Viral load of high-risk human papillomaviruses as reliable clinical predictor for the presence of cervical lesions

Running title: High viral load identifies cervical lesions

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Key words
Broad spectrum PCR, BSGP5+/6+-PCR/MPG, cervical cancer, human papillomavirus (HPV), Luminex, Multiplex HPV Genotyping, primary screening, real-time qPCR, sensitivity, specificity, viral load, VALGENT
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 over 7 orders of magnitude (on a log10 scale) in 999 consecutive BD-SurePath™ liquid-based cervical cytology samples from routine cervical screening enriched with ASC-US (n=100), LSIL (n=100) and HSIL (n=97) using type-specific multiplex real-time qPCR 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 to NIL/M in both the 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 cutoff for 14 Hr-HPV types, the sensitivity for prevalent CIN3+ remained at 100% for both assays compared to the minimal detection threshold. The specificity (corresponding to double negative cytology at subsequent screening episodes) increased substantially (qPCR, from 91.1 to 95.7%; BSGP5+/6+-PCR/MPG, from 79.8 to 96.2%).

Conclusions:
Compared to 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.
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 epidemiological association with cervical cancer: high-risk (Hr-HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), possibly high-risk (pHr-HPV: 26, 53, 67, 70, 73 and 82) and low-risk or undefined (Lr-HPV: e.g. 6, 11, 40, 42, 43 and 44) (3). 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 morphological 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 two clinically validated Hr-HPV tests Hybrid Capture 2 (HC2) and the GP5+/6+-PCR/EIA (5).

In order 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 semi-quantitative Hybrid Capture 2 (HC2) (7) and visual assessment of reverse line blot or dot blot signals (8), or truly quantitative singleplex or multiplex real-time quantitative PCR (qPCR) (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 demonstrate here the quantification ability of the BSGP5+/6+-PCR/MPG assay. The current paper 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 pathological 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: (a) 1,000 consecutive samples from women participating in cervical cancer screening and in addition (b) 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 histological verification, it is assumed, in the VALGENT protocol, that women, who have, at two 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 1st October 2006, 999 consecutive BD-SurePath liquid based cytology samples were collected during routine gynaecological 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 due to insufficient DNA material for PCR in Heidelberg (<5 µL).

**Cytology.** Thin-layer slide preparations were made with the fully robotic AutoCyte® PREP System (AutoCyte®; Tripath Imaging Inc.) and were prepared as described elsewhere (15). Cytology was read with prior knowledge of the qPCR HPV DNA data (16). The cytological results were classified according to the Bethesda system 2001 (4). Women with cytological 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 performed 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.

**Quantitative real-time 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, HPV53 E6, HPV56 E7, HPV58 E6, HPV59 E7, HPV66 E6, and HPV68 E7 as described recently (19). Real-time quantitative PCR for β-globin was used to verify the quality of DNA in the sample and to measure the amount of input DNA. Viral loads in each specimen were expressed as the number of HPV copies/cell as described (20).
BSGP5+/6+-PCR/MPG assay. The BSGP5+/6+-PCR/MPG assay comprises the BSGP5+/6+-PCR, which homogeneously amplifies all known genital HPV types generating biotinylated amplimers of ~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 analysed 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, Hilden, Germany). 0.2 to 0.5µM each of the BSGP5+ and 5´-biotinylated BSGP6+ primers, and 0.15µM 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 cutoff 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 PCR products are estimated to contain >10^6 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 non-descriptive viral load value (%HPVMFI / β-globin MFI).

BSGP5+/6+-PCR/MPG allowed the semi-quantitative analysis of HPV containing plasmid dilution series between 10 and 10,000 copies per PCR (21). Due to 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). In order 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 100ng/µL human placenta DNA (Supplementary Fig. S1). While HPV16 net MFI values reached the plateau phase with 1,000 to 10,000 copies per PCR, β-globin signals declined with increased HPV copy number input (Fig. S1). The HPV16/β-globin ratios, however, were able to quantify over 7 logs when β-globin primer concentrations between 0.1 and 0.2 µM were used.

**Statistical Analysis.** Statistical analyses were performed with the SAS software, version 9.2 (SAS Institute, Cary, NC, USA). Pairwise comparisons of the median viral loads in different groups were performed 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 two-sided, and P-values below 0.05 were considered statistically significant. The relation between viral load measurements performed with the two 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. 95 % confidence intervals 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 nine 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 correlations (p<0.0001) between the two 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 cutoffs for qPCR and BSGP5+/6+-PCR/MPG. Next we analysed 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 cutoff 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 cutoff 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 cutoff 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 cutoff values no significant differences in test accuracy (sensitivity, specificity and AUC) were observed between both methods.

