Comparison of Seegene Anyplex II HPV28 with the PGMY-CHUV Assay for Human Papillomavirus Genotyping

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The Anyplex II HPV28 (H28; Seegene) is a new semiquantitative real-time multiplex PCR assay for screening and genotyping 28 human papillomaviruses (HPV) in only 2 reaction wells. H28 was compared to the PGMY-CHUV assay (PG) with 309 archival DNA samples from cervical smears collected over 8 years in our laboratory. H28 and PG were fully concordant at the genotypic level on 228 (73.8%) out of 309 samples: 27 HPV negative and 201 HPV positive. The 201 fully concordant positive samples corresponded to single infections (n = 145) and to multiple infections (2 genotypes, n = 38; 3 to 5 genotypes, n = 18). The remaining 81 samples (26.2%) were either partially concordant (n = 64, 20.7%) or fully discordant (n = 17, 5.5%). While genotype-specific agreement was nearly perfect (κ = 0.877), HPV51 was significantly less well detected by H28 and the converse was observed for HPV40, -42, -54, and -68. Sequencing of PG amplicons confirmed HPV51 discordants and suggested the involvement of a possibly local HPV51 subtype. Mismatches in the PGMY09 primers to HPV68a explained most of the HPV68 discordants, confirming the specificity of H28 toward HPV68. With PG as a reference, the sensitivity and specificity of H28 were 93.4% and 99.0%, respectively. Considering H28 as a reference, the sensitivity and specificity of PG were 83.8% and 99.6%, respectively. H28 is a very sensitive and specific HPV genotyping assay suitable for research and clinical use as an adjunct to a clinically validated test. H28 semiquantitative readout ought to be evaluated for primary cervical cancer screening.

High-risk human papillomaviruses (HPV) are the causative agents of cervical cancer (1, 2). Molecular detection of high-risk HPV in cervical smears therefore is used as an adjunct to cytology to identify women at risk for cervical cancer (3–5). Assays that have been clinically validated identify high-risk genotypes as a whole (the hybrid capture II [HCII] assay) or distinguish HPV16 or HPV18 from the other high-risk types as a group (HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) (Abbott Real-Time high-risk HPV and Roche Cobas 4800 HPV tests). The Abbott and Roche tests have been validated against HCII using cervical intraepithelial neoplasia (CIN) grade 2 or higher as endpoints (6–8). They were also found in these studies to be suitable for primary cervical cancer screening according to published guidelines (9).

In contrast to the clinical application of partial genotyping exemplified by the Abbott and Roche assays mentioned above, comprehensive HPV genotyping is essential in epidemiology and for vaccine surveillance (10). Full HPV genotyping is also useful clinically to identify patients with persisting, type-specific high-risk HPV infections which are known to confer a higher risk of cancer progression than incident infections by the same high-risk type (11). Many published HPV genotyping assays rely on endpoint multiplex PCR followed by reverse hybridization against a panel of type-specific probes immobilized on membranes or beads (12–19). They target at least 25 genotypes: the 14 high-risk HPV genotypes, indicated above, as well as low-risk (HPV6, -11, -42, -44, -53, -54, and -70) or potentially high-risk (HPV26, -69, -73, and -82) genotypes. These assays, including ours, are time-consuming and necessitate some degree of expertise (20). Owing to their ease of use and objective readout, real-time PCR assays capable of detecting a higher number of HPV genotypes are desirable. Such coverage by real-time PCR is, however, challenging. Thus, published multiplex real-time PCR assays for HPV genotyping need at least 6 different PCR wells per sample to overcome the limitations of present chemistries and equipment, which distinguish at best 3 to 5 targets per well (21).

