Viral Load of High-Risk Human Papillomaviruses as Reliable Clinical Predictor for the Presence of Cervical Lesions

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Abstract

**Background:** Infections with high-risk human papillomaviruses (Hr-HPV) can cause malignant transformation of the human cervical epithelium. HPV DNA tests generally are very sensitive to detect cervical neoplastic lesions but also identify transient HPV infections. As a consequence, the specificity and positive predictive value are low.

**Methods:** We analyzed viral load of Hr- and possibly Hr-HPV types more than seven orders of magnitude (on a log10 scale) in 999 consecutive BD-SurePath liquid-based cervical cytology samples from routine cervical screening enriched with atypical squamous cells of undetermined significance (n = 100), low-grade squamous intraepithelial lesions (LSIL; n = 100), and high-grade squamous intraepithelial lesions (HSIL; n = 97) using type-specific multiplex quantitative real-time PCR and the BSGP5+/6+PCR/MPG assay. In the 36-month follow-up, 79 histologically verified CIN2+ and 797 double-negative cytology cases were identified.

**Results:** Viral loads in LSIL and HSIL were significantly increased compared with no intraepithelial lesion or malignancy in both the quantitative PCR (qPCR) and BSGP5+/6+PCR/MPG assay (P < 0.0001). The mean viral loads in LSIL and HSIL were not significantly different. Using a newly determined high viral load cut off for 14 Hr-HPV types, the sensitivity for prevalent CIN3+ remained at 100% for both assays compared with the minimal detection threshold. The specificity (corresponding to double-negative cytology at subsequent screening episodes) increased substantially (qPCR, from 91.1% to 97.5%; BSGP5+/6+PCR/MPG, from 79.8% to 96.2%).

**Conclusions:** Compared with DNA positivity alone, high Hr-HPV viral loads could reduce the amount of false positive results detected by the BSGP5+/6+PCR/MPG and qPCR by 81.4% and 52.1%, respectively.

**Impact:** Quantitative type-specific HPV DNA assays show high flexibility in defining thresholds that allow optimizing clinical accuracy for cervical cancer precursors. Cancer Epidemiol Biomarkers Prev; 22(3); 406–14. ©2013 AACR.

Introduction

Human papillomaviruses (HPV) are DNA viruses that infect cutaneous and mucosal epithelia. Until now, 120 HPV genotypes are characterized based on sequence information (1), with evidence for a larger number to exist (2). Currently, there are 51 known mucosal HPV types, which are further divided into three groups based on their epidemiologic association with cervical cancer: high-risk HPV (Hr-HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), possibly high-risk HPV (pHr-HPV: 26, 53, 67, 70, 73, and 82), and low-risk or undefined HPV (Lr-HPV: e.g., 6, 11, 40, 42, 43, and 44; 3). Cervical cancer (CxCa) develops from persistent Hr-HPV infections through precursor lesions, defined histologically as high-grade cervical intraepithelial neoplasia. Cytology, still the standard cervical cancer screening method, distinguishes between the classes “no intraepithelial lesion or malignancy” (NIL/M), “atypical squamous cells of undetermined significance” (ASC-US), “atypical glandular cells” (AGC), “atypical squamous cells of undetermined significance cannot exclude high-grade squamous intraepithelial lesions” (ASC-H), “low-grade squamous intraepithelial lesions” (LSIL), and “high-grade squamous intraepithelial lesions” (HSIL), based on microscopic morphologic criteria and the probability of underlying CIN3 lesion (4).

Guidelines for clinical validation of new Hr-HPV tests potentially usable in cervical cancer screening have been developed (5). Candidate Hr-HPV tests to be used for cervical screening should have, at least, a similar sensitivity and specificity for CIN2+ lesions as the 2 clinically
validated Hr-HPV tests Hybrid Capture 2 (HC2) and the GP5+/6+/PCR/EIA (5).

