To be acceptable for use in cervical cancer screening, a new assay that detects DNA of high-risk human papillomavirus (hrHPV) types must demonstrate high reproducibility and performance not inferior to that of a clinically validated HPV test. In the present study, a real-time quantitative PCR (qPCR) assay targeting the E6 and E7 genes of hrHPV was compared with Hybrid Capture 2 (hc2) in a Belgian cervical cancer screening setting. In women >30 years old, the sensitivity and specificity for intraepithelial neoplasias of grade 2 or worse (93 cases of cervical intraepithelial neoplasias of grade 2 or worse (CIN2+) and 1,207 cases of no CIN or CIN1) were 93.6% and 95.6%, respectively, and those of hc2 were 83.9% and 94.5%, respectively. The overall agreement of hrHPV positivity between the two runs of the qPCR test was 98.7% (95% CI, 97.5 to 99.4%), with a kappa value of 0.96 (95% CI, 0.83 to 1.00). The qPCR assay used in this study can be considered a reliable HPV assay that fulfills the clinical validation criteria defined for use in cervical cancer screening.
dizinsiche Diagnostik, Heidelberg, Germany) throughout the trial in accordance with the manufacturer’s instructions. A ratio of relative light units (RLU) to a standard positive control of $/H11022$ was considered positive. The qPCR assay was considered positive if at least 1 of the 13 high-risk types targeted by hc2 was present. The cumulative hrHPV load measured by the qPCR assay was defined as the sum of the type-specific loads of the 13 high-risk types.

Sensitivity was assessed with samples with histologically confirmed CIN2, whereas specificity was evaluated for women with an outcome of CIN1 or absence of CIN. The clinical sensitivity and specificity of the qPCR assay for CIN2 were compared to those of hc2 by using a noninferiority score test considering a relative sensitivity threshold of at least 0.90 and a specificity threshold of at least 98% (17). 

To determine the intrasystem reproducibility of the quantitative HPV genotyping assay, two portions (half of the extracted DNA of the original sample) of a set of 633 samples were retested by two independent laboratory technicians using the same reagent lot numbers after 6 weeks (RIATOL, Sonic Healthcare Benelux, Antwerp, Belgium). The two runs were performed with different PCR machines at two different locations (Antwerp and Hoboken). The concordance for the presence of high-risk types was assessed by the percent agreement and kappa values (13). The type-specific viral load agreement between the two runs was assessed by using Bland-Altman graphs, which plot the paired differences against the pairwise load means for each sample that is positive for a given hrHPV type by the qPCR assay (6). The plot further contains the limits of agreement, which correspond to $1.96$ standard deviations of the pairwise load differences. A horizontal line through the mean of the differences near the line of perfect agreement (through zero on the y axis) indicates good agreement. The change in sensitivity and specificity with different viral load cutoffs was assessed by receiver operating characteristic (ROC) curve analysis. Fitted maximum-likelihood ROC curves were estimated by assuming a binomial distribution of the underlying sensitivity and specificity using the MedCalc program (MedCalc Software, Mariakerke, Belgium) (22, 23). All other statistical analyses were performed with Stata version 10.0 (StataCorp, College Station, TX).

RESULTS

Out of the 1,300 liquid-based cervical smears from the controlled colposcopy trial, two sets were selected. The first set was for clinical sensitivity analysis (Fig. 1A) and included 93 smears from women (median age of 36 [range, 30 to 65] years) who had histologically confirmed CIN2 lesions (i.e., 42 with CIN2, 45 with CIN3, 1 with adenocarcinoma, and 5 with squamous cell carcinoma). The median follow-up time was 3.3 (range, 0 to 33) months. Women were identified through HPV testing (hc2 and/or qPCR assay), by cytology, or by the baseline colposcopy. A second set for clinical specificity analysis (Fig. 1B) included 1,207 representative smears from women (median age of 45 [range, 30 to 65] years) without a CIN2 diagnosis within a follow-up time of 28 months.

