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Cross-Sectional Comparison of an Automated Hybrid Capture 2 Assay and the Consensus GP5+/6+ PCR Method in a Population-Based Cervical Screening Program

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In this cross-sectional study, clinical performances of the hybrid capture 2 assay using an automated instrument (i.e., rapid capture system) (hc2-RCS) and the high-risk human papillomavirus GP5+/6+ PCR-enzyme immunoassay (EIA) test were compared using cervical scrape specimens from 8,132 women that participated in a population-based screening trial. The hc2-RCS test scored significantly more samples positive (6.8%) than the GP5+/6+ PCR-EIA (4.8%) ($P < 0.0005$). This could be attributed largely to a higher positivity rate by the hc2-RCS test for women with cytologically normal, borderline, or mild dyskaryosis. A receiver operator characteristics analysis of the semiquantitative hc2-RCS results in relation to different cytology categories revealed that these differences are owing to differences in assay thresholds. For women classified as having moderate dyskaryosis or worse who also had underlying histologically confirmed cervical intraepithelial neoplasia grade 3 or cervical cancer (≥CIN3), the hc2-RCS scored 97% (31/32) of samples positive, versus 91% (29/32) by GP5+/6+ PCR-EIA. However, this difference was not significant ($P = 0.25$). After increasing the hc2-RCS cutoff from 1.0 to 2.0 relative light units/cutoff value of the HPV16 calibrator (RLU/CO), no additional CIN3 lesions were missed by hc2-RCS, but the number of test-positive women with normal, borderline, or mild dyskaryosis was significantly decreased ($P < 0.0005$). However, at this RLU/CO, the difference in test positivity between hc2-RCS and the GP5+/6+ PCR-EIA was still significant ($P = 0.02$). The use of an RLU/CO value of 3.0 revealed no significant difference between hc2-RCS and GP5+/6+ PCR-EIA results, and equal numbers of smears classified as ≥CIN3 (i.e., 29/32) were detected by both methods. In summary, both assays perform very well for the detection of ≥CIN3 in a population-based cervical screening setting. However, adjustment of the hc2-RCS threshold to an RLU/CO value of 2.0 or 3.0 seems to produce an improved balance between the clinical sensitivity and specificity for ≥CIN3 in population-based cervical screening.

Nowadays, the role of a persistent infection with high-risk human papillomavirus (hrHPV) in the development of cervical cancer is undisputed (2, 18, 29). As a result, multiple studies have investigated the value of adding an hrHPV DNA test to the classical Pap smear to improve the efficacy of cervical cancer screening programs, the triage of women with ambiguous or borderline cervical smears, and the monitoring of women after treatment for high-grade cervical intraepithelial neoplasia (CIN) (5, 10, 30, 31).

However, only a limited number of assays that detect DNA of hrHPV types as a pool have proven to be of clinical value in longitudinal studies involving large cohorts of women. One of these involves the commercially available, FDA-approved hybrid capture 2 (hc2) test. This assay is based on the hybridization of target DNA with a cocktail of full-length RNA probes of 13 hrHPV types, which has an analytical sensitivity of about 450,000 human papillomavirus (HPV) copies per cervical scrape suspension (26). Digene recently introduced a rapid capture system (RCS) allowing high-throughput hc2 testing for population screening in an automated format. The hc2-RCS is a programmable 96-well microplate processor that integrates liquid handling, plate handling, incubation, shaking, and washing via software specifically designed to run the hc2 assay.

Another clinically validated hrHPV detection assay involves the hrHPV GP5+/6+ PCR-EIA, which tests for 14 hrHPV types in one assay in which PCR products are ultimately hybridized to a mixture of specific oligonucleotides. The application of the GP5+/6+ PCR-EIA assay on crude extracts has an analytical sensitivity which is estimated to be in the range of about 1,000 HPV copies per cervical scrape, with variations of about 10-fold, depending on the HPV type (24, 27). This assay has the advantage that direct genotyping is possible on the hrHPV-specific PCR products by reverse line blot analysis (27). Both hc2 and GP5+/6+ PCR show good to excellent interlaboratory reproducibility (8, 14). Despite their good clinical performances in terms of sensitivity and specificity in detecting cervical intraepithelial neoplasia grade 3 lesions or cervical cancer (≥CIN3) (5, 17, 20–22), the two methods have not been directly compared in large population-based studies. This is of particular importance, since there is now compelling evidence that there exists a differential risk posed by the different hrHPV types for cervical cancer (1, 4, 15). These findings ask for HPV typing as a follow-up test to distinguish those hrHPV-positive women that would benefit from more aggressive management on the basis of the HPV type present. Therefore, when hrHPV testing would be implemented in screening pro-

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grams, the advantages of the hc2-RCS (i.e., high-throughput and automated handling) and GP5+/6 PCR (i.e., easy genotyping) assays could be combined in a combination test in which hc2-RCS is applied first and then GP5+/6+ PCR genotyping as a reflex test on hc2-RCS-positive samples.