To reduce the number of women with normal cytology, who are subjected to unnecessary clinical follow-up based on a transient Hr-HPV infection, appropriate triage is required (6). HPV viral load has been proposed as a candidate, which potentially can discriminate significant from insignificant HPV infections. Viral load can be measured by a variety of methods, including the semiquantitative HC2 (7) and visual assessment of reverse line blot or dot blot signals (8), or truly quantitative singleplex or multiplex quantitative real-time PCR (qRT-PCR) (9, 10). The majority of cross-sectional studies reported an association of increasing viral load with advancing disease severity, which was strongest for HPV16, but less strong or even absent for other Hr-HPV types (8–11).

Contrary to the classical GP5+/6+/PCR/EIA which is not suited for quantifying HPV DNA (12), we show here the quantification ability of the BSGP5+/6+/PCR/MPG assay. This article is the first of a series of comparative studies, which aim assessing the role of HPV DNA assays that identify separate genotypes. It describes the detection of cytologically and histologically confirmed cervical intraepithelial lesions in relation to viral load and the set of target HPV types.

Materials and Methods

VALGENT project

The current study is part of the VALGENT (validation of HPV genotyping tests) project, which provides a comprehensive design to validate and compare general HPV tests (identifying Hr-HPV infection) and HPV genotyping assays (identifying some or all Hr-HPV types separately). The VALGENT protocol involves blinded HPV testing with different assays using a continuous series of archived cervical cell samples collected in the framework of cancer screening enriched with pathologic samples identified through screening as well but over a longer period (13). Through follow-up of screen-positive women and subsequent screening of screen-negative women, subjects with and without cervical cancer precursors are identified, allowing the evaluation of clinical sensitivity and specificity, respectively (13). Two groups of patient material are considered: (i) 1,000 consecutive samples from women participating in cervical cancer screening and in addition (ii) 100 women with a cytology result of ASC-US, 100 with LSIL, and 100 with HSIL, which are all derived from the screening population as well. By this enrichment, sufficient cases (≥60) with cervical precancer can be identified to assess clinical sensitivity for CIN2+ (14).

Clinical outcomes

We considered presence of CIN2+ and CIN3+, identified through the usual diagnostic work-up of screen-positive women, as thresholds of clinical disease outcome. Screen-test negative women are not submitted to diagnostic verification. As a proxy for absence of high-grade disease in the absence of histologic verification, it is assumed, in the VALGENT protocol, that women who have, at 2 subsequent screening rounds, a negative cytology result (NIL/M), are free of CIN2+. Therefore, in the current study, we considered the number of women from whom the continuous samples were taken with index NIL/M cytology and again NIL/M at the next screening as the denominator for the computation of clinical specificity (specificity A). A second specificity (B) was calculated by including in addition the women also belonging to the continuous screening samples with CIN1 histology at follow-up irrespective of the index cytology.

Clinical specimens

Starting from the October 1, 2006, 999 consecutive BD-SurePath liquid-based cytology samples were collected during routine gynecologic health checks from women in Flanders (Belgium). The study was supplemented by samples from patients with ASC-US (n = 100), LSIL (n = 100), and HSIL (n = 97). Study-specific patient identification codes were assigned and transmitted in such a manner that patient confidentiality was preserved. According to Belgian law (of May 20, 2004) no informed consent or any action of the patient was required for this retrospective study because anonymity of the patients is guaranteed. In the 36 months follow-up, 79 histologically verified CIN2+ (CIN2, n = 27; CIN3, n = 47; carcinoma, n = 5) and 797 double-negative cytology cases were identified. Among the 297 women who provided abnormal cervical cell samples, 15 samples (5 ASC-US, 8 LSIL, and 2 HSIL) with 3 confirmed CIN3 cases were excluded from the analysis because of insufficient DNA material for PCR in Heidelberg (<5 μL).

Cytology

Thin-layer slide preparations were made with the fully robotic AutoCyte PREP System7 (AutoCyte: Tripath Imaging Inc.) and were prepared as described elsewhere (15). Cytology was read with prior knowledge of the quantitative PCR (qPCR) HPV DNA data (16). The cytologic results were classified according to the Bethesda system 2001 (4). Women with cytologic abnormalities were managed according to Belgian follow-up guidelines (17).

