomes, and upregulation of E6 and E7 oncogenes, which can lead to abrogation of normal cell cycling events and tumor suppressor activity (7, 30, 31).

Large, randomized clinical trials have shown that HPV DNA testing has a higher sensitivity but lower specificity than cytology for detecting high-grade cervical lesions in primary screening (2, 5, 6, 12, 23, 24). As most HPV infections are transient, HPV DNA testing could result in follow-up of women with clinically insignificant infection, resulting in increased costs and patient anxiety. This is why an informed approach to HPV testing is imperative, with clinical contexts and reasons for testing clearly defined and justified, respectively.

Most commercially available HPV tests detect the presence of HPV DNA; however, it is possible to detect HPV mRNA transcripts coding for E6/E7 and thereby the presence of oncogene activity. A nucleic acid sequence-based amplification method detecting E6/E7 transcripts from the five most common HR HPV types in cervical carcinoma (types 16, 18, 31, 33 and 45) is commercially available from two companies (the PreTect HPV-Proofer [Norchip AS, Klokkarstua, Norway] and the NucliSens EasyQ [bioMerieux S.A., France]). The prevailing consensus is that upregulated expression of E6/E7 is necessary for the initiation and progression of cervical neoplasia. Detection of HPV oncogene activity through the detection of mRNA transcripts may therefore be a better indicator of HPV infection associated with increased risk of progression to neoplasia than detection of HPV DNA (14, 17, 18).

The aims of our study were to investigate the cross-sectional positivity of HR HPV DNA and E6/E7 mRNA expression in women with and without cervical neoplasia by using two commercial assays. A third broad-spectrum commercial genotyping

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assay was included so that type-specific analysis could be performed (on women with high-grade disease and on HPV-positive women with normal cytology). We also wanted to study the association between testing positive by the different methods and the severity of the cervical lesion.

MATERIALS AND METHODS

Study population. Women were recruited from four hospitals and nine gynecologists in private practice in Health Region East, Norway. Enrollment took place from 1 January 2005 to 31 December 2006. Included in the study were (i) 764 women 30 years or older attending routinely administered clinical services and with normal Pap smear cytology, normal cytological results from the preceding 2 years, and no previous history of treatment for cervical neoplasia and (ii) 655 women (no age criterion imposed) with histologically confirmed cervical intraepithelial neoplasia grade 2 or 3 (CIN2+), adenocarcinoma in situ (ACIS), or invasive carcinoma. A total of 623 of these patients were treated with conization. The median age among women with normal cytology was 51 years (range, 31 to 82 years), and it was 37 years (range, 17 to 76 years) for women with CIN2+.

The Pap smears were evaluated without knowledge of the HPV results by different, experienced cytotechnicians at the Department of Pathology, Akershus University Hospital. The smears were classified according to the criteria of the Bethesda Classification 2001 (26).

The histological analyses were performed on colposcopically directed biopsies and/or cone specimens. All specimens were reevaluated blindly by one experienced pathologist (A. K. Lie) and diagnosed according to the WHO classification (1). The specimen with the most severe lesion was chosen for analysis. Histology revealed CIN2 in 21.0% (135/643), CIN3 in 73.7% (474/643), ACIS in 1.6% (10/643), ACIS together with CIN2/3 in 1.7% (11/643), and invasive carcinoma in 2.0% (13/643) of the cases.

Collection of specimens for HPV testing. Cervical specimens were collected with a Cytobrush Plus (Medscan Medical AB, Sweden). For the normal cytological group a conventional Pap smear was taken first and the brush was transferred to a PreServ Cyt vial (Cytyc Corporation) for HPV testing. For the CIN2+ group, samples were transferred directly to the PreServ Cyt medium at the time of conization or at the time of biopsy within 2 months before conization. Cells were stored in PreServ Cyt medium for up to 21 days at room temperature or at 4°C before HPV testing.