This cross-sectional study involved a two-way comparison of the hc2-RCS and GP5+/6+ PCR hrHPV test on cervical scrapings in relation to the cytological results and histological outcome, the latter for women referred for colposcopy because of a cytology reading of moderate dyskaryosis or worse. To that end, cervical scrape specimens from 8,132 women that participated in an extension of a population-based screening (i.e., POBASCAM [5]) trial were analyzed by both methods. To ultimately explain discrepant test results between hc2-RCS and GP5+/6+ PCR, a further comprehensive analysis was performed involving possible PCR inhibition and viral parameters, such as viral type distribution and viral load.

Although the hc2-RCS test showed an overall higher positivity rate than the GP5+/6+ PCR-EIA, particularly cervical scrape specimens classified as normal, borderline, or mild dyskaryosis, adjustment of the hc2 cutoff point resulted in similar positivity rates for both methods. These results suggest that the clinical performances (i.e., clinical sensitivity and specificity for ≥CIN3) of both methods can be compatible, which is of importance when viral typing by GP5+/6+ PCR is envisaged as a follow-up test for hc2-RCS-positive women.

MATERIALS AND METHODS

Study population, collection of cervical samples, and cervical cytology. The study, initiated as an extension of the POBASCAM (population-based cervical screening trial Amsterdam) (5), started in April 2003, and the intake finished in April 2004. Women were recruited from the national screening program via the 242 general practitioners participating in the POBASCAM trial (5). POBASCAM was initiated to compare the efficacy of hrHPV testing in conjunction with cytology to that of sole classical cytology. The two-armed trial was carried out within the setting of the regular Dutch nationwide cervical screening program, in which women between 30 and 60 years old are invited with screening intervals of 5 years. Further details about POBASCAM have been described previously (5). Informed consent was obtained from all participating women. For this study, we collected scrape specimens from 10,051 women participating in an extension of the POBASCAM trial. We excluded women who had a history of abnormal cytology or CIN disease within the preceding 2 years. As a consequence, of the 10,051 women recruited, 8,132 were ultimately enrolled.

Cervical smears were taken using a Cervex brush or a cytobrush. After a conventional smear was made for cytological examination, the brush was placed in a vial containing 5 ml universal collection medium (UCM) (Digene Corporation) for hrHPV testing. Upon arrival in the testing laboratory, cervical samples were concentrated to 1 ml UCM by centrifugation of vials for 10 min at 4,000 × *g*, and 4 ml UCM supernatant was discarded. The pellet was resuspended in 1 ml UCM and stored at −80°C until use.

Cytomorphological analysis was performed according to the CISOE-A classification, which can be translated easily to the Bethesda 2001 classification (3). Briefly, smears were classified as inadequate, normal, borderline dyskaryosis, mild dyskaryosis, moderate dyskaryosis, severe dyskaryosis, suspected of carcinoma in situ, or suspected of invasive cancer. In the Bethesda classification, 2001 borderline/mild dyskaryosis equals ASC-US/ASC-H/LSIL, and moderate and severe dyskaryosis and suspected of carcinoma in situ are equal to high-grade squamous intraepithelial lesion (3, 25).

Women with cervical smears classified as moderate dyskaryosis or worse (high-grade squamous intraepithelial lesion according to the Bethesda classification) were immediately referred to the gynecologist for colposcopy-directed biopsy, and histological data for these women were used in this study. Biopsies were histologically classified as no CIN, CIN 1, 2, or 3, or cervical carcinoma. Histological follow-up data were retrieved from the Nationwide Pathology register.