Seegene recently commercialized the real-time PCR Anyplex II HPV28 kit (H28). H28 is aimed at genotyping 28 HPV in only 2 PCR wells per sample. This system therefore has the potential to greatly simplify HPV genotyping. For this reason, we decided to compare H28 with our in-house PGMY-CHUV (PG) assay described in Chapter 5 of the WHO HPV Laboratory Manual (10). PG has been found to be highly comparable to the commercially available linear array (LA; Roche) with some advantages in terms of sensitivity and specificity toward HPV56 and HPV52 (20). PG has also been repeatedly proficient in several laboratories within the WHO HPV Laboratory Network at frequencies at least as good as those of LA (22).

We show here that the present version of H28 overall is more sensitive than and as specific as PG. Among the high-risk genotypes, H28 detected significantly fewer cases of HPV51, possibly in a locally prevalent and subtype-dependent manner, and more cases of HPV68. The latter is a known consequence of the inability of the PGMY primers to efficiently target the HPV68a subtype (23). Supplementing PG with an additional set of an HPV68a-specific primer/probe corrected this defect, confirming the specificity of H28 toward HPV68.
MATERIALS AND METHODS

Samples. (i) Cervical smear DNA. A total of 298 DNA samples from our biobank were selected from all gynecological patients who submitted more than one cervical smear to our laboratory from May 1999 till December 2012 and who tested HPV positive at least once. An additional subset of 14 samples was also randomly selected among HPV-negative women with atypical squamous cells of undetermined significance (ASC-US). These DNA samples had been purified using MagNApure chemistry (Roche, Rotkreuz, Switzerland) as previously described (20).

A single sample was evaluated from each patient to increase genotype/subtype diversity. Out of the 312 samples, 3 were lost during storage, so 309 samples (99%) were left to be compared. These 309 samples represented 162 ASC-US, 80 low-grade squamous intraepithelial lesion (LSIL), 53 normal, and 14 high-grade squamous intraepithelial lesion (HSIL) samples. This mode of selection enriched for positives to ensure sufficient numbers of individual genotypes for statistical analysis.

(ii) WHO quality control panel. The 2011 WHO HPV proficiency panel was provided by EQUALIS AB (Uppsala, Sweden). It consisted of 43 DNA samples and of 3 cell samples. Only the DNA samples were tested with H28. They corresponded to single and to multiple infections containing 5 to 500 viral genome equivalents per reaction (5 μL DNA per reaction). The genomes represented in this panel were HPV6, -11, -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68a, and -68b.

Ethical Issues. This study was approved by our local ethics committee.

PGMY-CHUV assay (PG). PCR using the PGMY primers and reverse blotting hybridization (RBH) were performed as described in Chapter 5 of the WHO HPV Laboratory Manual and its validation study with 5 μL DNA in a single 50-μL PCR mixture containing 3 mM MgCl2 (10, 20). This assay allows genotyping of 31 HPV (6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 66, 68 [ME180, b subtype], 69, 70, 73, 82, 83, and 84).

Version 2 of PG (PGv2) was designed to resolve HPV68-discordant cases. Compared to PG, it uses an additional HPV68a-specific primer (RSMY09-L, 5'-CGTCCTAATGGGAATTGGTC-3'). This assay allows genotyping of 31 HPV (6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 66, 68 [ME180, b subtype], 69, 70, 73, 82, 83, and 84).

Anyplex II HPV28. H28 was performed as recommended by the manufacturer (Seegene, Seoul, South Korea) with 5 μL DNA in each of the two 20-μL reaction mixtures with primer set A or B. H28 uses HPV-specific dual priming oligonucleotides (DPO) for multiplex (real-time) PCR (24).

In addition, it uses a primary HPV type-specific probe (pitcher) carrying an artificial 5'-extension and a secondary, artificial, fluorescently labeled probe (catcher) specific to each pitcher. For a given HPV, the catcher is activated by the pitcher’s 5’ extension oligonucleotide upon its release from the bound pitcher by the exonucleolytic activity of the polymerase.