Total nucleic acid extraction. To allow one extraction for both mRNA and DNA, the manual DNA extraction protocol (AmpliLute; Roche/Qiagen) supplied with the Amplicor HPV test was replaced by the semiautomatic NucliSense miniMag (bioMerieux) or automatic easyMag (bioMerieux) total nucleic acid extraction protocol recommended by the PreTect HPV-Proofer test manufacturer. Briefly, 5 ml of each cell sample in PreServ Cyt medium was pelleted by centrifugation. In cases with visible blood, only 3 ml of the cell sample was used, and in cases with few visible cells 10 ml of the cell sample was used. For the miniMag procedure, 1 ml of lysis solution and 100 μl of elution buffer were used, and for the easyMag procedure 2 ml of lysis solution and 55 μl elution buffer were used. Isolated nucleic acid was kept cold and analyzed within 4 hours following extraction or stored at −80°C until analysis.

Validation of the nucleic acid extraction procedure. To compare the performance of easyMag extraction with AmpliLute extraction, 66 samples with high-grade lesions were extracted in parallel by both methods. The DNA concentrations in the extracts were determined using an in-house real-time beta-globin PCR for absolute quantification, using a dilution of human DNA with known concentrations as a standard (data not shown). Undiluted and diluted extracts were compared, as undiluted AmpliLute extracts were replaced by 1:10-diluted easyMag extracts in the modified Amplicor test.

HPV DNA testing. The Amplicor HPV test (Roche Diagnostics, Switzerland) detects the following HPV DNA genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The Amplicor test does not include genotyping, and a positive result of the test is interpreted as the presence of one or more of the above genotypes. As the AmpliLute manual extraction protocol was replaced by the automatic extraction protocol described above using a larger input of sample (5 ml versus 250 μl), 5 μl total nucleic acid was diluted with 45 μl PCR-grade water instead of using 50 μl undiluted Amplilute-extracted DNA in each PCR mixture. All other steps in the analyses were performed according to the manufacturer's recommendations.

HPV DNA genotyping. HPV DNA genotyping was performed with the Linear Array HPV assay (Roche Diagnostics, Switzerland) in women with normal cytology and positive HPV test and in all women with histologically confirmed CIN2+. This assay detects 37 different genotypes, including HR, probably HR, low risk, and HPV types not yet classified (Table 1) (21). This was done retrospectively using the same extracts used for the Amplicor HPV test and PreTect HPV-Proofer test. As for the Amplicor test the input for the PCR was 5 μl extract added to 45 μl PCR-grade water. All other steps including analysis were as recommended by the manufacturer.

TABLE 1. HPV genotypes detected with the Linear Array

Classification[a]	HPV genotypes detected
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably high risk	26, 53, 66, 68, 73, 82, IS39
Low risk	6, 11, 40, 42, 54, 61, 70, 72, 81, CP 6108
Not yet classified	55, 62, 64, 67, 69, 71, 83, 84

[a] The classifications are based on IARC recommendations (21).

HPV mRNA testing. The PreTect HPV-Proofer (Norchip, Norway) detects HPV 16, 18, 31, 33, and 45 E6/E7 full-length mRNA transcripts. Briefly, 5 μl undiluted isolated nucleic acid was analyzed according to the manufacturer's instructions using the Lambda FL 600 fluorescence reader (Bio-Tek Instruments, Inc.) and the PreTect analysis software (Norchip; Norway). Samples that were HPV mRNA negative, internal control negative, or internal control indeterminate (signal between 1.4 and 1.7), as well as samples that were HPV indeterminate, were reextracted and reanalyzed using up to 10 ml PreServ Cyt sample as recommended by the manufacturer (PreTect HPV-Proofer user guide version 1105 720001 and earlier versions; Norchip). Samples that tested indeterminate twice were considered negative if the internal control was positive.

