Evaluation of the Performance of the Novel PapilloCheck® HPV Genotyping Test by Comparison With Two Other Genotyping Systems and the HC2 Test

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The novel PapilloCheck® genotyping test was compared with SPF10 PCR LiPav1 and PGMY09/11 on hybrid capture 2 (HC2)-pretested samples. From results of 826 cervical samples detection rates and kappa values for the tests were calculated using a HPV type consensus definition. With PapilloCheck® HPV types 53, 56, and 33 were found with a sensitivity of 100%. The lowest detection rate was observed for HPV 35 (72.2%). The SPF10 PCR LiPav1 was found to be 100% positive for HPV 18, 31, 53, 56, and 35 and lowest for HPV 59 (81%). The PGMY09/11 system detected only HPV 59 at 100% detection rate and showed lowest sensitivity for HPV 56 (40.5%). Multiple infection rates ranged from 25.8% (PGMY09/11 PCR-LBA), over 39.5% (PapilloCheck®) to 55.9% (SPF10 PCR LiPav1). In samples with higher viral DNA load detection rates and concordance between the genotyping tests increases. The kappa values in comparison to the HPV consensus type ranged from $k = 0.21$ to $k = 0.82$ for comparing SPF10 PCR with the HPV consensus type, while values for PGMY09/11 PCR ranged from $k = 0$ to $k = 0.96$ and were best for the PapilloCheck® ($k = 0.49$–0.98). Detection rates for the identification of high-grade cervical intraepithelial neoplasia (CIN2+) ranged from 93.7% (PGMY09/11 PCR) to 98.4% (PapilloCheck®, SPF10 PCR, HC2). In conclusion, this study shows that the PapilloCheck® give comparable results to established PCR methods. However, these results also show a necessity for the standardization of genotype-specific HPV detection assays. ***J. Med. Virol. 82:605–615, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: PapilloCheck; papillomavirus; cervical cancer; genotype test; HPV

INTRODUCTION

Cervical cancer remains the second most frequent female malignancy in the world. Molecular and epidemiologic studies have clearly demonstrated that a persistent infection with HPV is a necessary risk factor for the development of cervical intraepithelial lesions and invasive carcinoma, whereas the absence of HPV infection reduces the risk to a minimum [Ho et al., 1995; Walboomers et al., 1999; Bosch et al., 2002; Clifford et al., 2003].

Consequently the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 have been classified as class I carcinogens for humans by the IARC [International Agency for Research on Cancer Lyon, Cogliano et al., 2005]. In addition four types were described with proposed high-risk potential (53, 68, 73, 82; and seven HPV types are considered as low-risk types [6, 11, 40, 42, 43, 44/55, 70; Munoz et al., 2003]. Since the causal link between the infection of high-risk HPVs and cancer development has been well established [Bosch et al., 2002], there is a general consensus among the HPV research community that the addition of HPV DNA testing could increase the efficacy of the present cytological screening programs. [Cuzick et al., 2006; Kjaer et al., 2006; Schiffman et al., 2007; Dillner et al., 2008].

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At present the hybrid capture 2 (HC2) system (Qiagen GmbH, Hilden, Germany), Gaithersburg MD, USA) assay and the Cervista test (Hologic, Madison, WI) is the only test for the detection of HPV DNA approved by U.S. Food and Drug Administration for cervical cancer screening. The HC2 assay uses an RNA probe cocktail to detect 13 high-risk and 5 low-risk HPV types; however, the test does not distinguish individual genotypes and cannot identify infection with multiple genotypes [Iftner and Villa, 2003].

Recent studies have, however, provided firm evidence for a difference in the carcinogenic potential between the different high-risk HPVs [Bulkmans et al., 2005; Castle et al., 2005; Lai et al., 2007], The independent prognostic significance of HPV 18 positivity in early-stage cervical cancer was confirmed [Lai et al., 2007] and the prevalent HPV 16 infection (HPV 16+) was associated with a very high absolute risk of cervical intraepithelial neoplasia 3 (CIN3) over a 2-year period [Castle et al., 2005]. These and other studies point to the importance of a reliable HPV genotyping test in addition to the high-risk HPV-positive or -negative screening. Similarly, clinical trials conducted to test the efficacy of prophylactic vaccines that target two carcinogenic HPV types, HPV 16 and HPV 18 as well as the low-risk HPV 6 and 11, require accurate detection of type-specific HPV infections associated with cancer and precancerous lesions.

The most widely used PCR-based protocols employ consensus primers that amplify a highly conserved region of the L1 gene, and are potentially capable of detecting all mucosal HPV types [Bernard et al., 1994; Iftner and Villa, 2003]. Among these are the single pair of consensus primers GP5+/6+ [Jacobs et al., 1997] and the MY09/11 degenerate primers [Manos et al., 1989] and their multi-primer derivatives, the PGMY09/11 primers [Gravitt et al., 2000].

Recently, a novel CE-marked test based on the detection of the E1-region has been made commercially available (PapilloCheck®) that solves the problem of multiple HPV type-detection by using a low-density microarray format. The PapilloCheck® (Greiner Bio-One GmbH, Frickenhausen, Germany) PCR test amplifies a 350 bp fragment of the E1 ORF. The amplified products are then hybridized to specific DNA probes fixed on a DNA-chip. The assay allows the simultaneous identification of 18 high-risk/probable high-risk and 6 low-risk HPV types. Information on the performance of this novel test system in clinical trials or practice is scarce [Kovács et al., 2008; Jones et al., 2009]. We therefore attempted to evaluate the performance of the PapilloCheck® test system with a defined HC2-pretested cervical sample set described before [Klug et al., 2008] by comparison with two other genotyping methods.