Hc2-RCS. Hc2 was performed using the High-Risk HPV DNA test executed on the RCS. This test detects 13 hrHPV types (i.e., HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68). For hc2-RCS testing, 500 μl of UCM sample was mixed with 75 μl of guanidine-hydrochloride (8 M) and 250 μl denaturation reagent with indicator dye, briefly shaken, and denaturated for 45 min at 65°C. The samples were further processed on the RCS according to the recommendations of the manufacturer (Digene Corporation). Ultimately, of each denaturated sample, 75 μl was used for testing. Samples with relative light unit per cutoff value (RLU/CO) of ≥1 were initially scored hc2 positive.

HPV PCR testing. Sample material was prepared for GP5+/6+ PCR testing as follows: 150 μl UCM sample material was centrifuged for 10 min at 4,000 × *g*, and the pellet was resuspended in 1 ml Tris-HCl (pH 8.0). DNA was released after "freezing and boiling" of this 1-ml sample, and subsequently, 10 μl of this sample material was used as input in the PCR. The GP5+/6+ PCR and subsequent EIA readout system using a probe cocktail of 14 h-HPV types (i.e., HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) were performed essentially as described previously (27). The cutoff value of the GP5+/6+ PCR-EIA was calculated as three times the average EIA value of four negative blanks that are included in each PCR run. Reverse line blot was used to genotype HPV-positive samples, which can detect up to 27 additional HPV types besides the above-mentioned 14 hrHPV types (27).

Type-specific HPV E7 PCR was performed as described previously (29) for the following types: HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68.

HPV 16 DNA load assessment. Quantification of HPV16 type-specific DNA load and the amount of cells in a scrape sample was performed by real-time PCR using the LightCycler instrument (13, 23). Viral load assessment was performed on a subset of samples that were GP5+/6+ PCR positive for HPV16 that revealed either a hc2-RCS negative ($n = 11$) or hc2-RCS positive ($n = 12$) test result.

Statistics. For comparison of the positivity rates of hc2-RCS and GP5+/6+ PCR and their relation to cytologic and histologic parameters, the McNemar test was used. The agreement was determined using the kappa value. To determine whether differences in performance of hc2-RCS and GP5+/6+ PCR can be attributed to assay threshold differences, the receiver operator characteristics curve (i.e., ROC curve) was computed for the semiquantitative hc2-RCS using moderate dyskaryosis or worse as the outcome measure. All HPV16 load values obtained by real-time PCR were log normalized. For baseline parameters, we computed median and range to describe the variety in the measurements. One-way analysis-of-variance (ANOVA) analysis was used to compare the mean HPV16 and β-globin loads between the hc2-negative and the hc2-positive groups. *P* values of 0.05 or less were considered statistically significant. All analyses were performed using SPSS 11.5 software.

TABLE 1. GP5+/6+ PCR and hc2-RCS test results

hc2-RCS assay result	No. of specimens tested by GP5+/6+ PCR		Total no. of specimens
	Positive	Negative	
Positive	358	197	555
Negative	35	7,542	7,577
Total	393	7,739	8,132

RESULTS

Hc2-RCS and GP5+/6+ PCR test results in relation to cytology. A total of 8,132 cervical scrape specimens were analyzed by two hrHPV detection methods, i.e., hc2-RCS and GP5+/6+ PCR-EIA. The hc2-RCS test scored 555 (6.8%) samples positive overall, whereas the GP5+/6+ PCR assay revealed positivity for 393 (4.8%) samples (Table 1). The two tests gave concordant test results for 358 positive and 7,542 negative samples, with a good overall level of agreement (kappa = 0.74). A total of 232 samples had discrepant test results between hc2-RCS and the GP5+/6+ PCR assay. The number of hc2-RCS positive but GP5+/6+ PCR-negative samples ($n = 197$) was significantly higher than the number of

TABLE 2. GP5+/6+ PCR and hc2-RCS results in relation to cytology

Cytology result	No. of specimens[a]				Total no. of specimens	*P* value[b]
	Positive by GP and hc2-RCS	Negative by GP and hc2-RCS	Positive by GP but negative by hc2-RCS	Negative by GP by positive by hc2-RCS		
Normal, borderline, or mild dyskaryosis	301	7,453	33	190	7,977	<0.0005
Moderate dyskaryosis or worse	52	3	1	4	60	0.38
Total	353	7,456	34	194	8,037	<0.0005

[a] GP, GP5+/6+ PCR.
[b] McNemar statistics.

hc2-RCS negative, GP5+/6+ PCR-positive samples ($n = 35$; McNemar $P < 0.0005$).