Statistical analyses. We compared the percentages of test positives according to the assay used. Statistical analyses were performed using two-by-two contingency tables with two-sided *P* values calculated with a Pearson chi-square test. Fisher's exact test and McNemar's test were used for comparisons of paired proportions. *P* values of <0.05 were considered statistically significant. We also calculated percent agreement between the different HPV assays, and values for Cohen's κ statistic were used as indicators of concordance; κ values of <0.20 indicated poor agreement, 0.21 to 0.40 fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement, and >0.80 indicated nearly perfect agreement. Kappa values were calculated for agreement between assays on detection of HPV 16, 18, 31, 33, and 45. Data analyses were performed by using SPSS software (version 16.0). The relative sensitivity and specificity were calculated based on valid test results on 736 women with histologically confirmed CIN2+ and 643 women with normal cytology.

Ethics. The Regional Committee for Ethics in Medical Research, East Region, Norway (676-04239), Norwegian Social and Health Directorate (05/163), and Norwegian Data Inspectorate (07/00975-2/SVE) approved the study. Written informed consent was obtained from all study participants.

RESULTS

Validation of nucleic acid extraction procedure. Based on the beta-globin real-time PCR performed on 66 samples with high-grade lesions, the mean DNA concentration in undiluted AmpliLute extracts was found to be 25 ± 30 ng/μl (mean ± standard deviation). The mean DNA concentration in 1:10-diluted easyMag extracts was found to be 10 ± 9 ng/μl. Subsequent analysis of the same samples with the Amplicor test revealed 100% agreement regarding beta-globin gene detection (66 out of 66 samples were beta-globin positive) and 86.4% (95% confidence interval, 76.1 to 92.7) agreement regarding HPV detection (57 out of 66 samples revealing the same result). A total of 38 HPV-positive samples were detected combining both extractions. Five of these were only positive when extracted using the AmpliLute procedure, and four were only positive when extracted using the easyMag procedure. In conclusion, the agreement regarding detection of the internal control was 100% and for HPV DNA it was substantial (Cohen's κ, 0.74). Mean DNA concentrations used as input to the Amplicor test were similar, but DNA concen-

TABLE 2. Outcomes of HPV testing with PreTect HPV-Proofer, Amplicor, and Linear Array in the normal cytology group

Comparison test and result	Result with PreTect HPV-Proofer		
	% Negative (*n*)	% Positive (*n*)	Total % (*n*)
Amplicor			
Negative	89.4 (658)	2.3 (17)	91.7 (675)
Positive	7.3 (54)	1.0 (7)	8.3 (61)
Total	96.7 (712)	3.3 (24)	100.0 (736)
Linear Array			
Not tested	89.4 (658)	0 (0)	89.4 (658)
Negative	2.0 (15)	2.2 (16)	4.2 (31)
Positive	5.3 (39)	1.1 (8)	6.4 (47)
Total	96.7 (712)	3.3 (24)	100.0 (736)

TABLE 3. Outcome of HPV testing by PreTect HPV-Proofer, Amplicor, and Linear Array in the CIN2+ group

Comparison test and result	Result with PreTect HPV-Proofer		
	% Negative (*n*)	% Positive (*n*)	Total % (n)
Amplicor			
Negative	3.1 (20)	0.5 (3)	3.6 (23)
Positive	32.8 (211)	63.6 (409)	96.4 (620)
Total	35.9 (231)	64.1 (412)	100.0 (643)
Linear Array			
Negative	0.8 (5)	0.8 (5)	1.6 (10)
Positive	35.1 (226)	63.3 (407)	98.4 (633)
Total	35.9 (231)	64.1 (412)	100.0 (643)

trations in the easyMag extracts appeared to be more uniform than the AmpliLute extracts. Based on these results we concluded that the total nucleic acid automatic extraction method could replace the more laborious manual AmpliLute extraction method.