A number of studies comparing different methods for HPV typing applied to a common sample set already noted considerable differences in the type specific sensitivities as well as the ability to detect multiple infections between the individual test systems. GP5+/6+ PCR was described to have a 1,000-fold lower sensitivity to detect HPV 53 and 61 than MY09/11 PCR and vice versa the latter one for HPV 35 [Qu et al., 1997]. A comparison of the PGMY09/11 reverse line blot with the SPF10-PCR test revealed a higher detection rate for HPV 42, 56, and 59 for the PGMY09/11 test, whereas the SPF10-PCR test detected significantly more HPV 31 and 52 [van Doorn et al., 2002].

Such test differences can significantly affect conclusions drawn from epidemiologic surveys. Therefore, care has to be taken when performing an epidemiological analysis to define type-specific prevalence rates or establishing probe cocktails for screening tests and the performance of the different HPV typing methods has to be taken into account when interpreting the results. In addition PCR-based HPV tests are difficult to design to allow the setting of a cut-off point. This is decisive if such a test can be used as a stand-alone HPV test in cervical cancer screening as it needs a clinical sensitivity comparable to the HC2 test. Tests with a higher sensitivity for the detection of HPV DNA than the HC2 test will detect a large number of latent infections that are clinically irrelevant and lead when used not in combination with a pre-screening test with a clinically defined cut-off to an overtreatment of women participating in cervical cancer screening [Snijders et al., 2003].

In this study we tested the performance of the PapilloCheck® HPV genotyping test in comparison to the PGMY09/11 PCR and LiPav1 methods on 826 samples. Analytical detection rates and kappa values for the three genotyping assay were calculated. Moreover, detection rates in cases and controls were calculated for 306 patients, from whom histological results were available.

MATERIALS AND METHODS

Study Population

Cervical smears of 881 women were selected from two large cohort studies of Germany [n = 609; Petry et al., 2003; Klug et al., 2007] and Denmark [n = 272; Kjaer et al., 2006]. The samples were chosen to be able to optimally compare the performance of the different test systems. Besides the HC2 positive group of samples (87%) the HC2-negative samples represented largely "problematic" samples that either had abnormal results by cytology or colposcopy or the HC2 result was in between 0.7 and 1.0 RLU/CO. The latter one represents samples that are weakly HPV positive but minimally below the cut-off of the HC2 test, which is a clinically defined cut-off and not the actual threshold of the HC2 analytical sensitivity for detection of HPV DNA. All cervical samples were collected in STM (HC2 sample collection device kit, Qiagen GmbH) and denatured for the following HC2 test using the HR probe after which the DNA was extracted within 2 months and the samples were stored again at −20°C.

DNA Extraction

From 200 µl of the denatured STM sample DNA was extracted with phenol–chloroform in an elution volume of 100 µl. DNA extraction and all pre- and post-PCR procedures were carried out in separate rooms and cabinets. Buffer and blanc controls were included in the extraction protocol to monitor contamination events. All samples were tested for integrity of DNA by using the PapilloCheck® HPV genotyping test sample control function and by testing for the presence of the β-globin gene using the PGMY09/11 PCR line blot assay (LBA).

HPV Typing

For HPV typing three different PCR-based tests were used: PapilloCheck® (Greiner Bio-One GmbH) the premarket version of the HPV linear array (PGMY09/11 PCR LBA, Roche Molecular Diagnostics, Somerville, CA) and SPF10 LiPav1 (DDL, Leiden, Netherlands). While two assays aim to identify HPV types by amplifying the L1 gene (PGMY09/11 LBA and the SPF10 LIPA), PapilloCheck® detects a specific region at the 3′-end of the E1 gene. The premarket version of the HPV linear array (PGMY09/11 PCR LBA, Roche Molecular Diagnostics) detects 38 different HPV types, the SPF10 LiPav1 (DDL, Leiden) detects 25 different HPV types and the PapilloCheck®(Greiner Bio-One GmbH) detects 24 different HPV types (see below).

The consensus primers used for the SPF10-PCR assay [Kleter et al., 1998, 1999] amplify a smaller fragment (65 bp, compared to 150 bp for the GP primers and 450 bp for MY09/11 and PGMY primers) of the L1 gene, which increases the sensitivity of the test. With the help of these primers HPV DNA present in the patient sample can be amplified and the type be identified by reverse blotting against type specific oligonucleotides. Another procedure the HPV DNA Chip [Hwang et al., 2003] uses GP5+/6+ primers to amplify the HPV DNA in the presence of fluorescence labeled nucleotides, which is subsequently hybridized to type-specific oligonucleotides immobilized on glass slides and the resulting hybrids are detected by scanning with a laser device. In addition a number of consensus PCRs have been developed to amplify highly conserved regions of the L1 or E1 gene followed by cycle sequencing to identify the type. The latter methods have considerable limitations in detecting multiple HPV types in one sample [Klug et al., 2008].

Hybrid Capture 2 (Qiagen GmbH)

The high-risk probe cocktail of the HC2 test (Qiagen GmbH), which detects at least the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and other types due to cross reactivity [Castle et al., 2002] was used to test all samples following the manufacturer's instructions. Samples were considered positive, when they attained or exceeded the FDA-approved threshold of 1.0 pg HPV DNA/ml, which corresponds to 1.0 relative light units (RLU/CO).