The 5’ extension is complementary to the 3’ end of the matched catcher. Its binding to the catcher primes DNA synthesis by the polymerase and conversion of the single-stranded catcher to a double-stranded form with concomitant release of quenching and emission of fluorescence. The catchers are labeled with a common fluorophore for a given set of 3 genotypes. Each catcher, and hence its corresponding HPV, can be distinguished from the others sharing the same fluorophore by melting curve analysis at 3 specific steps during the real-time PCR. Since the catchers are totally artificial, their melting temperatures can be well defined and attributed to the HPV type that each catcher represents. This design constitutes the TOCE system, which has already been implemented for the detection of respiratory viruses (25). In addition, knowledge of the step at which the melting curve becomes positive allows us to semiquantify the viral load, from low (+; positive after 40 PCR cycles), to intermediate (+; positive within 31 to 39 cycles), to high (++; positive before 31 cycles). H28 thus distinguishes semiquantitatively 28 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, and 82) in only 2 reactions by taking advantage of the 5 dyes that can be resolved on the CFX96 real-time PCR instrument (Bio-Rad; provided together with the H28 kits by BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Data recording and interpretation were automated with the Seegene viewer software according to the manufacturer’s instructions.

DNA sequencing and phylogenetic analysis. HPV51- and HPV68-discordant cases were assessed by DNA sequencing of PG amplicons and comparison to the nonredundant nucleotide acid GenBank database as described in Estrade et al. (20). DNA sequencing was also performed with the HB0M1 primer for HPV51 (13).

Phylogenetic analysis of HPV51 PG amplicons was performed with the tree builder tool of Geneious (Geneious version 7.0.5; Biomatters LTD, New Zealand) using an HPV16 PG amplicon (GenBank accession number AU003027) as an outgroup with 1,000 replicates. The noninformative PGMY primer sequences were removed from the amplicon sequences prior to alignment and tree building.

RESULTS

Evaluation of H28 with the 2011 quality control panel from WHO. All 43 DNA samples were correctly identified by H28 (not shown; sensitivity and specificity of 100%). H28 therefore was proficient for the genotyping of the 17 HPV types and subtypes represented in this panel, including those at the lowest concentration of 5 to 50 viral genome equivalents per reaction. Two genotype-sample combinations were missed by PG in comparison (HPV18 at 5 copies per reaction, single infection; HPV31 at 50 copies per reaction, multiple infections). PG was not proficient only for HPV68a, which cannot be efficiently detected with the PGMY primers (23).

Evaluation of H28 with PG. After restricting the analysis to the 26 genotypes shared by H28 and PG, 293 samples out of 309 were concordantly screened by both assays as HPV positive (n = 266) or as HPV negative (n = 27), and 16 were discordant (agreement = 94.8%; k = 0.742, considered strong) (Table 1). A total of 282 samples were recorded positive by either assay. Of them, 201 (71.3%) were fully concordant at the genotypic level, even with multiple infections containing up to 5 genotypes (single infections, n = 145 samples; double infections, n = 38 samples; 3 to 5 genotypes, n = 18 samples), 64 were partially discordant (double infections, n = 32 samples; 3 to 8 genotypes, n = 32 samples), and 17 were fully discordant. Among the 17 fully discordant samples, 6 H28-negative sam-
samples were recorded as single infections by PG (HPV51, n = 4 samples; HPV53 and HPV70, n = 1 sample each), 10 PG-negative samples were recorded as single infection (n = 8 samples; HPV18, -40, -45, -53, -54 twice, -56, -68) or double infections (n = 2 samples; HPV16 and -53, HPV42 and -68) by H28, and 1 was fully discordant among the 266 H28/PG-positive samples (HPV82 with PG and HPV59 with H28).