To compare the clinical value of the hc2-RCS and GP5+/6+ PCR assays, their results were first related to the cytomorphologic findings. The 8,132 smears comprised 95 (1.2%) samples classified as inadequate, 7,841 (96.4%) as normal, 116 (1.4%) as borderline dyskaryosis, 20 (0.2%) as mild dyskaryosis, 28 (0.3%) as moderate dyskaryosis, 22 (0.3%) as severe dyskaryosis, and 10 (0.1%) suspected of carcinoma in situ. For further analysis, the adequate smears were categorized into two groups, i.e., normal, borderline, or mild dyskaryosis and moderate dyskaryosis or worse. Hc2-RCS and GP5+/6+ PCR test results in relation to these cytologic categories are given in Table 2. Only in women with normal, borderline, or mild dyskaryosis, the hc2-RCS positivity rate was significantly higher than that of the GP5+/6+ PCR (McNemar, normal, borderline, or mild dyskaryosis, $P < 0.0005$; moderate dyskaryosis or worse, $P = 0.38$). The semiquantitative nature of the hc2-RCS assay allowed further exploration of whether these differences in performance of the hc2-RCS and GP5+/6+ PCR tests can be explained by differences in assay thresholds. Therefore, the hc2-RCS RLU/CO values were used to plot positivity rates for moderate dyskaryosis or worse and negativity rates for normal, borderline, or mild dyskaryosis in an ROC curve, and these rates were compared to that of the GP5+/6+ PCR (Fig. 1). An increase of the hc2-RCS threshold to 3.2 RLU/CO resulted in positivity and negativity rates similar to those of the GP5+/6+ PCR. This value did not differ meaningfully when different cytological categories (i.e., normal cytology versus borderline dyskaryosis or worse and normal or borderline dyskaryosis versus mild dyskaryosis or worse) were used for ROC analysis (data not shown).

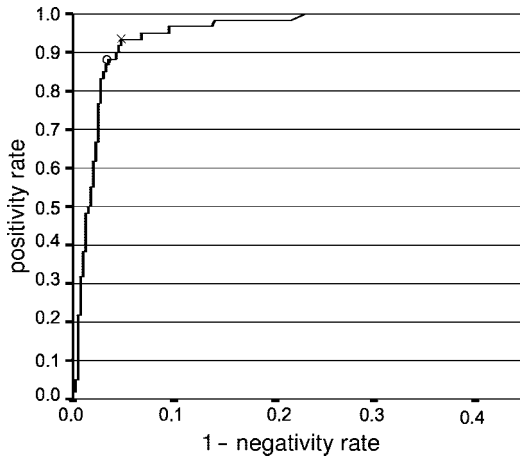

FIG. 1. ROC curve for hc2-RCS using the cytology threshold of moderate dyskaryosis or worse. Positivity rates of hc2-RCS for smears representing moderate dyskaryosis or worse were plotted versus "1 − negativity rate" of the hc2-RCS test for normal, borderline, or mild dyskaryosis for increasing threshold values of the hc2-RCS test. The area under the curve was 0.976. The "x" indicates the hc2-RCS threshold value of 1.0 RLU/CO. The "o" marks the point for the GP5+/6+ PCR-EIA positivity and negativity values (i.e., 88.3% and 95.8%, respectively), which equals an hc2-RCS threshold value of 3.2 RLU/CO.

Hc2-RCS and GP5+/6+ PCR test results in relation to histology. In this cross-sectional study, histologic data could be collected only from women with moderate dyskaryosis or worse, since these women were directly referred for colposcopy. Data from colposcopy-directed biopsy were available from 48 women, 46 of whom had an underlying CIN lesion. The latter comprised 1 CIN1 lesion, 13 CIN2 lesions, and 32 CIN3 lesions. The mean time interval between cytological diagnosis and biopsy was 2.1 months (range, 0 to 8 months). GP5+/6+ PCR-EIA and hc2-RCS positivity rates in relation to histology are summarized in Table 3. Hc2-RCS scored 31 of 32 ≥CIN3 cases positive and GP5+/6+ PCR two fewer cases, resulting in sensitivities for ≥CIN3 of 97% (95% confidence interval, 93.8 to 100%) and 91% (95% confidence interval, 85.1 to 95.8), respectively. However, this difference was not statistically significant ($P = 0.25$).