PGMY09/11 PCR Line Blot Assay (Roche Molecular Diagnostics)

The premarket version of the HPV Linear Array [PGMY09/11 PCR LBA; Roche Molecular Diagnostics; Gravitt et al., 2000] recognizes the following HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 81, MM9 = 73, MM4 = 82, MM7 = 83, MM8 = 84, IS39, and CP6108 (cand.83). The oligonucleotide primer mix for the PCR contains five forward and 13 backward non-degenerated, biotinylated consensus oligonucleotide primers. The average size of the amplified HPV sequence is 450 bp. As sample control, the primer pair B_PC04 and B_GH20 for the detection of β-globin is used. The hybridization strips and the required buffers and detection solutions were provided by Roche Molecular Systems, Inc. (Somerville, CA).

All PCR reactions were performed with 10 µl input DNA in a final volume of 100 µl and 7.5 Units of the hot start AmpliTaq Gold polymerase (Roche Molecular Systems, Inc.). The activation step of the enzyme for 9 min at 95°C, was followed by 40 cycles of 1-min denaturation at 95°C, 1-min primer annealing at 55°C and 1 min of primer extension reaction at 72°C. The PCR was carried out on a Perkin Elmer PCR system 9600 (PerkinElmer, Waltham, MA) as this test system was established for this device.

The amplification reaction was controlled with an agarose gel and ethidium bromide staining. From the remaining volume a 75 µl aliquot was denatured and hybridized to a detection strip at 53°C for 30 min in a waterbath, followed by a washing step at 53°C for 15 min. Detection was performed by using streptavidin-conjugated horseraddish peroxidase (HRP-SA) and the substrate solutions A and B at room temperature. Bands indicating the presence of individual HPV types were read by eye and interpreted according to the manual and finally documented by photography.

SPF10 PCR Line Probe Array v1 (DDL, Leiden)

The SPF10 PCR followed by reverse line probe assay [LiPA v1; DDL, Leiden; Kleter et al., 1998, 1999] recognizes the following HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 56, 58, 59, 66, 68/73, 54, 53, 34, 43, 44, 74, 70. The hybridization strips and the required buffers and detection solutions were provided by DDL (Leiden). All PCR reactions were performed with 5 µl input DNA and biotinylated primers in a final volume of 50 µl using 1,5 Units of the AmpliTaq Gold hot start polymerase (Roche Molecular Systems, Inc.) and an initial activation step of 94°C for 9 min, followed by 40 cycles of 30 sec of denaturation at 94°C, by 45 sec of primer annealing at 52°C and 45 sec of primer extension at 72°C. The PCR was carried out on a MJ Thermocycler PTC 200 (Bio-Rad Laboratories GmBH, München, Germany). The SPF10-PCR line probe array v1 test (LiPav1; DDL, Leiden) amplifies a short (65 bp) fragment in the L1 region using four forward and two reverse primers [Kleter et al., 1998, 1999]. A 10 µl

aliquot of the PCR reaction was taken, denatured and hybridized at 49°C for 60 min to one detection strip, followed by multiple washing steps. Detection was performed by using the substrate solutions and bands were identified by naked eye and interpreted according to the manual.

PapilloCheck® HPV Genotyping Assay (Greiner Bio-One GmbH)

The PapilloCheck® HPV genotyping assay (Greiner Bio-One GmbH) recognizes the following HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44/55, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82. Therefore the test recognizes all thirteen HPV types recently classified as class I carcinogenic with respect to cervical cancer by the WHO [Cogliano et al., 2005], seven HPVs considered as low-risk types (6, 11, 40, 42, 43, 44/55, 70) and four types with proposed high-risk potential [53, 68, 73, 82; Munoz et al., 2003]. Additionally the human ADAT1 (t-RNA-specific adenosine desaminase1) gene is amplified and used as a control for the integrity of the purified DNA. The PapilloCheck® chips and all reagents were provided by Greiner Bio-One GmbH. All PCR reactions were performed with 5 µl input DNA in the presence of nucleotides with a fluorescent label in a final volume of 25 µl using 1 Unit of the AmpliTaq Gold hot start polymerase (Roche Molecular Systems, Inc.). An initial activation step of 95°C for 10 min was followed by 40 cycles of 30 sec of denaturation at 95°C, by 25 sec primer annealing of 55°C and 45 sec of primer extension at 72°C. The average size of the amplified product is 350 bp.

After the amplification reaction, additional 15 cycles of 30 sec at 95°C and 45 sec of 72°C were performed to achieve a single-stranded product for the following hybridization step. The PCR was carried out on a MJ Thermocycler PTC200 (Bio-Rad Laboratories BmBH). Five microliters of the PCR product and 30 µl hybridization buffer were used for the hybridization on the low-density microarray plastic HTA12 chip. A volume of 25 µl of the PCR-hybridization mix were applied to every well on the chip and incubated for 15 min at room temperature in a humid atmosphere. Twelve hybridization reactions can be performed simultaneously on one chip. Hybridization is followed by three washing steps (first for 10 sec at room temperature; second for 60 sec at 50°C; third for 10 sec again at room temperature). After this the chip was dried under a stream of compressed air. The chip was automatically scanned and analyzed using the CheckScanner™ and the CheckReport™ software (Greiner Bio-One), respectively.

Histology

Biopsy and/or endocervical curettage was taken during colposcopy for 306 women. For statistical analysis histology was considered negative for 243 women who had cervical intraepithelial neoplasia (CIN) 1 or less (79.4%). Sixty-three women were diagnosed with CIN2 or worse (20.6%) and regarded as cases.