Risk of cytological abnormality associated with viral load. The distribution of Hr-HPV loads assessed in baseline cervical samples by cytological category are depicted in scatter dot plots (Fig. 3). Using this approach, viral loads in ASC-US, LSIL and HSIL were significantly increased compared to
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 to 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 to 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 CIN0 (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 cutoff increased the specificity for absent disease strongly compared to HPV DNA positivity defined at the lowest cutoff irrespective of the inclusion criteria used for specificity calculation (Table 2). At the same time, the sensitivity for 49 CIN3+ remained constant at 100% for BSGP5+/6+-PCR/MPG and qPCR (Table 2). Compared to 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 two quantitative HPV genotyping assays, the BSGP5+/6+-PCR/MPG (21, 22, 25) and qPCR (9, 19) using newly defined cutoffs for high viral load. Compared to 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 cutoffs, while the sensitivity remained similarly high. In contrast to previous reports (26), 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, 27, 28). In our study, we demonstrated 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 to 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 cutoffs. While the high viral load assessment could be valuable to optimize clinical accuracy for cervical cancer precursors, low viral load/technical cutoffs could be applied to the two assays in order 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 CxCa has been reduced since introduction of the Papanicolaou (Pap) test in the 1970’s. Despite this success, screening is far from perfect. The Pap test depends on subjective judgement of the degree of nuclear atypia. A single Pap test fails to detect cervical abnormalities in a substantial proportion of women with cervical

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precancer (29) and is characterised by low reproducibility (30, 31). Hr-HPV DNA testing is accepted as an adjunct modality to cytological testing for women over 30 years of age in the United States (32). 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 over 95%. While 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 (33) could be performed to identify women with abnormal cytology who need direct referral for colposcopy-directed biopsy. In this way, up to 81% of Hr-HPV positive but healthy women could be saved from extensive follow-up, which would result in a significant increase in specificity and positive predictive value 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 two 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 since it is shown in well conducted RCTs that hrHPV negative women have a lower risk of developing CIN3 or cervical cancer compared to 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 ten 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 histological 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 favour for the qPCR assay, since
cytologists, colposcopists and histologists, aware of the knowledge of qPCR status may have
influenced finding small regressive CIN2+, which is a form of over-diagnosis.

We are aware of the possibility that the use of the high viral load cutoff 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 cutoffs with the performance of the
GP5+/6+-PCR/EIA assay, in order to see whether high viral load will fulfil the requirements of the
guidelines for the clinical validation of a diagnostic Hr-HPV DNA test (5). Of interest, a general cutoff
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 to 284 ng/µL) seemed not to invalidate the cutoff 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 cutoffs might have to be
modified by using suitable plate controls in order 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 to
presence or absence of HPV DNA in exfoliated cells. While 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.
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Author contributions

M.A. and M.P. initiated and coordinated the study. C.D. and I.B. provided extracted clinical specimens and performed the qPCR and cytology. J.B. performed histological and cytological analyses. M.S performed viral load experiments and cutoff definitions, and wrote the initial draft of the manuscript. M.S. and J.A. performed statistical analyses. All authors contributed and agreed to the final manuscript.

Potential conflict of interest

MS and MP were supported by Qiagen and CD by Genticel. MS and MP are listed on a DKFZ patent application to the European Patent Office (Europe patent application EP11161675.1).


34. IARC. Agents Classified by the IARC Monographs, Volumes 1–100. [cited; Available from: http://monographs.iarc.fr/ENG/Classification/ClassificationsGroupOrder.pdf


Table 1. Association of HSIL with high viral loads versus DNA positivity alone stratified for HPV16, Hr- or pHr-HPV types