When the hc2-RCS threshold value was arbitrarily increased to 2.0 RLU/CO, the number of positive scrape specimens for women with ≥CIN3 remained the same as at 1.0 RLU/CO,

TABLE 3. GP5+/6+ PCR and hc2-RCS results in relation to histology[a]

Intake (n[b])	No. (%) of GP5+/6+ PCR-positive specimens	No. (%) of hc2-RCS-positive specimens	*P* value[c]
No CIN (2)	1 (50)	1 (50)	—[d]
CIN 1 (1)	1 (100)	1 (100)	—
CIN 2 (13)	12 (92)	12 (92)	1.00
CIN 3 (32)	29 (91)	31 (97)	0.25
Total (48)	43 (90)	45 (94)	0.73

[a] Mean time between cytology and biopsy: 2.1 months (range, 0 to 8 months).
[b] n, number of samples.
[c] McNemar.
[d] —, no discrepant cases; not possible to determine *P* value.

TABLE 4. Positivity rates of GP5+/6+ PCR and hc2-RCS at different RLU/CO thresholds in relation to cytology and histologically confirmed ≥CIN3

Assay	No. (%) of specimens from women with[a]:		
	Normal, borderline, or mild dyskaryosis	Moderate dyskaryosis or worse	≥CIN3
GP5+/6+ PCR-EIA	340 (4.3)	54 (90)	29 (91)
Hc2-RCS			
RLU/CO 1.0	499 (6.3)[b]	56 (93)[e]	31 (97)[e]
RLU/CO 2.0	371 (4.7)[c]	56 (93)[e]	31 (97)[e]
RLU/CO 3.0	333 (4.2)[d]	54 (90)[e]	29 (91)[e]

[a] For normal, borderline, or mild dyskaryosis, $n = 7,977$; for moderate dyskaryosis or worse, $n = 60$; for ≥CIN3, $n = 32$.
[b] $P < 0.0005$ compared to positivity rate of GP5+/6+ PCR (McNemar).
[c] $P = 0.02$ compared to positivity rate of GP5+/6+ PCR (McNemar); $P < 0.0005$ compared to positivity rate at RLU/CO 1.0 (McNemar).
[d] No significant difference in positivity rate compared to that of GP5+/6+ PCR (McNemar, $P = 0.76$); $P < 0.0005$ compared to positivity rate at RLU/CO 1.0 (McNemar).
[e] No significant difference in positivity rate compared to that of GP5+/6+ PCR (McNemar).

TABLE 5. Potential cross-reactivities of the hc2-RCS probe with other HPV types

HPV type(s)	No. of specimens containing type
30	3
42	3
53	6
53, 67	1
54, 70, 81, LR[a]	1
67	6
70	7
70, 39	1
72	1
82	5
LR[a]	1
Total	35

[a] LR, low-risk HPV, not further specified.

whereas a significant reduction in test positivity was obtained for women with normal, borderline, or mild dyskaryosis (i.e., 4.7% versus 6.3% at 1.0 RLU/CO; McNemar $P < 0.0005$) (Table 4). Nevertheless, at an RLU/CO value of 2.0, hc2-RCS positivity among women with normal, borderline, or mild dyskaryosis was still significantly higher than that with GP5+/6+ PCR (McNemar, $P = 0.02$). With use of an RLU/CO value of 3.0, the hc2-RCS positivity rate for women with normal, borderline, or mild dyskaryosis was no longer significantly different from that of the GP5+/6+ PCR (4.2% versus 4.3%; McNemar $P = 0.76$), while both tests scored the same number of women with ≥CIN3 positive.

Analysis of hc2-RCS positive, GP5+/6+ PCR-negative samples. It is unlikely that the extra positivity scored by hc2-RCS with smears read as normal, borderline, or mild dyskaryosis is owing to low copy numbers of hrHPV that fall below the detection limit of the PCR assay, since the analytical sensitivity of the GP5+/6+ PCR is higher than that of hc2-RCS. To support this notion, we determined the HPV16 load by real-time PCR for 23 samples harboring a single HPV16 infection as determined by GP5+/6+ PCR followed by reverse line blot genotyping. One subset of these samples had tested negative with the hc2-RCS test ($n = 11$), and the other subset had tested positive with the hc2-RCS test ($n = 12$). Indeed, the HPV16 DNA load in the hc2-RCS negative group (median, 1.0×10^5; range, 1.3×10^4 to 1.5×10^6 copies/scrape specimen) was significantly lower (ANOVA, $P < 0.0005$) than the HPV16 load in the hc2-RCS positive group (median, 4.9×10^6; range, 1.0×10^6 to 8.7×10^7 copies/scrape specimen). These numbers were not influenced after stratification for cytology.