Statistical Analysis

In total, cervical smears of 881 women pretested by HC2 were available for the analysis after DNA extraction. Samples were excluded from the analysis if they tested negative for the integrity of cellular DNA by using the PapilloCheck® HPV genotyping test sample control function (N = 34) or by testing for the presence of the β-globin gene with the PGMY09/11 PCR LBA (N = 10) or in both (N = 4). In addition those samples were excluded that revealed an amplification product visible in the agarose gel but a negative result on the detection strip of the PGMY09/11 LBA (N = 6) or an unclear SPF10 result (N = 1). In total 826 samples were included in the final analysis, where sensitivities and kappa values were calculated for the different tests used in the study. Detection rates for the three genotyping assays were calculated using a HPV type consensus definition. For each sample a HPV consensus type result was created on the basis of at least two genotyping tests that gave the same typing result. Hence, a HPV type test result of an individual genotyping test was considered to be true positive or true negative, if at least two genotyping tests would give a concordant result for that particular HPV type. In 139 samples the HPV consensus result was "HPV negative." For 653 samples a consensus HPV type could be generated. In the case of 34 samples every individual genotyping test had another HPV result and the consensus type was called "HPV positive."

RESULTS

Testing of the 826 samples by the HC2 test using the high-risk cocktail probe resulted in 716 samples being HC2 positive (RLU/CO $\geq$ 1) and 110 samples that were HC2 negative. Ninety-nine of the 110 HC2 negative samples were either conspicuous in cytology, colposcopy or the HC2 result was in between 0.7 and 1.0 RLU/CO. For the comparative study the original HC2 results were used.

The HPV detection rate of the different genotyping methods in HC2 positive samples ranged from 87.1% (PGMY09/11 PCR) over 87.6% (PapilloCheck®) to 96.4% (SPF10 PCR) (Table I).

The detection rate in HC2 negative samples (RLU/CO < 1.0) ranged from 13.6% (PGMY09/11 PCR) over 34.5% (PapilloCheck®) to 76.4% (SPF10 PCR) with the PapilloCheck® giving overall results with closest similarity to the consensus. Kappa values for the genotyping tests by comparing their results to HC2 results ranged from moderate for PGMY09/11 PCR (k = 0.57) and PapilloCheck® (k = 0.44) to poor (SPF10 PCR K = 0.26). Also the kappa value calculated for the comparison of the consensus HPV type result with HC2 was moderate (k = 0.54). This can be explained by the findings that 63 samples that were positive by HC2 (RLU/CO $\geq$ 1) were negative in the HPV consensus type. The RLU/CO values of these 63 samples were for 30 samples between 1 and 2 RLU/CO, for 23 samples between 2 and 10 RLU/CO and 10 samples had a RLU/CO above 10. Of those 63 samples 18 were negative in all

TABLE I. Comparison of Three Different Genotyping Tests With the HC2 and the HPV Consensus Definition

HC2	PapilloCheck pos		PapilloCheck neg		HC2 total	k (95% CI)
RLU						
≥1.0	627	87.6%	89	12.4%	716	
<1.0	38	34.5%	72	65.5%	110	
	665	80.5%	161	19.5%	826	0.44 (0.36–0.53)
HC2	PGMY09/11 PCR-LBA pos		PGMY09/11 PCR-LBA neg		HC2 total	k (95% CI)
≥1.0	624	87.1%	92	12.8%	716	
<1.0	15	13.6%	95	86.4%	110	
	639	77.4%	187	22.6%	826	0.57 (0.49–0.64)
HC2	SPF10 PCR-LiPavi pos		SPF10 PCR-LiPavi neg		HC2 total	k (95% CI)
≥1.0	690	96.4%	26	3.6%	716	
<1.0	84	76.4%	26	23.6%	110	
	774	93.7%	52	6.3%	826	0.26 (0.15–0.36)
HC2	HPV consensus type pos		HPV consensus type neg		HC2 total	k (95% CI)
≥1.0	653	91.2%	63	8.8%	716	
<1.0	34	30.9%	76	69.1%	110	
	687	87.3%	139	12.7%	826	0.54 (0.46–0.62)

K, kappa value; CI, confidence interval.
All percentages are calculated with N = 826 as denominator.

three genotyping tests and therefore most probably represent false positive results of the HC2 test. Thirteen of these 18 samples had a HC2 result between 1 and 2 RLU/CO, 4 between 2 and 10 RLU/CO and 1 had a RLU/CO of 13.4. On the other hand 34 samples were positive in the HPV consensus type and negative by HC2 with 14 samples positive in all three genotyping tests that most probably represent HC2 false negative results (Table I). The multiple infection rate as detected with the different genotyping tests ranged from 25.8% in the case of PGMY09/11 PCR-LBA, over 39.5% in the case of PapilloCheck® to 55.9% in the case of SPF10 PCR LiPa. The rate of multiple infections with the HPV consensus type was 27.8% (Table II). The highest rate of single infections was observed in samples tested with the PGMY09/11 PCR-LBA (74.2%) and the lowest in samples tested by the SPF10 PCR-LiPav1. As a general phenomenon the SPF10 PCR-LiPav1 detected more HPV types in individual samples as all other tests with 21.6% of all samples having three different HPV types and 7% of samples having four or more HPV types present. Interestingly the rate of samples with double infections was almost the same for the PapilloCheck® (27.2%) and the SPF10 PCR-LiPav1 and the rate of samples detected of having four or more HPV types was very similar for the PapilloCheck® (3.6%) and the PGMY09/11 PCR-LBA (2.8%).