Isolation of DNA from cervical cells

DNA isolation from liquid-based cytology leftover and standardization for PCR was conducted as previously described (18, 19). At Riatol, the isolated DNA was divided in 5 aliquots and one aliquot was sent to Heidelberg for HPV genotyping using the BSGP5+/6+/PCR/MPG assay.

qRT-PCR analysis of HPV DNA

One DNA aliquot of the 1,296 samples was tested using 3 double- and 4 triplex TaqMan-based qPCR assays targeting type-specific sequences of viral E6 or E7 genes: HPV6 E6, HPV16 E7, HPV18 E7, HPV31 E6, HPV33 E6, HPV35 E6, HPV39 E7, HPV45 E7, HPV51 E6, HPV52 E7,
isoforms were titrated during the amplification of 10-fold data. To strengthen this effect, amplification competition of between 10 and 10,000 copies per PCR (21). Because of analysis of HPV containing plasmid dilution series showed reduced amplification of further observed that specimens with high viral loads in each specimen were expressed as the number of HPV copies per cell as described (20).

**BSGP5+/6+-%PCR/MPG assay**

The BSGP5+/6+-%PCR/MPG assay comprises the BSGP5+/6+-%PCR, which homogenously amplifies all known genital HPV types generating biotinylated amplimers of approximately 150 bp from the L1 region (21) and a Multiplex HPV Genotyping (MPG) assay with bead-based xMAP Luminex suspension array technology, which is able to simultaneously identify all 51 genital HPV types including Hr-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a/b, and pHr-HPV types 26, 53, 67, 70, 73, 82, and the β-globin gene (21, 22). Recently, the prevalence and viral load of 51 genital HPV types and 3 subtypes as determined by BSGP5+/6+-%PCR/MPG has been reported using the same clinical specimens as in the present study (23).

Isolated DNA from the 1,281 liquid-based cytology samples with sufficient DNA material for Heidelberg was analyzed blinded to qPCR and original cytology by the BSGP5+/6+-%PCR/MPG assay with some modifications. HPV amplification was carried out using the Multiplex PCR Kit (Qiagen). Note that, 0.2–0.5 μmol/L each of the BSGP5+ and 5’-biotinylated BSGP6+ primers, and 0.15 μmol/L each of the β-globin primers MS3 and 5’biotinylated MS10 were added to the PCR mixture. The results were expressed in median fluorescence intensities (MFI) of ≥100 beads per sample. The cut off value (5 net MFI) to define HPV DNA positivity was applied as described previously (24). For the colony PCR, *Escherichia coli* DH5α, transformed by high-copy-number plasmids containing the viral genome, replaced the template DNA. Depending on the amount of transferred bacteria, colony PCRs are estimated to contain >10⁶ HPV copies per PCR. Quantification of HPV signals was accomplished by computing for each positive reaction the relative HPV MFI signal (%HPVMFI) by dividing the measured HPV MFI value with the maximum value detected of this HPV type using colony PCR products. Finally, the relative MFI (%) was divided by the measured β-globin MFI value to form a nondescriptive viral load value (%HPVMFI/β-globin MFI).

BSGP5+/6+-%PCR/MPG allowed the semiquantitative analysis of HPV containing plasmid dilution series between 10 and 10,000 copies per PCR (21). Because of amplification competition of β-globin with HPV, it was further observed that specimens with high viral loads showed reduced amplification of β-globin (unpublished data). To strengthen this effect, β-globin primer concentrations were titrated during the amplification of 10-fold dilution series of HPV16 containing plasmid DNA in a background of 100 ng/μL human placenta DNA (Supplementary Fig. S1). Although HPV16 net MFI values reached the plateau phase with 1,000–10,000 copies per PCR, β-globin signals declined with increased HPV copy number input (Supplementary Fig. S1). The HPV16/ β-globin ratios, however, were able to quantify more than 7 logs when β-globin primer concentrations between 0.1 and 0.2 μmol/L were used.