The genotype-specific comparison of H28 with PG is presented in Table 2. While the agreement was nearly perfect (total, 98.71%; \( \kappa = 0.877 \)) considering all 26 genotypes, there was a statistically significant higher number of genotype-sample combinations found by H28 that were not found by PG (n = 76) than of those found by PG and not by H28 (n = 28, \( P < 0.0001 \)). This was contributed mainly by the low-risk viruses (total L, 7 versus 38, \( P < 0.0001 \), agreement = 98.18%, \( \kappa = 0.826 \)) in comparison with the high-risk viruses (total H, 21 versus 38, \( P = 0.0372 \), agreement = 98.94%, \( \kappa = 0.899 \)). Consistent with this observation, the proportion of concordant genotype-sample combinations from the high-risk group (280/339) was significantly higher (\( P = 0.0065 \), by Fisher’s exact test) than from the low-risk group (114/159). Individually, the majority of genotypes exhibited agreement values above 99%, except HPV40, -42, -51, -53, -54, -56, -58, -66, -70, and -71.

### TABLE 1 Comparison of H28 and PG for screening HPV-positive and HPV-negative cases

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Risk</th>
<th>Total no. of samples</th>
<th>% agreement</th>
<th>SD</th>
<th>Int.</th>
<th>McNemar value</th>
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<td>NEG</td>
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<tr>
<td>NEG</td>
<td>27</td>
<td>10</td>
<td>93.4%</td>
<td>0.084</td>
<td>np 1.000</td>
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<tr>
<td>POS</td>
<td>6</td>
<td>266</td>
<td>99.6%</td>
<td>0.074</td>
<td>np 1.000</td>
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<tr>
<td>Total</td>
<td>33</td>
<td>276</td>
<td>98.71%</td>
<td>0.012</td>
<td>np 1.000</td>
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*PG, PGMY-CHUV; H28, Anyplex HPV28; NEG, negative; POS, positive. A test was considered HPV positive if it was positive for at least one of the 26 genotypes shared by both assays (see Materials and Methods). Agreement, 293/309 (~94.8%; \( \kappa = 0.742 \)) considered as a strong agreement; \( P = 0.453 \) (McNemar).

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### TABLE 2 Genotype-specific comparison of H28 and PG

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Risk</th>
<th>No. of genotype-sample combinations</th>
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*Only the 26 genotypes shared by both assays were considered for analysis.

\( a \) L, low risk; H, high risk.

\( b \) \(-/-\), negative with both assays; \(-/+\), H28 negative and PG positive; \(+/-\), H28 positive and PG negative; \(+/+\), positive with both assays.

\( c \) Interpretation of the kappa values. we, weak; st, strong; np, near perfect; pe, perfect.

\( d \) \( P < 0.05 \), two-sided McNemar’s test.

\( e \) The total number of samples was 309, giving 8,034 genotype-sample combinations. PG, PGMY-CHUV; H28, Anyplex HPV28. Sensitivity of H28 using PG as a reference, 394/422 = 93.4%; specificity of H28 using PG as a reference, 7,536/7,612 = 99.0%; sensitivity of PG using H28 as a reference, 394/470 = 83.8%; specificity of PG using H28 as a reference, 7,336/7,564 = 99.6%.
DISCUSSION

Overall, H28 performed very similarly to PG. However, H28 detected more positives, suggesting that it truly is more sensitive than PG. This conclusion is supported by H28 correct identification of all genotypes even at the lowest concentration (5 to 50 viral genome equivalents per reaction) in the 2011 WHO proficiency panel samples and in the 2013 panel (S. A. Nordbø, personal communication). Although it was proficient with both panels, PG was in comparison less sensitive in 3 samples involving HPV18 (5 genome equivalents, 2011 panel), HPV31 (50 genome equivalents, 2011 panel), and HPV56 (50 genome equivalents, 2013 panel).