In samples with higher viral DNA load as suggested by higher HC2 RLU/CO values the detection rates of the different genotyping methods and the concordance between the genotyping tests increases (Fig. 1). It can be clearly seen that the number of samples where all genotyping tests disagree in their results decreases from 15% (n = 10) in samples with a HC2 RLU/CO value <0.5 (n = 65) to below 1% (n = 2) in samples with RLU/CO values above 100 (n = 172). At the same time the concordance between all tests increases from 28% (n = 18) in samples with a HC2 RLU/CO value <0.5 (n = 65) to 93% (n = 160) in samples with RLU/CO values above 100 (n = 172) (Fig. 1).

In the PGMY09/11 PCR and the PapilloCheck® the most common type found is HPV 16, with detection rates of 17.7% and 19.4% respectively, whereas SPF10 PCR detected HPV 31 as the most frequent type found in 30.0% of the samples. The next most frequent types detected by PapilloCheck® are HPV 31 (14.4%), HPV 39 (9.3%), HPV 51 (9.1%), and HPV 52 (7.5%), by PGMY09/11 PCR HPV 52 (9.3%), HPV 31 (9.0%), HPV 51 (6.8%), and HPV 39 (5.7%), and by SPF10 PCR HPV 16 (26.8%), HPV 18 (16.7%), HPV 51 (16.6%), and HPV 52 (13.3%) as shown in Table III. The much higher prevalence of HPV 18 as detected by the SPF10 PCR is due to the extremely high sensitivity of the SPF10 PCR for HPV 18 with a detection threshold of 10 viral genome copies [Klug et al., 2008].

Next the detection rate as well as kappa values were calculated by comparing the individual genotyping test results with the consensus HPV type for 19 individual HPV types (Table IV). The HPV consensus type was created on the basis of at least two genotyping tests that gave the same typing result. Hence, a HPV type test result of an individual genotyping test was considered to

TABLE II. Detection of Single and Multiple HPV Infections by Individual Genotyping Assays

	Single (%)	2 HPV types (%)	3 HPV types (%)	4 or more HPV types (%)
PapilloCheck®	60.5	27.2	8.7	3.6
PGMY09/11 PCR-LBA	74.2	18.2	4.9	2.8
SPF10 PCR-LiPav1	44.1	27.4	21.6	7.0
HPV consensus type	72.1	19.2	7.1	1.5

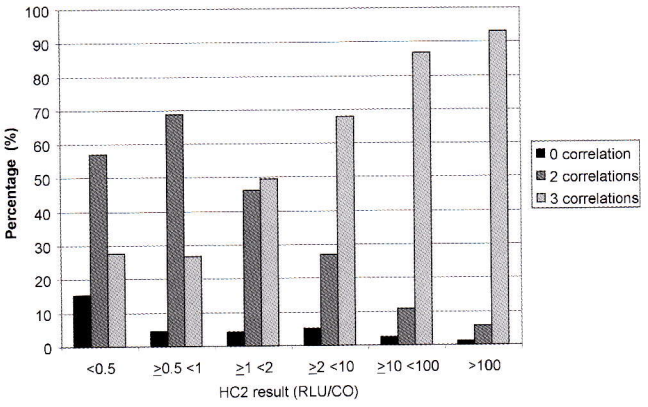

Fig. 1. Concordance of HPV type result in correlation to HC2 RLU values. The figure shows the concordance of the HPV genotyping results between the three genotyping tests with regard to the RLU values obtained by HC2 testing. White bars means non-concordant results; gray bars means two test results are concordant; black bars means all three test results are concordant. The percentages were calculated for the stratified RLU/CO values with following denominators: <0.5 RLU/CO N = 65; ≥0.5 < 1.0 N = 45; ≥1.0 < 2.0 N = 97; ≥2.0 < 10.0 N = 200; ≥10.0 < 100.0 N = 247; ≥100.0 N = 172.

be true positive or true negative, if at least two genotyping tests would give a concordant result for that particular HPV type. In 139 samples the HPV consensus result was "HPV negative." For 653 samples a consensus HPV type could be generated. In the case of 34 samples every individual genotyping test had another HPV result and the consensus type was called "HPV positive." This comparison revealed low sensitivities for the detection of HPV 56 (40.5%), HPV 11 (66.7), and HPV 31 (67.0%) and some weakness for the detection of HPV 51 (78.1%), HPV 39 (78.6%), and HPV 66 (76.9%) by using the PGMY09/11 system. Only in the case of HPV 59 a sensitivity of detection of 100% was achieved. Using the PapilloCheck® allowed for seven HPV types (HPV 6, 11, 33, 40, 42, 53, 56) a 100% sensitivity of detection while the sensitivities for detection of HPV 35 (72.2%) and HPV 45 (85.4%) were the lowest. Using the SPF10 PCR allowed to detect nine HPV types (HPV 6, 11, 18, 31, 35, 40, 53, 56, 70) with a 100% sensitivity, while lower sensitivities were observed for the detection of HPV 59 (81.0%) and especially for HPV 42 (56.3%). The kappa values were generally lower for the comparison of the results obtained with the SPF10 PCR with the HPV consensus type ranging from k = 0.21 to k = 0.82, while values obtained for the PGMY09/11 PCR ranged from k = 0 to k = 0.96 and were best for the PapilloCheck® ranging from k = 0.49 to k = 0.98 (Table IV).