**Statistical analysis**

Statistical analyses were conducted with the SAS software, version 9.2 (SAS Institute). Pairwise comparisons of the median viral loads in different groups were conducted by the Mann–Whitney test for both HPV assays. The rare categories, AGC and ASC-H, were excluded from this assessment. In case of multiple infections, only the HPV type with highest viral load was included. All tests were 2-sided, and *P*-values below 0.05 were considered statistically significant. The relation between viral load measurements conducted with the 2 assays, was evaluated with the Pearson and Spearman correlation coefficients. Discordant samples (positive with one but negative with the other assay) were included in these calculations. The HPV assay characteristics included: presence of Hr-HPV DNA, pHr-HPV DNA, viral load, and type of assay. The terms “TP,” “TN,” “FP,” and “FN” were used for true positive, true negative, false positive, and false negative, respectively. Note that, 95% confidence intervals (CI) for test accuracy parameters were computed considering binomial distributions.

**Results**

**Viral load comparison of BSGP5+/6+-%PCR/MPG with qPCR**

The correlation between viral loads obtained from the 1,281 specimen is shown in the XY scatter plots in Fig. 1 for 9 types. As expected, plots showing a polynomial rather than a linear shape showed reduced Pearson but increased Spearman coefficients. Both, the plots as well the correlation coefficients indicated highly significant (*P* < 0.0001) between the 2 viral load measurements for single as well as multiple infections over a wide range of HPV copy numbers. In addition, assay-specific differences in the analytical sensitivity for distinct HPV types could be observed indicating a higher analytical sensitivity for BSGP5+/6+-%PCR/MPG for the majority of HPV types (Fig. 1).

**Determination of high viral load cut offs for qPCR and BSGP5+/6+-%PCR/MPG**

Next we analyzed the viral load data of (p)Hr-HPV types in CIN2+ (*n* = 76), CIN3+ (*n* = 49), and absent disease (*n* = 797; corresponding to double-negative cytology at subsequent screening episodes) by Receiver Operating Characteristic (ROC) curve analysis to determine assay-specific general high viral load cut off that could be applied to all Hr- and pHr-HPV types. In case of multiple infections, only the Hr- and pHr-HPV type with highest viral load was included in the analysis.
The ROC curve analysis revealed for the qPCR a cut off of 0.46 copies/cell with a sensitivity for CIN2+ of 100% and a specificity for absent disease of 95.6%, with an area under the ROC curve (AUC) of 0.993 (95% CI 0.989–0.997; Fig. 2). For BSGP5+/6+/PCR/MPG a cut off of 0.0007 units with a sensitivity for CIN2+ of 98.7% (100% for CIN3+) and a specificity for absent disease of 95.4% was determined, with an AUC of 0.992 and a 95% CI between 0.988–0.997 (Fig. 2). For the given cut off values no significant differences in test accuracy (sensitivity, specificity, and AUC) were observed between both methods.

**Risk of cytologic abnormality associated with viral load**

The distribution of Hr-HPV loads assessed in baseline cervical samples by cytologic category are depicted in scatter dot plots (Fig. 3). Using this approach, viral loads in ASC-US, LSIL, and HSIL were significantly increased compared with NIL/M in both the qPCR and BSGP5+/6+/PCR/MPG assay (P < 0.0001, Mann–Whitney test; Fig. 3). Viral loads in LSIL and HSIL were also significantly increased compared with ASC-US in both assays (P < 0.0001). Viral loads between LSIL and HSIL did not differ in the qPCR and BSGP5+/6+/PCR/MPG assay (P = 0.97 and P = 0.29, respectively).

The risk of HSIL cytology by virological status (positive for HPV16, Hr-HPV, pHr-HPV, or high-load for HPV16, Hr-HPV, pHr-HPV) is shown in Table 1. For both assays, high viral load of HPV16 or any Hr-HPV showed a higher relative risk for prevalent HSIL compared with DNA positivity that was significant for BSGP5+/6+/PCR/MPG.