To further explain the occurrence of hc2-RCS-positive, GP5+/6+ PCR-negative cases, we addressed the influence of other potential possibilities: (i) false-negative GP5+/6+ PCR result owing to (a) inadequate crude samples for PCR due to the presence of PCR inhibitors or (b) viral integration events disrupting the GP5+/6+ PCR primer binding region in L1; (ii) false-positive hc2-RCS results owing to cross-reactivity of the hc2-RCS with other HPV types.

In order to address the first possibility, all 197 GP5+/6+ PCR-EIA negative, hc2-RCS-positive samples were retested by β-globin PCR. Only 11 (5.6%) of these samples tested negative and therefore can be considered invalid for PCR. Of the three CIN3 cases that tested negative by GP5+/6+ PCR, one case, which was also negative by hc2-RCS, was inadequate for PCR testing on the crude extract due to inhibition of the PCR. This sample, however, revealed HPV16 positivity by GP5+/6+ PCR after DNA extraction.

To determine possible integration of the HPV genome in the GP5+/6+ primer region, type-specific E7 PCR was performed on the crude extracts of the other two GP5+/6+ PCR-negative ≥CIN3 cases that were hc2-RCS positive. Both tested positive by E7 PCR and are likely to contain integrated virus with disrupted L1. These included one case containing HPV16 and one case with HPV18.

Of the remaining 186 samples that were β-globin PCR positive, the GP5+/6+ PCR was repeated, and PCR products were subjected to overall HPV typing by reverse line blotting to determine the level of cross-reactivity of the hc2-RCS test. A total of 35 (17.8%) samples were found to contain HPV types not present in the hc2-RCS probe (Table 5). Most of these cases fell in the category of normal cytology. The level of cross-reactivity of GP5+/6+ PCR could not be determined in this study, since hc2-RCS is unable to determine the HPV genotype of an infection.

Analysis of GP5+/6+ PCR-positive, hc2-RCS-negative samples. Of the 35 GP5+/6+ PCR-positive, hc2-RCS-negative samples, 32 (91%) harbored an HPV type that is present in the hc2-RCS probe (Table 6). HPV types that were most often missed by hc2-RCS were HPV16, HPV45, HPV56, and HPV31. Most of these cases (28/32) were cytologically normal or borderline dyskaryotic.

In the 23 samples used for HPV 16 load analysis by real-time PCR (as detailed above), we also determined the amount of cells per scrape by β-globin gene real-time PCR. Interestingly, the amount of cells per scrape in the hc2-RCS positive smears (median, 4.0×10^6; range, 2.8×10^5 to 2.3×10^7 cells/scrape) was significantly higher than that found in the hc2-RCS negative group (median, 1.2×10^6; range, 1.5×10^5 to 8.6×10^6 cells/scrape; ANOVA, $P = 0.04$). The smaller amount of DNA

TABLE 6. Distribution of HPV types in GP5+/6+ PCR-positive but hc2-RCS-negative cases

HPV type(s)	No. of specimens containing type
16	8
16, 31	1
18	2
31	4
33	2
35	1
45	7
52	1
56	4
56, 66, 73	1
58	1
Total	32

in these smears may have contributed to the negative test results of the hc2-RCS. However, clinical follow-up data need to be gathered before conclusions can be made as to what extent these cases reflect clinically relevant infections.

DISCUSSION

In this study we compared the performances of the hc2-RCS test and the hrHPV GP5+/6+ PCR-EIA in relation to cytologic and histologic parameters of women participating in a population-based screening trial. The overall agreement between both tests was good and was comparable to that found by Kulmala et al. (2004) between the manual hc2 and GP5+/6+ PCR assays (i.e., kappa 0.67) (16).