A direct test-to-test evaluation was performed by pairwise comparison of the individual genotyping tests and kappa calculation for the class I carcinogenic HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66; Table V). The lowest kappa value was found by comparing SPF10 PCR and PapilloCheck® test results for HPV 18 (k = 0.34), while the highest kappa value was found for HPV 59 (k = 0.90) by comparing PapilloCheck® with PGMY09/11 PCR (Table V). Interestingly the type specific kappa values from the pairwise comparison of PapilloCheck® with PGMY09/11 PCR was for eleven HPV types higher than the type specific kappa values found by comparing PapilloCheck® with SPF10 PCR or PGMY09/11 PCR with SPF10 PCR. In contrast the kappa values for HPV 56 (k = 0.61) and HPV 66 (k = 0.66) were higher for PapilloCheck® in comparison to the SPF10 PCR (Table V; Fig. 2).

From 306 women histological results were available. For those positivity of the detection of cervical intraepithelial neoplasia (CIN2+) (case definition) were calculated. Positivity for the detection of CIN2+ ranged from 93.7% for PGMY09/11 PCR to 98.4% for the PapilloCheck®, the SPF10 PCR and the HC2. Positivity in controls (≤CIN1) ranged between 90.5% for the SPF10 PCR, over 75.7% for the HC2, and 76.1% for the PapilloCheck® to 69.1% for the PGMY09/11 (Table VI).

To address the problem if differences observed in the HPV detection rate between individual genotyping test are due to the different genomic regions that are amplified by PCR, we re-examined samples being positive in tests amplifying a portion of the viral L1 gene (PGMY09/11 PCR, SPF10 PCR) and negative in the PapilloCheck® that amplifies the 3′-end of the E1 gene and vice versa. Of all 826 samples tested we observed 30 cases in which the L1 tests (SPF10 and PGMY09/11) were HPV positive and the PapilloCheck® gave negative HPV results. To verify this result we performed another PCR reaction from the E1-gene using different primer pairs as the PapilloCheck® assay [CP4/CP5 and CP5/PPF1; Iftner et al., 2003] and obtained in 29 cases an amplification product that was confirmed in 25 cases to be HPV DNA by direct sequencing. In seven cases E1-PCR [Iftner et al., 2003] and sequencing revealed HPV types not detectable by the PapilloCheck® assay. The other 18 HPV types were 17 common high-risk types and one low-risk type. Out of those, 10 samples had HC2 results in between RLU/CO values of 1.0–2.0, which is close to the clinical defined cut-off of the HC2 test of 1.0 RLU/CO. Fourteen samples gave the same results in sequencing the E1 region as PCR tests based on the amplification of the L1 region, in eight samples all results were different and in the remaining three samples the result from E1 sequence was different from the common result of the PCR tests based on the amplification of the L1 region. The remaining sample negative by E1-PCR had an original HC2 result just above the test cut-off level (RLU/CO value of 1.04) and the consensus between SPF10 PCR and PGMY09/11 for this sample was HPV 16. On the other hand eight samples were negative by the PGMY09/11 PCR and the SPF10 PCR, while the PapilloCheck® gave a positive HPV result. Of these samples five were HC2 negative and the other three samples had a HC2 result of 2.6 RLU/CO, 7.7 RLU/CO, and 18.6 RLU/CO, respectively.

DISCUSSION

In this study, the performance of a novel commercially available CE-marked test system (PapilloCheck®) was

TABLE III. Type-Specific Prevalence as Defined bye Each Test

HPV type	Classification	PapilloCheck®	PGMY09/11	SPF10
HPV 16	1	19.4%	17.7%	26.8%
HPV 18	1	4.0%	4.7%	16.7%
HPV 31	1	14.4%	9.0%	30.0%
HPV 33	1	4.4%	3.5%	7.3%
HPV 35	1	1.6%	2.2%	4.8%
HPV 39	1	9.3%	5.7%	9.9%
HPV 45	1	4.5%	4.5%	9.6%
HPV 51	1	9.1%	6.8%	16.6%
HPV 52	1	7.5%	9.3%	13.3%
HPV 56	1	7.4%	1.8%	5.5%
HPV 58	1	6.1%	5.3%	6.1%
HPV 59	1	2.5%	2.8%	2.5%
HPV 66	1	5.9%	4.1%	6.5%
HPV 53	Potential high risk	6.1%	5.2%	10.8%
HPV 68	Potential high risk	5.6%	2.7%	1.5%
HPV 73	Potential high risk	1.5%	3.5%	0.1%
HPV 82	Potential high risk	1.9%	0.6%	N/A
HPV 6	Low risk	0.6%	0.0%	2.2%
HPV 11	Low risk	0.6%	0.2%	3.0%
HPV 40	Low risk	0.4%	0.0%	0.7%
HPV 42	Low risk	5.8%	1.9%	1.1%
HPV 43	Low risk	0.8%	N/A	1.2%
HPV 44	Low risk	N/A	N/A	3.0%
HPV 54	Low risk	N/A	1.9%	2.2%
HPV 61	Low risk	N/A	0.6%	N/A
HPV 70	Low risk	4.1%	3.0%	7.4%
HPV 72	Low risk	N/A	0.1%	N/A
HPV 81	Low risk	N/A	0.7%	N/A
CP108	Low risk	N/A	0.4%	N/A
IS39	N/A	N/A	0.1%	N/A
HPV 34	N/A	N/A	N/A	0.5%
HPV 55	N/A	N/A	1.2%	N/A
HPV 57	N/A	N/A	0.1%	N/A
HPV 62	N/A	N/A	2.2%	N/A
HPV 64	N/A	N/A	0.1%	N/A
HPV 67	N/A	N/A	0.7%	N/A
HPV 71	N/A	N/A	0.1%	N/A
HPV 74	N/A	N/A	N/A	4.4%
HPV 83	N/A	N/A	1.1%	N/A
HPV 84	N/A	N/A	1.6%	N/A
HPV 91	N/A	N/A	0.8%	N/A
HPV 44/55	N/A	3.1%	N/A	N/A
HPV 56/74	N/A	N/A	N/A	0.7%
HPV 39/68/73	N/A	N/A	N/A	0.1%
HPV 68/73	N/A	N/A	N/A	10.8%