**Accuracy for clinical outcomes**

High viral load was significantly more frequent in women with CIN1, CIN2, or CIN3+ than in women with double-negative cytology or CINO (P < 0.0001; Fig. 4). Viral loads did not differ between CIN1 and CIN2+ in both the qPCR and BSGP5+/6+/PCR/MPG assay (P = 0.81 and P = 0.89, respectively).
Using the general high viral load cut off increased the specificity for absent disease strongly compared with HPV DNA positivity defined at the lowest cut off irrespective of the inclusion criteria used for specificity calculation (Table 2). At the same time, the sensitivity for CIN3+ remained constant at 100% for BSGP5+/6+-PCR/MPG and qPCR (Table 2). Compared with DNA positivity, Hr-HPV high viral loads could reduce the amount of FP results detected by the BSGP5+/6+-PCR/MPG by 81.4% and by 52.1% in qPCR (Table 2). Thus, high viral load detection using qPCR or BSGP5+/6+-PCR/MPG appeared to allow a very sensitive and specific diagnosis of CIN3+.

Discussion

In a cross-sectional study involving women participating in cervical cancer screening we evaluated clinical test characteristics of 2 quantitative HPV genotyping assays, the BSGP5+/6+-PCR/MPG (21, 22, 24) and qPCR (9, 19) using newly defined cut offs for high viral load. Compared with DNA positivity alone, high viral loads of HPV 16 but also from all other pooled Hr- and pHr-HPV types, were more predictive for cervical lesions and CIN2+/3+ than HPV detection alone. The specificity of the assays increased substantially using high viral load cut offs, although the sensitivity remained similarly high. In contrast to previous reports (25), a single measurement appeared to predict the presence of prevalent cervical lesions with high specificity and sensitivity, reducing the amount of HPV DNA false-positive women by up to 81% among women with NIL/M at baseline and follow-up.

Previous viral load studies did not address the problem of multiple infections (10). We developed an algorithm for treating multiple infections that takes into account only the Hr-HPV type with the highest viral load. Methodical differences for assessing viral load probably have led to
inconsistent data for non-HPV16 types (10, 26, 27). In our study, we showed the suitability of the BSGP5+/6+/PCR/MPG assay to not only genotype all Hr- and pHr-HPV types but also to quantify their loads in a single reaction. Compared with the 3 double and 4 triplex qPCR assays, both assays showed an excellent clinical sensitivity for CIN3+ and clinical specificity using the novel algorithm for treating multiple infections and the newly set high viral load cut offs. Although the high viral load assessment could be valuable to optimize clinical accuracy for cervical cancer precursors, low viral load/technical cut offs could be applied to the 2 assays to follow-up patients after treatment or to monitor the efficiency of vaccines. In addition, the detection of low copy numbers is interesting for epidemiology and when applying the test to less optimal sample material.

In countries with cervical cancer precursor screening programs, the incidence of cervical cancer has been reduced since introduction of the Papanicolaou (Pap) test in the 1970s. Despite this success, screening is far from perfect. The Pap test depends on subjective judgment of the degree of nuclear atypia. A single Pap test fails to detect cervical abnormalities in a substantial proportion of women with cervical precancer (28) and is characterized by low reproducibility (29, 30). Hr-HPV DNA testing is accepted as an adjunct modality to cytologic testing for women over 30 years of age in the United States (31). However, the high analytical sensitivity of newer HPV tests leads to the identification of additional transient low-load HPV infections. As a consequence, the specificity and positive predictive value (PPV) for lesions remains low. Viral load assessment, as described in this study, would be highly beneficial to the cervical cancer precursor screening, because it appears to detect cervical lesions with a sensitivity and specificity of more than 95%. Although it cannot discriminate between different grades of lesions, it would reduce substantially the number of false-positive women with no lesion. In practice, our data open possibilities to test women in primary screening with a validated quantitative HPV assay that preferably detects all Hr-HPV types. For those that test positive for high viral loads, reflex cytology or HPV RNA pattern detection (32) could be conducted to identify women with abnormal cytology who need direct referral for colposcopy-directed biopsy. In this way, up to 81% of Hr-HPV

<table>
<thead>
<tr>
<th>Table 1. Association of HSIL with high viral loads versus DNA positivity alone stratified for HPV16, Hr-, or pHr-HPV types</th>
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<tbody>
<tr>
<td><strong>Assay</strong></td>
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<tr>
<td>BSGP5+/6+/PCR/MPG</td>
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*aPositivity, positivity for at least one of the types; high load, above cut off for high viral load for at least one of the types analyzed; AGC and ASC-H excluded from analyses.