HPV detection in women with normal cytology. In women with normal cytology, 3.7% (28/764) of the cases were excluded because both the internal control for DNA and/or RNA quality and HPV were negative, leaving 736 with valid test results. A total of 10.6% (78/736) tested positive for HR HPV (DNA and/or mRNA). The Amplicor test was positive in 8.3% (61/736) and the PreTect HPV-Proofer test was positive in 3.3% (24/736) (Table 2). Concordant results between Amplicor and PreTect HPV-Proofer were found in 90.3% (665/736). The HPV-positive cases ($n = 78$) were genotyped using Linear Array, and 47 cases tested positive (60%). HPV was more frequently detected using the Amplicor test or Linear Array than with PreTect HPV-Proofer (Pearson chi-square test, $P < 0.001$) (Table 2). By Linear Array multiple infections with two or more genotypes were detected in 1.4% (10/736) of all women and in 12.8% (10/78) of women with positive samples, but by PreTect HPV-Proofer no women were determined to have multiple infections.

HPV detection in women with high-grade cervical neoplasia. In women with CIN2+, 1.8% (12/655) of the cases were excluded because the internal control for DNA and/or RNA quality and HPV were negative, leaving 643 with valid test results. A total of 97.0% (624/643) tested positive for HR HPV (HPV DNA and/or HPV mRNA). Amplicor was positive in 96.4% (620/643), Linear Array was positive in 98.4% (633/643), and PreTect HPV-Proofer was positive in 64.1% (412/643) (Table 3). In women with CIN2+ HPV was detected in 99.4% (639/643) when all HPV types detected by linear array were considered. Agreement between Amplicor and PreTect HPV-Proofer was found in 66.7% (429/643) (Table 3) and between Linear Array and PreTect HPV-Proofer in 64.1% of the samples (412/643) (Table 3). Concordant results for the three HPV assays were present in 63.8% (410/643).

In total, Linear Array detected the presence of 34 different HPV genotypes in women with CIN2+, and the distribution of the HR HPV genotypes is shown in Fig. 1. HPV 16 was the most common HPV type, detected in 51.3% (330/643) of the women, followed by HPV 31, 33, 52, 18, 51, 58, and 45. HPV 16 and/or 18 was detected in 58.0% (373/643). Probable HR HPV genotypes were detected in 13.5% (87/643), low-risk HPV genotypes in 18.0% (116/643), and genotypes that have not yet been classified in 13.2% (85/643) of the women. Linear Array detected multiple infections in 52.6% of the cases (338/643). HPV 6/11 was detected in 2.0% (13/643), together with other HPV types in most of the cases (1.7% [11/643]).

The distribution of HPV genotypes detected by the PreTect HPV-Proofer is shown in Table 4. HPV 16 was the most prevalent genotype, found in 42.3% (272/643) of the women, followed by HPV 33 (13.2%), HPV 45 (6.1%), HPV 18 (5.3%), and HPV 31 (2.3%). HPV 16 and/or 18 were detected in 47.1% (303/643) of the women. Multiple infections with two or more genotypes were detected in 5% (33/643) of the specimens by PreTect HPV-Proofer. Of the 33 specimens with multiple infections detected by HPV-Proofer, Linear Array results showed at least one of the same genotypes.

For women with CIN2+ the HR HPV genotype profile detected by Linear Array compared to PreTect HPV-Proofer is shown in Table 4. Agreement between the two tests was poor to moderate for HPV 31 (κ value, 0.18) and substantial for HPV 18, 16, 33, and 45 (κ values, 0.68 to 0.81). In women who were Amplicor positive and PreTect HPV-Proofer negative ($n = 211$), genotyping with Linear Array revealed an HR HPV genotype not included in the mRNA test in 41.2% (87/211). There was 96.7% concordance between Amplicor and Linear Array test results.