The sensitivity and specificity values of the qPCR assay for CIN2 were 93.6% (87/93; 95% confidence interval [CI], 86.5 to 97.6%) and 95.6% (95% CI, 94.3 to 96.7%), respectively. The sensitivity and specificity values of hc2 for CIN2 were 83.9% (78/93; 95% CI, 74.8 to 90.7%) and 94.5% (95% CI, 93.0 to 95.7%), respectively. Both the clinical sensitivity and specificity for CIN2 of the quantitative HPV genotyping assay were not inferior to those of the hc2 ($P < 0.0001$ and $P < 0.0001$, respectively). Moreover, the quantitative HPV genotyping assay was not only superior to hc2 with respect to the sensitivity of CIN2 detection, with a sensitivity difference of 9.7% (CI, 0.1 to 19.2%) ($P = 0.029$; McNemar’s test), but also more specific than hc2, with a specificity difference of 1.6% (CI, 1.3 to 1.6%) ($P = 0.016$; McNemar’s test). An overview of the real-time qualitative HPV assay results stratified by CIN2 disease status is given in Table 1.

The reproducibility was very high for the 13 pooled high-risk
types considered together (98.7% [95% CI, 97.5 to 99.4%]), as well as for each HPV type separately, between 98 and 100%. The positive agreement for results that were hrHPV positive in the first or second run was 93% (95% CI, 87 to 97%). The kappa value was 0.96 (95% CI, 0.83 to 1.00) for hrHPV and >0.87 for separate hrHPV types.

The average number of hrHPV infections per sample did not differ in samples where both runs yielded a positive hrHPV result. For the reproducibility of viral load measurements (log10 of viral load), the analysis was restricted to samples where both measurements were positive. The Bland-Altman plots of the 13 hrHPV types are displayed in Fig. 2. The purple horizontal line through the average difference of log loads was always located near the green line of perfect agreement (Fig. 2).

The correlation between the viral loads measured by hc2 (expressed in RLU) and the qPCR assay (log of the sum of viral loads of 13 hrHPV types) is shown in Fig. 3. The correlation was \( r = 0.70 \) (95% CI, 0.60 to 0.78; \( P < 0.0001 \)) for samples with a positive hrHPV qPCR assay results (\( n = 140 \)) (Fig. 3A). The distribution of the cumulative viral load in all of the CIN2+ cases is also shown for hc2-negative (\( n = 13 \)) and hc2-positive cases (\( n = 78 \)) in Fig. 3B. ROC curve analysis for these hc2 cases showed a cutoff for CIN2+ at 2.35 log hrHPV copies/cell with a sensitivity of 90.5% and a specificity of 69.2% with an area under the ROC curve of 0.791 and a 95% CI between 0.691 and 0.871.

The ROC curves for both tests are displayed in Fig. 4. The sensitivities of hc2 and the qPCR assay for CIN2+ jointly rise with

<table>
<thead>
<tr>
<th>Status and qPCR result</th>
<th>No. of smears with hc2 result of:</th>
<th>Total no. of smears</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrHPV−</td>
<td>hrHPV+</td>
</tr>
<tr>
<td>CIN2</td>
<td>1,130</td>
<td>24(^a)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>1,140</td>
<td>67</td>
</tr>
<tr>
<td>CIN2+</td>
<td>2</td>
<td>4(^b)</td>
</tr>
<tr>
<td></td>
<td>13(^c)</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^a\) Of the 24 hc2 hrHPV-positive/qPCR hrHPV-negative specimens, 5 were positive by qPCR for genotypes that are not included in the 13 HPV types, i.e., HPV53 (\( n = 1 \)), HPV66 (\( n = 2 \)), and HPV67 (\( n = 2 \)).

\(^b\) All four patients were CIN2, three patients were positive for HPV types not included in the 13 HPV types with the qPCR, namely, two HPV53-positive patients and one HPV66 patient, and all three patients had a high viral load levels.

\(^c\) All of the 13 hc2 hrHPV-negative/qPCR hrHPV-positive patients (3 HPV16; HPV16 and HPV18; HPV16, HPV31, and HPV53; HPV31; 2 HPV53; HPV51; HPV51 and HPV52; HPV52; and 2 HPV56) had a low mean viral load level (2.11 log HPV copies/cell, which was significantly lower than the mean viral load level of the 74 hc2 hrHPV-positive/qPCR hrHPV-positive patients (3.43 log HPV copies/cell; \( P = 0.0003 \)).