<table>
<thead>
<tr>
<th>Assay</th>
<th>HPV status 1</th>
<th># of HPV types 2</th>
<th>NIL/M, ASCUS, LSIL (n=1163)</th>
<th>HSIL (n=110)</th>
<th>sum NIL/M, ASCUS, LSIL and HSIL.</th>
<th>HSIL in each HPV status (%)</th>
<th>Relative risk: high load vs positivity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSGP5+/6+-PCR/MPG</td>
<td>positivity</td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no hpv</td>
<td>719 (61.8)</td>
<td>1 (0.9)</td>
<td>720 (0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 pos.</td>
<td>111 (9.5)</td>
<td>49 (44.5)</td>
<td>160 (30.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 13 pos.</td>
<td>401 (34.5)</td>
<td>106 (96.4)</td>
<td>507 (20.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 20 pos.</td>
<td>444 (38.2)</td>
<td>109 (99.1)</td>
<td>553 (19.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high load</td>
<td>no hpv or low load</td>
<td>933 (80.2)</td>
<td>2 (1.8)</td>
<td>935 (0.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 pos.</td>
<td>33 (2.8)</td>
<td>37 (33.6)</td>
<td>70 (52.9)</td>
<td></td>
<td>1.73 (1.25 - 2.38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 13 pos.</td>
<td>206 (17.7)</td>
<td>101 (91.8)</td>
<td>307 (32.9)</td>
<td></td>
<td>1.57 (1.25 - 1.99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 20 pos.</td>
<td>230 (19.8)</td>
<td>108 (98.2)</td>
<td>338 (32.0)</td>
<td></td>
<td>1.62 (1.29 - 2.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td>positivity</td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no hpv</td>
<td>856 (73.6)</td>
<td>6 (5.5%)</td>
<td>862 (0.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 pos.</td>
<td>66 (5.7)</td>
<td>50 (45.5)</td>
<td>116 (43.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 13 pos.</td>
<td>287 (24.7)</td>
<td>104 (94.5)</td>
<td>391 (26.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 15 pos.</td>
<td>307 (26.4)</td>
<td>104 (94.5)</td>
<td>411 (25.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high load</td>
<td>no hpv or low load</td>
<td>925 (79.5)</td>
<td>7 (6.4)</td>
<td>932 (0.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16 pos.</td>
<td>35 (3.0)</td>
<td>39 (35.5)</td>
<td>74 (52.7)</td>
<td></td>
<td>1.22 (0.91 - 1.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 13 pos.</td>
<td>225 (19.3)</td>
<td>103 (93.6)</td>
<td>328 (31.4)</td>
<td></td>
<td>1.18 (0.94 - 1.49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>any of 15 pos.</td>
<td>238 (20.5)</td>
<td>103 (93.6)</td>
<td>341 (30.2)</td>
<td></td>
<td>1.19 (0.95 - 1.51)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 positivity: positivity for at least one of the types; high load: above cutoff for high viral load for at least one of the types analysed, AGC and ASC-H excluded from analyses.

2 number of HPV types analysed: no HPV: negative for the HPV type(s) analysed; 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 (34).
Table 2. Accuracy of BSGP5+/6+ PCR/MPG and the qPCR assay according to the number of targeted HPV types and viral load cutoff

<table>
<thead>
<tr>
<th>PCR outcome</th>
<th>nb. of HPV</th>
<th>sensitivity for CIN2+</th>
<th>specificity A</th>
<th>specificity B</th>
<th>reduction of FP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>BSGP5+/6+ positivity</td>
<td>13 types by HC2</td>
<td>100 (76/76)</td>
<td>80.3 (640/797)</td>
<td>78.7 (648/823)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 types by GP5+/6+ EIA</td>
<td>100 (76/76)</td>
<td>79.8 (636/797)</td>
<td>78.3 (644/823)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 by IARC V. 100B</td>
<td>100 (76/76)</td>
<td>77.2 (615/797)</td>
<td>75.6 (622/823)</td>
<td></td>
</tr>
<tr>
<td>high load</td>
<td>13 types by HC2</td>
<td>97.4 (74/76)</td>
<td>96.2 (767/797)</td>
<td>94.7 (770/823)</td>
<td>80.9</td>
</tr>
<tr>
<td></td>
<td>14 types by GP5+/6+ EIA</td>
<td>97.4 (74/76)</td>
<td>96.2 (767/797)</td>
<td>94.7 (770/823)</td>
<td>81.4</td>
</tr>
<tr>
<td></td>
<td>20 by IARC V. 100B</td>
<td>98.7 (75/76)</td>
<td>95.4 (760/797)</td>
<td>93.7 (771/823)</td>
<td>79.7</td>
</tr>
<tr>
<td>qPCR positivity</td>
<td>13 types by HC2</td>
<td>100 (76/76)</td>
<td>91.3 (728/797)</td>
<td>89.8 (759/823)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 types by GP5+/6+ EIA</td>
<td>100 (76/76)</td>
<td>91.1 (726/797)</td>
<td>89.6 (737/823)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 by IARC V. 100B</td>
<td>100 (76/76)</td>
<td>90.5 (721/797)</td>
<td>88.8 (731/823)</td>
<td></td>
</tr>
<tr>
<td>high load</td>
<td>13 types by HC2</td>
<td>100 (76/76)</td>
<td>95.7 (763/797)</td>
<td>94.4 (777/823)</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>14 types by GP5+/6+ EIA</td>
<td>100 (76/76)</td>
<td>95.7 (763/797)</td>
<td>94.4 (777/823)</td>
<td>52.1</td>
</tr>
<tr>
<td></td>
<td>15 by IARC V. 100B</td>
<td>100 (76/76)</td>
<td>95.6 (762/797)</td>
<td>94.2 (775/823)</td>
<td>53.9</td>
</tr>
</tbody>
</table>