HPV test results according to severity of cervical disease. Amplicor was positive in 95.6% cases of CIN2 (129/135), in 97.0% cases of CIN3/ACIS (480/495), and in 84.6% of cases of invasive carcinoma cases (11/13) (Table 5). Two invasive carcinomas were Amplicor negative. mRNA testing revealed oncogene expression from HPV 45 in one of these cases, and results were negative in the other. PreTect HPV-Proofer was positive in 50.4% of the women with CIN2 (68/135), in 67.5% with CIN3/ACIS (334/495), and in 76.9% of the women with invasive carcinomas (10/13). Three invasive carcinomas tested negative with PreTect HPV-Proofer, and genotyping with Linear Array revealed HPV 11, 33, 81, and 56. The HPV11-positive invasive carcinoma was classified as a condylomatous type of squamous cell carcinoma, a newly described type in the WHO 2004 classification. The mRNA test was significantly more often positive in the CIN3+ lesions compared to CIN2 lesions (Pearson chi-square test, $P < 0.0001$). Detection of HPV DNA, however, was not associated with histology grade. The relative cross-sectional sensitivity and specificity were calcu-

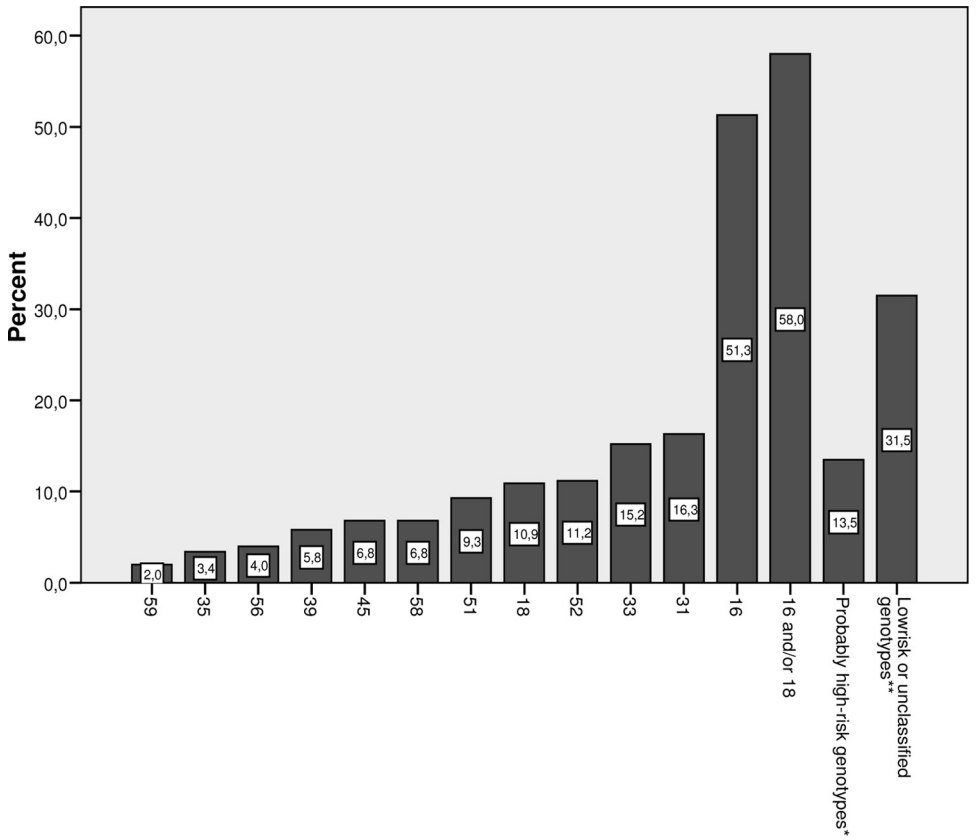

FIG. 1. Distribution of genotypes in positive tests among 643 women with CIN2+ detected by linear array. *, probably high-risk HPV genotype (25, IS 39, 53,66, 68, 73, and 82); **, HPV low-risk genotype (6, 11, 40, 42, 54, 61, 70, 72, 81, and CP 6108) or unclassified genotype (55, 62, 64, 67, 69, 71, 83, and 84).

lated (Table 6) and revealed the highest sensitivity for HPV DNA testing and the highest specificity for HPV mRNA testing.