Higher sensitivity rather than lower specificity of H28 was also supported by its not recording systematic double infections involving the same genotypes during this work. Based on these results, we concluded that the H28-positive/PG-negative results were indeed true positives. It therefore seemed reasonable to also consider H28 as a reference against which to compare PG. Under this condition, the sensitivity of PG was lower, at 83.8% (versus 93.4% for H28 using PG as a reference), and the specificity was slightly higher, at 99.6% (versus 99.0%). Enrichment of samples from patients known to have been positive at least once by PG introduced a selection bias that may have lessened the difference in sensitivity between H28 and PG. This selection bias was minimal, however, considering that more than 67% of the genotype-sample combinations (data not shown) were confirmed as HPV positives independently of the assay.

The reevaluation of the HPV51 discordants confirmed our results except for one case, which could not be amplified on retesting at Seegene using different PCR assays. This sample (R50) was recorded as weakly positive by PG with stochastic amplification. In contrast, the remaining 3 HPV51 subtypes from discordant single infections clustered phylogenetically and exhibited mismatches affecting the H28 primers/probe (Seegene, personal communication). These HPV51 subtypes were not represented in nucleic acid databases. They therefore were likely to be suboptimally detected by H28, whose sensitivity relies on type-specific primers and probes strictly adapted to published sequences (25). It remains to be determined, however,
whether the H28 false-negative HPV51 subtypes are specific to our study population or distributed worldwide.

The PGMY primers used in PG are degenerate to identify potentially unknown genotypes (13). Broad-range detection benefits from this approach at the cost of sensitivity, especially with low viral loads in multiple infections (20). The trends for discordance with low viral loads and with multiple-infection status between PG and H28 regarding HPV40, -42, and -54 are in line with PGMY degeneracy. At the extreme, the lack of sensitivity of PGMY-based assays toward HPV68a is due to three mismatches within the PGMY09-L primer, as determined by DNA sequencing of a rare HPV68a PG-positive case from our biobank. This observation allowed designing the HPV68a-specific RSMY09-L primer used in PGv2. PGv2 is currently under prospective validation to ensure that RSMY09-L does not negatively affect the detection of other genotypes. At the time of manuscript submission, PGv2 was not inferior to PG for HPV genotyping (n = 315 samples, of which 7 contained HPV68a). PGv2 was, in addition, fully proficient with the 2013 WHO quality control panel, including HPV68a (not shown). The limitation of PGMY-based HPV genotyping assays regarding HPV68a should be taken into account for data evaluation comparing the pre- and postvaccination eras, especially when addressing type replacement possibly involving HPV68a.

H28 thus appears very efficient for epidemiological studies using fresh cervical smears. Like PG, it is, however, too sensitive for clinical purposes, if not used as an adjunct to a clinically validated assay. The locations of the H28 primers/probes within the HPV genomes are Seegene’s proprietary information. It therefore is impossible to predict whether this assay would be suitable for epidemiological studies using fixed and paraffin-embedded high-grade tumor material, since sensitivity may be affected by the size and genomic location of the amplicons (26). Validation of H28 with such samples therefore would be of interest. Clinical evaluation of H28 with CIN2 and more (CIN2+) or CIN3+ endpoints would also be of interest, because the semiquantitative readout that its technology allows may be suitable for primary cervical cancer screening using clinically relevant HPV genotype-specific detection thresholds (27). If feasible, H28 would address not only HPV type-specific persistence but also clinically relevant infections.

In conclusion, H28 is a very sensitive and specific HPV screening and genotyping assay which may simplify and standardize HPV genotyping. It requires less manpower and expertise than PG or other assays based on reverse hybridization and could be used for large-scale testing thanks to its automation capacity and objective reading.

ACKNOWLEDGMENTS
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The H28 kits and the Bio-Rad CFX96 real-time PCR instrument were provided by Bühlmann Laboratories for the duration of the study. All other costs were covered by R.S.’s own funding.

P. Shaw critically reviewed the manuscript.

We have no conflicts of interests related to BÜHLMANN Laboratories or to Seegene.

ADDENDUM IN PROOF
Seegene now offers a research-use-only (RUO) kit for detection of the HPV51 subtypes that were missed by H28.

REFERENCES

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