All percentages are calculated with N = 826 as denominator.

compared with two different genotyping methods on HC2-pretested cervical swabs. The rationale of this study was to identify differences in the outcome of results. When the three HPV genotyping assays used in this study were compared, HPV typing results were found to be inhomogeneous between the different test systems. However, this is rather due to the different sensitivities of the three methods, defined by the size of the amplification product of their respective PCR reactions, as the HPV detection rate and the concordance between all tests increases with higher viral load. This also explains the differences in the multiple infection rates observed, as the most sensitive test (LiPav1) detected 55.9% of all samples to contain multiple HPV types. However, there are clearly also differences in the detection rates of specific HPV types between the three genotyping methods that are more related to the format of the specific primers in each test system than to a general lower sensitivity for HPV detection.

In order to further analyze the performance of each genotyping assay a consensus genotype was defined for each sample on the basis of at least two genotyping tests with same typing result. This allowed to define detection rates as well as to determine kappa values for nineteen individual HPV types commonly detectable by all three genotyping systems. This revealed some deficiencies for the detection of HPV 56, 11, and 31 by using PGMY09/11 PCR, for detection of HPV 35 by using PapilloCheck® and for HPV 42 and 59 by using the LiPav1 test, which is reflected in the respective kappa values. Interestingly the two tests (PGMY09/11 and PapilloCheck®) with a comparable sensitivity for HPV detection as the HC2 assay, revealed for most HPV types higher kappa values

TABLE IV. Type-Specific Test Parameters in Comparison to the HPV Consensus Type

	Consensus		SPF10			PGMY09/11				PapilloCheck®			
HPV type	N total	Positive	Positive	+/+	Detection rate (%)	Kappa (CI = 95%)	Positive	+/+	Detection rate (%)	Kappa (CI = 95%)	Positive	+/+	Detection rate (%)	Kappa (CI = 95%)
HPV 16	826	152	221	148	97.4	0.74 (0.68–0.79)	146	139	91.4	0.92 (0.88–0.95)	160	139	91.4	0.87 (0.82–0.91)
HPV 31	826	109	248	109	100.0	0.52 (0.45–0.59)	74	73	67.0	0.77 (0.70–0.84)	119	106	97.2	0.92 (0.88–0.96)
HPV 52	826	71	110	70	98.6	0.75 (0.67–0.82)	77	67	94.4	0.90 (0.84–0.95)	62	62	87.3	0.93 (0.88–0.97)
HPV 51	826	64	137	63	98.4	0.58 (0.50–0.67)	56	50	78.1	0.82 (0.74–0.90)	75	62	96.9	0.88 (0.82–0.94)
HPV 39	826	56	82	54	96.4	0.76 (0.68–0.84)	47	44	78.6	0.84 (0.77–0.93)	77	55	98.2	0.81 (0.74–0.89)
HPV 53	826	48	89	48	100.0	0.68 (0.58–0.74)	43	39	81.3	0.85 (0.77–0.93)	50	48	100.0	0.98 (0.95–1.00)
HPV 45	826	41	79	40	97.6	0.64 (0.54–0.75)	37	35	85.4	0.89 (0.82–0.97)	37	35	85.4	0.89 (0.82–0.97)
HPV 66	826	39	54	38	97.4	0.81 (0.72–0.90)	34	30	76.9	0.81 (0.72–0.91)	49	36	92.3	0.81 (0.72–0.90)
HPV 18	826	38	138	38	100.0	0.39 (0.29–0.49)	39	37	97.4	0.96 (0.91–1.00)	33	33	86.8	0.93 (0.86–0.99)
HPV 58	826	39	50	37	94.9	0.82 (0.73–0.91)	44	38	97.4	0.91 (0.84–0.98)	50	38	97.4	0.85 (0.76–0.93)
HPV 56	826	37	55	37	100.0	0.79 (0.70–0.89)	15	15	40.5	0.57 (0.40–0.73)	61	37	100.0	0.74 (0.64–0.84)
HPV 70	826	32	61	32	100.0	0.67 (0.56–0.78)	25	25	78.1	0.87 (0.78–0.97)	34	31	96.9	0.94 (0.87–1.00)
HPV 33	826	28	60	25	89.3	0.55 (0.42–0.68)	29	25	89.3	087 (0.78–0.97)	36	28	100.0	0.87 (0.78–0.95)
HPV 59	826	21	21	17	81.0	0.80 (0.67–0.94)	23	21	100.0	0.95 (0.89–1.00)	21	20	95.2	0.95 (0.88–1.00)
HPV 35	826	18	40	18	100.0	0.61 (0.46–0.76)	18	17	94.4	0.94 (0.86–1.00)	13	13	72.2	0.84 (0.69–0.98)
HPV 42	826	16	9	9	56.3	0.72 (0.51–0.92)	16	23	75.0	0.75 (0.57–0.92)	48	16	100.0	0.49 (0.33–0.64)
HPV 11	826	3	25	3	100.0	0.21 (0.00–0.43)	2	2	66.7	0.80 (0.41–1.00)	5	3	100.0	0.75 (0.41–1.00)
HPV 6	826	3	18	3	100.0	0.28 (0.02–0.54)	0	0	0.0	0.00 (0.00–0.07)	5	3	100.0	0.75 (0.41–1.00)
HPV 40	826	2	6	2	100.0	0.50 (0.07–0.92)	0	0	0.0	0.00 (0.00–0.07)	3	2	100.0	0.80 (0.41–1.00)