DISCUSSION

The aims of our study were to compare relatively new commercially available assays for detection of HPV in Norwegian women with and without high-grade cervical neoplasia as the baseline for longitudinal analyses. HR HPV was detected in 10.6% of women above the age of 30 with normal cytology and 8.3% tested positive with Amplicor, which is in agreement with other European studies using HC II or consensus PCR (3, 9, 10, 17). Among the specimens from women with normal cytology, a significantly higher number were positive by Amplicor than by PreTect HPV-Proofer ($P < 0.001$). The reason for this may be that more genotypes are included in the DNA test (13 versus 5 genotypes) and/or that the chemistry behind the mRNA test renders it more specific for the detection of clinically significant infection. Those with HPV E6/E7 mRNA-negative detection in HPV DNA-positive samples can be interpreted as HPV carriers without active viral transcription. However, it may be that transcriptional activity occurs but at levels insufficient for PreTect HPV-Proofer detection. Surprisingly, with PreTect HPV-Proofer samples tested positive in 2.3% of the Amplicor-negative cases. This could be have been caused by a false-positive mRNA test (oncogene expression

TABLE 4. Distribution of HPV genotypes detected by PreTect HPV-Proofer and Linear Array in the CIN2+ group ($n = 643$)

HPV Genotype(s)	PreTect HPV-Proofer		Linear Array		% with positive results in both tests[a]	*P* value	κ value
	% Positive (*n*)	% Negative (*n*)	% Positive (*n*)	% Negative (*n*)			
16	42.3 (272)	57.7 (371)	51.3 (330)	48.7 (313)	79.7 (267)	<0.001	0.79
18	5.3 (34)	94.7 (609)	10.9 (70)	89.1 (573)	48.6 (34)	<0.001	0.63
31	2.3 (15)	97.7 (628)	16.3 (105)	83.7 (538)	12.1 (13)	<0.001	0.18
33	13.2 (85)	86.8 (558)	15.2 (98)	84.8 (545)	71.0 (76)	<0.001	0.80
45	6.1 (39)	93.9 (604)	6.8 (44)	93.2 (599)	69.4 (34)	<0.001	0.81
16/18	47.1 (303)	52.9 (340)	58.0 (373)	42.0 (270)	78.8 (298)	<0.001	0.75

[a] Percent (number) of HPV-positive women who tested positive on both PreTect HPV-Proofer and Linear Array.

TABLE 5. Relationship between morphology and HPV testing

Morphology (*n*)	PreTect HPV-Proofer		Amplicor		*P* value, Proofer vs Amplicor	Linear Array		*P* value, Proofer vs Linear Array
	% Positive (*n*)	% Negative (*n*)	% Positive (*n*)	% Negative (*n*)		% Positive (*n*)	% Negative (*n*)	
Normal[a] (736)	3.3 (24)	96.7 (712)	8.3 (61)	91.7 (675)	<0.0001	60.3 (47)	39.7 (31)	0.001
CIN2 (135)	50.4 (68)	49.6 (67)	95.6 (129)	4.4 (6)	0.091	98.5 (133)	1.5 (2)	0.992
CIN3/ACIS (495)	67.5 (334)	32.5 (161)	97 (480)	3 (15)	<0.0001	98.8 (489)	1.2 (6)	0.072
Carcinoma (13)	76.9 (10)	23.1 (3)	84.6 (11)	15.4 (2)	0.326	84.6 (11)	15.4 (2)	0.4
CIN2+ (643)	64.1 (412)	35.9 (231)	96.4 (620)	3.6 (23)	<0.0001	98.4 (633)	1.6 (10)	0.35

[a] For women with normal cytology, genotyping with Linear Array was performed only in cases with a positive HPV test (positive by Amplicor and/or PreTect HPV-Proofer).

not associated with cervical neoplasia), lack of specificity of PreTect HPV-Proofer, or false-negative DNA tests due to a breakpoint in the L1 region during HPV integration. We intend to follow these women with repeat cytology and HPV testing after 12 months; if HPV infection is persistent and/or cytology is positive by colposcopy, biopsy will be performed.