a positivity: positivity for at least one of the types; high load: above cutoff for high viral load for at least one of the types analysed

b number of HPV types analysed: 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+/6+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+/6+-PCR/MPG according to (34)

c sensitivity (% nb. of TP/nb. women)

d specificity A (% nb. of TN/nb. women without disease) for identifying women with two consecutive negative cervical cytology results

e specificity B (% nb. of TN/nb. women without disease) for identifying women with two consecutive negative cervical cytology results, or any women belonging to the 999 continuous screening samples with <=CIN1 histology at follow-up

f reduction of FP using high viral load instead of positivity as outcome among women with two consecutive negative cervical cytology results (% nb. of FP “positivity”/nb. of FP “high load”)
Figure 1. Correlation of viral load determined by BSGP5+/6+-PCR/MPG and qPCR with Pearson (P) and Spearman (S) correlation coefficients, for HPV types 16, 18, 31, 33, 35, 39, 45, 51 and 52. Viral loads detected in single infections are depicted by white dots, and multiple infections by black dots. Negatives in qPCR and BSGP5+/6+-PCR/MPG were set to $10^{-4}$ and $10^{-5}$, respectively.
Figure 2. ROC curve analysis for detection of CIN3+ by BSGP5+/6+-PCR/MPG (A) and by qPCR (B).
Figure 3. Comparison of viral loads for any Hr- and pHr-HPV type in different cytological grades. For multiple infections, the HPV type with the highest viral load is plotted. AGC and ASC-H are excluded from analyses. The red horizontal lines represent the median, and the interquartile range. The high viral load cutoff is indicted by the dotted line. Negatives in qPCR and BSGP5+/6-PCR/MPG were set to $10^{-6}$. 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 the analysis both assay analyses. HPV types 26, 67, 70, 73 and 82 were additionally included for BSGP5+/6+-PCR/MPG analysis.
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 horizontal lines represent the median and the interquartile range. High viral load cutoffs for the respective assays are indicated by the dotted line. Negatives in qPCR and BSGP5+/6+-PCR/MPG were set to $10^6$. 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+/6+-PCR/MPG analysis.
Figure 1

- HPV16
  - single infection: P: 0.58 (<.0001)
  - multiple infection: S: 0.82 (<.0001)

- HPV18
  - P: 0.99 (<.0001)
  - S: 0.78 (<.0001)

- HPV31
  - P: 0.40 (<.0001)
  - S: 0.86 (<.0001)

- HPV33
  - P: 0.53 (<.0001)
  - S: 0.66 (<.0001)

- HPV35
  - P: 0.72 (<.0001)
  - S: 0.82 (<.0001)

- HPV39
  - P: 0.42 (<.0001)
  - S: 0.81 (<.0001)

- HPV45
  - P: 0.65 (<.0001)
  - S: 0.26 (<.0001)

- HPV51
  - P: 0.14 (<.0001)
  - S: 0.82 (<.0001)

- HPV52
  - P: 0.10 (<.0002)
  - S: 0.83 (<.0001)

qPCR (copies / cell)

- qPCR (copies / cell)
- qPCR (copies / cell)
Figure 3

Viral load (copies/cell)

Viral load (%HPV-MFI/10^6 cells MFI)

NILM
ASC-US
LSIL
HSIL

qPCR

BSGP5+6+PCR/MFG
Figure 4

Viral load (copies/cell) vs. viral load (% HPV AVER/MFI).
Viral load of high-risk human papillomaviruses as reliable clinical predictor for the presence of cervical lesions

Markus Schmitt, Christophe E Depuydt, Ina Benoy, et al.

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