![FIG 2 Bland-Altman plots displaying the viral load agreement of each of the 13 hrHPV types between the first and second qPCR assay assessments according to the HPV type. The green horizontal line corresponds to perfect agreement, the purple line corresponds to the average difference (Diff) in type-specific log loads, and the red lines correspond to the 95% limits of agreement.](http://jcm.asm.org/ on October 25, 2017 by guest)
a lower viral load cutoff up to approximately 73% at a specificity of approximately 97%. The sensitivity of the qPCR assay continues to rise steeply to a maximum of 94% at a specificity of 96%. However, the hc2 ROC curve shows a less steep slope and any further sensitivity increase is accompanied by a substantial loss of specificity.

An overview of the ROC curve analysis of both hc2 and the qPCR assay for the detection of CIN2+ is given in Table 2. Comparison of ROC curves for a given sensitivity of 95%, however, showed that the qPCR assay was significantly more specific (25.9%). Also for a given specificity of 95%, the qPCR assay showed significantly better sensitivity (15.2%) than hc2 in the detection of CIN2+ (Table 2).

**DISCUSSION**

The aim of the present study was to compare the clinical accuracy of a type-specific real-time qPCR assay with the clinically validated reference HPV test, hc2, with samples from women coming for routine cervical cancer screening and included in a controlled colposcopy trial. The results show higher clinical sensitivity (93.6% versus 83.9%) and specificity (95.6% versus 94.5%) of the qPCR assay (0.0001 HPV copies/cell) than of hc2 (>1 RLU) for CIN2+.

Indeed, at a sensitivity of 95%, hc2 was approximately 25% less specific than the qPCR assay, and at a specificity of 95%, hc2 was also 15% less specific. These differences could be explained, on the one hand, by the nature of the assay. Indeed, the qPCR assay is more sensitive than liquid hybridization and also more specific because there is no cross-reaction among the different type-specific E6/E7 qPCR assays, in contrast to the known cross-reactivity with low-risk HPV types exhibited by the high-risk probes of hc2 (8). Furthermore, on the other hand, during recent years, we improved the clinical sensitivity for detection of CIN2+. This improvement in sensitivity may have been the result of modification of the sampling device (Cervex-Brush Combi, yielding a 50-fold greater viral load) left in the transport liquid (10); this brush could be better at sampling squamo-columnar junction cells at the transition zone, where neoplastic lesions preferentially occur (14); and the enrichment of the cell suspension used to make a liquid-based cytology specimen and the BD FocalPoint assisted cytological interpretation with knowledge of the HPV status. We demonstrated that prior knowledge of the HPV status improves the sensitivity of cytology for CIN2+ detection (5). The efficacy of using a clinically more sensitive assay has recently been well illustrated by four randomized controlled trials comparing primary HPV screening with cytology screening (4). These randomized trials consistently showed, in the second screening round, a significant reduction in the incidence of CIN3+ and even of invasive cancer (CIN3 precedes cervical cancer) by HPV screening, reducing the number of women dying from cervical cancer.

In the present study, ROC curve analysis showed a 3-log-lower clinical threshold for the qPCR assay (~0.04 log HPV copies/cell) than hc2 (2.35 log HPV copies/cell) for the detection of CIN2+. Further, ROC curve analysis also showed that the clinical threshold for hc2 was lower (0.77 RLU) than that given in the product insert (>1 RLU), corroborating the improvement of our clinical sensitivity.

Concerning transferability to other laboratories, we recommend the use of a cell enrichment method and correction for the number of cells before performing DNA extraction, instead of taking a fixed volume from the original vial. Furthermore, we encourage the use of primers targeting the E6 or E7 region and not
L1, as L1-negative cancers have been described (27). And finally, we recommend the use of individual type-specific and normalized (for the number of cells) HPV tests which allow monitoring of individual patients over time (12). Such serial measurements without clinical threshold constraints (9, 12, 16), in comparison to a fixed single viral load threshold (24, 25), could allow earlier detection of CIN2+ and improve clinical sensitivity.

Whether the use of the qPCR assay would result in less cancer than that of hc2 and whether it would allow the safe extension of screening intervals cannot be derived from this cross-sectional validation study. However, this study clearly indicates that the qPCR assay fulfills and even exceeds the requirements defined in the international guidelines for HPV DNA assays that can be used for cervical cancer screening (17).

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We have no conflicts of interest to declare.

REFERENCES