In our study PreTect HPV-Proofer was positive in 3.3% of the women with normal cytology, which is higher than reported from another, larger cross-sectional Norwegian study where PreTect HPV-Proofer tested positive in 1.7% (68/3970) of women above the age of 30 with normal cytology (17). Castle et al. tested women in a routine screening program with the Aptima HPV assay (which can detect E6/E7 mRNA from 14 carcinogenic HPV types) and found that 8% (10/125) of women with normal cytology tested positive (4). It could be argued therefore (notwithstanding the analytical sensitivities of the two mRNA assays) that the smaller type range of the PreTect HPV-Proofer has contributed to the lower detection rate.

Due to the lower detection of HPV mRNA in women with normal cytology, it may constitute a better first-line screen compared to HPV DNA testing, provided the sensitivity for significant disease is not compromised and clinically significant infections are not missed. Longitudinal follow-up (including that associated with mRNA-negative/DNA-positive women) from this study should elucidate the prospective performance of the tests.

HR HPV was detected in 97.0% of women with histologically confirmed CIN2+. A significantly higher number of women with CIN2+ were HPV DNA positive rather than HPV mRNA positive. Concordant results for the three HPV tests were found in 63.8%. There are several explanations for the different outcomes of the tests. The different assays are not uniform with regard to the analytical sensitivity, use of template, and the spectrum of detectable genotypes. PreTect HPV-Proofer detects transcripts and oncogene activity from 5 out of the 13 HR HPV types included in the Amplicor test. The Linear Array, which detects 37 different genotypes, has lower analytical sensitivity than the Amplicor test. The concordance between Amplicor and the Linear Array in the CIN2+ group was 96.7%, which is almost the same as in the study of Steven et al. (97.8%) (27).

TABLE 6. Sensitivities and specificities for the three tests

Test	% Sensitivity[a]	% Specificity[b]
Amplicor	96.4	91.7
Proofer	64.1	96.7
Linear Array	98.4	NA[c]

[a] Based on 643 women with histologically confirmed CIN2+.
[b] Based on 736 women with normal cytology.
[c] NA, not available. Specificity could not be measured using the Linear Array as only some ($n = 78$ HPV positive) of the 736 women with normal cytology were tested via this technique.

We detected an HR HPV genotype not included in the mRNA test in 41.2% of the women with CIN2+ (i.e., positive Amplicor and negative PreTect HPV-Proofer). HPV DNA testing will not discriminate between active and latent or transient infections, while mRNA testing may be more likely to. It is estimated that only 12 to 31% of CIN3 lesions will progress to invasive carcinomas if they are left untreated (15, 16, 22), so it could be that the HPV mRNA-negative/DNA-positive CIN2+ cases were those infections associated with regressing lesions. However, this will be impossible to confirm, since Norwegian women with CIN2+ lesions are routinely treated with conization.

In our study 96.4% of women with CIN2+ tested positive with Amplicor, which is in accordance with the large POBASCAM and ARTISTIC trials, in which HPV DNA testing was performed with PCR or hybrid capture 2 (2, 9). The Amplicor test was negative in 23 women with CIN2+, and among these, 3 patients tested positive with PreTect HPV-Proofer. As discussed earlier, the reason for this may be false-negative DNA tests associated with viral integration.