Differences in performances of hc2-RCS and GP5+/6+ PCR could mainly be explained by differences in assay thresholds. Still, the higher positivity rate of the hc2-RCS at an RLU/CO value of 1.0 compared to that of the GP5+/6+ PCR in our study is somewhat surprising, given the lower analytical sensitivity of the hc2 assay. The latter was supported by higher viral load values in HPV16 PCR-positive samples that were also hc2/RCS positive than in those that were hc2-RCS negative. However, we collected evidence that part of the hc2-RCS positivity in GP5+/6+ PCR-negative samples can be attributed to some cross-reactivity of the hc2-RCS assay with HPV types that are not covered by the probes of the hc2-RCS test. Similar results have been obtained in other studies (6, 12). A much smaller proportion of hc2-RCS-positive/GP5+/6+ PCR-negative samples could be explained by PCR failures due to PCR inhibitors in the crude extracts, while the presence of integrated viral DNA with a disrupted GP5+/6+ region is likely to be a rare event that only occasionally may occur in high-grade lesions. A major part of hc2-RCS-positive/GP5+/6+ PCR-negative test results, which mainly involved scrapings from women with normal, borderline, or mild dyskaryosis and weakly positive hc2/RCS values, is likely to reflect a certain level of background noise when the hc2-RCS assay is applied at an RLU/CO value of 1.0. However, our data indicate that a more optimal signal-to-noise ratio of the hc2-RCS method can be obtained by adjusting the threshold of this assay to levels at which the results of this test better match those of GP5+/6+ PCR. Moreover, since an interlaboratory reproducibility evaluation by Castle et al. (2004) revealed that the reproducibility of an hc2-positive test is lowest for women with normal cytology, particularly those with RLU/CO values between 1 and 3, adjustment of the hc2 thresholds is likely to also increase the reproducibility of the assay (8).

Our results seem to contradict the findings of Kulmala et al. (2004), who detected a higher positivity rate with GP5+/6+ PCR than with the manual hc2 assay (i.e., 33.8% and 27.9%, respectively). This difference may be explained by the fact that their study population generally involved younger women displaying a much higher HPV prevalence rate that may have masked the potential hc2 noise. On the other hand, the overall hc2-RCS positivity rate (i.e., 6.8%) in our study is in the same range as that obtained with the manual hc2 in the HART study (i.e., 7.6%), which involved women of a population-based screening cohort with a similar age distribution (10).

In this study a threshold of 2.0 RLU/CO for the hc2-RCS test would result in an increased clinical specificity for women with ≥CIN3, while no additional lesions ≥CIN3 were missed. Therefore, in case the hc2 is used as a primary screening tool, we, like others (7, 10), feel that the assay threshold easily can be increased to 2.0 RLU/CO, since this would result in a higher specificity, thereby minimizing the unnecessary follow-up of women with transient infection. Alternatively, when genotyping by GP5+/6+ PCR is envisaged as a reflex test for hc2-RCS-positive women without cytological abnormalities, adaptation of the hc2-RCS cutoff is less crucial, since in that scenario, the cutoff of the GP5+/6+ PCR assay would be decisive for further management. Still, it should be realized that definitive figures about clinical sensitivity and specificity for ≥CIN3 (24) and consequently the clinically most informative RLU/CO threshold can be calculated only when all follow-up information on women with normal, borderline, or mild dyskaryosis has been gathered.

Conversely, Ordi et al. (19) advocated the use of 1.0 RLU/CO, since their data showed that increasing the cutoff level would not lead to an increase in specificity. However, the women in their study were selected on the basis of cytological abnormality (ASC-US or worse). For such a high-risk population, specificity is rather low regardless of which cutoff level is used. We even feel that in case the hc2-RCS cutoff is increased to 2.0 or 3.0 RLU/CO, the clinical specificity of hrHPV testing for ≥CIN3 still needs improvement, since a substantial number of women that score hc2 positive at these cutoff values do not have or develop ≥CIN3. This may be achieved by further stratifying HPV-positive women by genotyping (4), viral load assessment (11, 28) and/or analysis of the presence of E6/E7 mRNA (9).

Most importantly, when sensitivity and specificity to detect lesions ≥CIN3 are considered equally important aspects of overall accuracy, both assays studied herein are similarly accurate, provided that the hc2-RCS cutoff is slightly adapted. For primary and secondary screening, this opens possibilities for a combination test of hc2-RCS and GP5+/6+ PCR, in which the hc2-RCS test, being easy and robust and therefore an ideal tool for application in large screening programs, is used first and the GP5+/6+ PCR as a reflex test (i.e., testing only of hc2-RCS-positive samples) for genotyping of hc2-RCS-positive women. The latter assay requires special skills and a more stringent infrastructure to reduce PCR-related contamination

risks and therefore preferably should be performed in well-equipped, specialized centers.

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