*bNumber of HPV types analyzed: no HPV, negative for the HPV type(s) analyzed; any of 13 pos., positive for any of the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 according to the types included in HC2; any of 15 pos., additional types 53 and 66 included in qPCR; any of 20 pos., additional pHr-HPV types 26, 53, 66, 67, 70, 73, and 82 included in BSGP5+/6+/PCR/MPG according to (33).
Table 2. Accuracy of BSGP5+/-PCR/MPG and the qPCR assay according to the number of targeted HPV types and viral load cut off

<table>
<thead>
<tr>
<th>PCR Outcome</th>
<th>No. of HPV</th>
<th>Sensitivity for CIN2+</th>
<th>Sensitivity for CIN3+</th>
<th>Specificity A</th>
<th>Specificity B</th>
<th>Reduction of FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSGP5+/-6+</td>
<td>13 types</td>
<td>By HC2</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>80.3 (640/797)</td>
<td>78.7 (648/823)</td>
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<td></td>
<td>14</td>
<td>By GP5+/-6+ EIA</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>79.8 (636/797)</td>
<td>78.3 (644/823)</td>
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<td></td>
<td>20</td>
<td>By IARC V. 100B</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>77.2 (615/797)</td>
<td>75.6 (622/823)</td>
</tr>
<tr>
<td>High load</td>
<td>13</td>
<td>By HC2</td>
<td>97.4 (74/76)</td>
<td>100 (49/49)</td>
<td>96.2 (756/797)</td>
<td>94.7 (797/823)</td>
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<td></td>
<td>14</td>
<td>By GP5+/-6+ EIA</td>
<td>97.4 (74/76)</td>
<td>100 (49/49)</td>
<td>96.2 (756/797)</td>
<td>94.7 (797/823)</td>
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<td></td>
<td>20</td>
<td>By IARC V. 100B</td>
<td>98.7 (75/76)</td>
<td>100 (49/49)</td>
<td>95.4 (760/797)</td>
<td>93.7 (771/823)</td>
</tr>
<tr>
<td>qPCR</td>
<td>13</td>
<td>By HC2</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>91.3 (728/797)</td>
<td>89.8 (739/823)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>By GP5+/-6+ EIA</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>91.1 (726/797)</td>
<td>89.6 (737/823)</td>
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<tr>
<td></td>
<td>15</td>
<td>By HC2</td>
<td>100 (76/76)</td>
<td>100 (49/49)</td>
<td>90.5 (721/797)</td>
<td>88.8 (731/823)</td>
</tr>
<tr>
<td>High load</td>
<td>13</td>
<td>By HC2</td>
<td>95.7 (753/797)</td>
<td>100 (49/49)</td>
<td>94.5 (757/797)</td>
<td>94.4 (777/823)</td>
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<td></td>
<td>14</td>
<td>By GP5+/-6+ EIA</td>
<td>95.7 (753/797)</td>
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<td>94.4 (777/823)</td>
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<tr>
<td></td>
<td>15</td>
<td>By HC2</td>
<td>95.6 (752/797)</td>
<td>100 (49/49)</td>
<td>94.2 (756/797)</td>
<td>94.2 (775/823)</td>
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*POSitivity, positivity for at least one of the types; high load, above cut off for high viral load for at least one of the types analyzed.

**Number of HPV types analyzed: 13 types, positive for any of the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 according to the types included in HC2; 14 types, positive for any of the 13 HPV types and HPV66 according to the types included in GP5+/-PCR/EIA; 15 types, additional types 53 and 66 included in qPCR; 20 types, additional pHr-HPV types 26, 53, 66, 67, 70, 73, and 82 included in BSGP5+/-PCR/MPG according to (33).

*Sensitivity (%, no. of TP/no. women with disease).

*Specificity A (%, no. of TN/no. women without disease) for identifying women with 2 consecutive negative cervical cytology results.

*Specificity B (%, no. of TN/no. women without disease) for identifying women with 2 consecutive negative cervical cytology results, or any women belonging to the 999 continuous screening samples with <CIN1 histology at follow-up.