There is a lack of data on mRNA testing in clinical contexts. Cross-sectional Norwegian studies have shown that mRNA transcripts from HPV types 16, 18, 31, 33, or 45 can be detected in 77% of women with histologically verified CIN2+ and in 89% of invasive squamous cell carcinomas, compared to 94.5% and 92%, respectively, by HPV DNA testing (11, 14, 17). These studies support our results that HPV detection in preinvasive lesions will differ depending on whether you use mRNA methods with fewer genotypes or HPV DNA detection methods with a broad spectrum of genotypes. The Aptima HPV assay, a Gen-Probe test detecting E6/E7 mRNA for 14 carcinogenic HPV types, showed a prevalence of 92.4% in women with CIN2+ (4). Adding extra (probably) oncogenic HPV types in mRNA HPV tests may negatively influence the specificity of the test for high-grade lesions (13, 25). The performance of the PreTect HPV-Proofer assay is clearly influenced by the choice and number of genotypes included in the assay. It remains to be documented whether mRNA assays need to be intrinsically quantitative to be effective. In determining the optimal genotype mix for an mRNA test, indeed any HPV test is a contentious area. There will have to be a

compromise between including more rare HPV types to maximize sensitivity and detecting large numbers of what could be clinically irrelevant infections. Defining the appropriate analytical sensitivity for clinical utility is equally challenging.

According to the known prevalence of HPV types in invasive cervical carcinomas, more than 80% of the potential cases can be detected by the PreTect HPV-Proofer assay. The IARC pooled analysis of 3,085 invasive cervical carcinomas revealed that the five most common HPV genotypes were, in descending order of frequency, HPV 16, 18, 45, 31, and 33 (20). These genotypes were detected in 82.9% of the cases, which corresponds well with a Norwegian study of 204 women diagnosed with squamous cell carcinomas (11). In that previous study the five most common HPV genotypes were 16, 18, 31, 33, and 45.

DNA and mRNA testing may be employed together for screening to take advantage of the higher sensitivity and specificity, respectively, of the tests, and patients are then referred for a biopsy if both tests are positive. If only HPV DNA is positive, the patient may be retested for HPV DNA at a later date and then referred for colposcopy if persistently positive. mRNA testing alone for screening appears to be too insensitive, at least for the currently evaluated PreTect HPV-Proofer assay. Moreover, as HPV vaccination becomes more common and the prevalence of HPV vaccine types is reduced, there will be a requirement to reconsider/recalibrate HPV assays in line with the shifting dynamics of HPV type-specific prevalence and associated disease.

Accurate geographical data on HR HPV genotype distributions have implications not only for follow-up protocols in cervical cancer screening programs but also for assessing the expected impact of an HPV 16/18 vaccine program on CIN2+. In our study, 58.8% of the women with CIN2+ tested positive for HPV 16 and/or 18 as detected by either Linear Array or PreTect HPV-Proofer. This result corresponds with a recent meta-analysis which showed that HPV 16 and/or 18 was detected in 52% of women with high-grade precursor lesions (25a).

So far only one study has investigated the predictive values of HPV DNA versus mRNA testing in triage (28). This study revealed that PreTect HPV-Proofer has the highest specificity and the lowest sensitivity, which seems to be in accordance with our findings. At this stage we cannot calculate positive or negative predictive values from our study, due to the absence of histology results from the normal cytology group.

In conclusion, the detection of HPV varied according to the assay used, and the concordance between the tests was low. Our results indicate that mRNA testing may be a biomarker for progression of cervical neoplasia, but further data are needed to confirm this. mRNA testing for the five HR HPV types described may be a more specific approach and appropriate for risk evaluation. It is not clear whether the increased specificity of mRNA testing via the PreTect HPV-Proofer is driven by truly detecting transcripts or by detecting a more limited range of HPV types. Consensus on the number and types of genotypes that should be included in a diagnostic test to achieve the best sensitivity and specificity has not been reached and will likely evolve as interventions such as HPV vaccination become more common.

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