*Reduction of FP using high viral load instead of positivity as outcome among women with 2 consecutive negative cervical cytology results (%, no. of FP “positivity”/no. of FP “high load”).

Figure 4. Comparison of viral loads for any Hr- and pHr-HPV type in CIN1, CIN2, CIN3+, and double-negative NIL/M at baseline and follow-up. For multiple infections, only the HPV type with the highest viral load is included. The grey horizontal lines represent the median and the interquartile range. High viral load cut offs for the respective assays are indicated by the dotted line. Negatives in qPCR and BSGP5+/-PCR/MPG were set to 10^-6. Note that, 14 Hr-HPV types comprising types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68a/b, and the pHr-HPV type 53 were included in both assay analyses. HPV types 26, 67, 70, 73, and 82 were additionally included for BSGP5+/-PCR/MPG analysis.
positive but healthy women could be saved from extensive follow-up, which would result in a significant increase in specificity and PPV of Hr-HPV testing for HSIL and CIN2+ in cervical screening programs.

A strong point of the study is that it is part of the VALGENT project which includes also the comparison of the 2 evaluated tests with the GP5+/6+ PCR EIA (14) on the same samples (13). The GP5+/6+ PCR (as well as the HC2 assay) is accepted as clinically validated because it is shown in well-conducted randomised controlled trials that Hr-HPV-negative women have a lower risk of developing CIN3 or cervical cancer compared with women with negative cervical cytology. The validation of BSGP5+/6+PCR/MPG as well as other HPV genotyping tests against these criteria will be reported separately. VALGENT incorporates a powerful study design, providing sufficient power to assess sensitivity of HPV tests for cervical precancer, avoiding testing of tens of thousands of screening samples and providing a clear and operational definition of the group used for computation of specificity (13).

Limitation of the study must be seen in the fact that histologic data was missing for some women lost to follow-up, and that cytology and histology was read during clinical routine without deliberately blinding the qPCR DNA data. The latter may have led to a favor for the qPCR assay, because cytologists, colposcopists, and histologists, aware of the knowledge of qPCR status may have influenced finding small regressive CIN2+, which is a form of overdiagnosis.

We are aware of the possibility that the use of the high viral load cut off may miss low-load but progressive Hr-HPV infections in women that will develop CIN3 before the next screening interval. However, this study design issue is also inherent to the current guidelines defining the minimal equivalency criteria for HPV tests that can be used in primary cervical cancer screening (14).

It will be important to compare the predefined high viral load cut offs with the performance of the GP5+/6+PCR/EIA assay, to see whether high viral load will fulfill the requirements of the guidelines for the clinical validation of a diagnostic Hr-HPV DNA test (5). Of interest, a general cut off could be applied to all Hr- and pHr-HPV types. In addition, varying DNA input in the PCR (median 43.2 ng/μL, range 1.5–284 ng/μL) seemed not to invalidate the cut off definition (data not shown). However, due to different synthesis batches of reagents, such as primers, probes, or PCR kits, quality controls and internal standards will be required to not only monitor a failure of the reaction but also to determine the efficiency of the whole process. By that, herein defined cut offs might have to be modified by using suitable plate controls to ensure optimal clinical performance.

In conclusion, quantitative type-specific HPV DNA assays show flexibility in defining thresholds and targeting HPV types, which could optimize clinical accuracy for cervical cancer precursors. The analysis of Hr-HPV DNA viral load offers a strong benefit on clinical specificity compared with presence or absence of HPV DNA in exfoliated cells. Although viral load cannot discriminate between low- and high-grade cervical lesions, it detects any cervical abnormality with a clinical sensitivity close to that of DNA detection assays. Therefore, it appears to be well justified to further validate viral load assessment and to explore their potential use in primary cervical screening.

Disclosure of Potential Conflicts of Interest

J. Bogers is employed (other than primary affiliation; e.g., consulting) in the University of Antwerp as a professor and is a consultant/advisory board member of Advisory. M. Pawlita and M. Schmitt have a commercial research grant from Qiagen and have ownership interest (including patents) and is listed on a DKFZ patent application to the European Patent Office (Europe patent application EP11161675.1). No potential conflicts of interest were disclosed by the other authors.

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References


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