Percentage values of HPV positivity are calculated with N = 826 as denominator; Positive (+/+) means test results that were identical in the individual test system and the consensus type; CI, confidence interval.

when compared with each other than when compared with the highest sensitive LiPav1 test. One caveat of this kind of analysis is that the consensus genotype is based on the concordance of a test result achieved with genotyping systems with different analytical sensitivities, which may cause a bias against the superior sensitive test system in relation to the kappa values as presented in Table IV.

To address the point if the observed test differences might be due to the use of different genomic regions amplified by the individual test systems, samples that were positive in the amplification of the L1 region, but negative for the amplification of the E1 region were re-examined and vice versa. In 25 of 30 cases with positive L1-amplification and negative E1 results it was confirmed that the E1 region was not deleted because of an integration event, but was rather not detected by the E1-based PapilloCheck® assay. In contrast, we found eight samples to be negative by both PGMY09/11 PCR and the highly sensitive LiPav1 test, indicating loss of this part of the genomic region, while PapilloCheck® was positive in all cases indicating conservation of the E1-region. This was again confirmed by E1-PCR and sequencing, which identified in two cases high-risk HPV types.

To investigate the clinical value of the different tests, the detection rate in cases and controls was determined on a subset of 306 samples with histological results. This comparison showed that all tests, except for the PGMY09/11 PCR (with 93.7%) had a detection rate of >98% to detect high-grade disease (cervical intraepithelial neoplasias 2 or higher). Interestingly all HPV test systems revealed a high detection rate in the controls (≤CIN1). The high detection rate in the control group here is in contrast to studies where tests were used in population-based screening trials and tests like the HC2 had a specificity of ≥95%, meaning a low detection rate in the non-case (control) group. This difference can be explained by the low percentage of samples being true HPV negative in the selected samples used for the test comparison study. This is the reason, why we did not calculate the clinical sensitivity and specificity of the different test systems and rather called it detection rate as the assumed clinical specificity, would be different if the tests would have been used in a population based screening trial with a larger percentage of negative samples.

For the detection of women with persistent infections genotyping tests appear superior to those tests that stratify infected women only in groups infected with high-risk or low-risk types. It has been shown that certain carcinogenic types are associated with an extreme high absolute risk for cervical intraepithelial neoplasias 3 or cancer, like HPV 16 and 18 [Kjaer et al., 2002; Castle et al., 2005; Khan et al., 2005; Berkhof et al., 2006; Bulk et al., 2006]. Genotyping tests could therefore be of high value for the individual risk stratification of women found to be persistently infected by those very high-risk types. Those women would then need to undergo a more intense follow up than women positive for other high-risk HPV types.

TABLE V. Type-Specific Agreement in the Assays

	Kappa (95% CI)		
	PapilloCheck®/SPF10	PapilloCheck®/PGMY	SPF10/PGMY
HPV 16	0.63 (0.57–0.69)	0.79 (0.74–0.85)	0.67 (0.61–0.73)
HPV 18	0.34 (0.24–0.45)	0.88 (0.80–0.96)	0.38 (0.27–0.48)
HPV 31	0.48 (0.40–0.55)	0.69 (0.61–0.77)	0.37 (0.28–0.45)
HPV 33	0.49 (0.35–0.64)	0.76 (0.63–0.88)	0.47 (0.31–0.62)
HPV 35	0.47 (0.28–0.67)	0.77 (0.60–0.94)	0.57 (0.40–0.74)
HPV 39	0.63 (0.54–0.72)	0.67 (0.57–0.77)	0.62 (0.52–0.73)
HPV 45	0.55 (0.43–0.68)	0.77 (0.66–0.88)	0.56 (0.44–0.68)
HPV 51	0.52 (0.43–0.61)	0.71 (0.62–0.80)	0.46 (0.36–0.55)
HPV 52	0.68 (0.60–0.76)	0.82 (0.75–0.89)	0.67 (0.59–0.75)
HPV 56	0.61 (0.50–0.73)	0.38 (0.20–0.55)	0.41 (0.23–0.59)
HPV 58	0.72 (0.61–0.82)	0.80 (0.70–0.89)	0.77 (0.68–0.77)
HPV 59	0.76 (0.61–0.91)	0.90 (0.81–0.99)	0.77 (0.62–0.91)
HPV 66	0.66 (0.54–0.77)	0.63 (0.50–0.76)	0.64 (0.51–0.77)

CI, confidence interval.

TABLE VI. Positivity Rates in Cases and Controls

	Disease status ≥CIN2	Disease status ≤CIN1		Positivity in cases (%)	Positivity in controls
PapilloCheck®					
Positive	62	185	247		
Negative	1	58	59		
	63	243	306	98.4	76.1
PGMY09/11					
Positive	59	168	227		
Negative	4	75	79		
	63	243	306	93.7	69.1
SPF10					
Positive	62	220	282		
Negative	1	23	24		
	63	243	306	98.4	90.5
HC2					
Positive	62	184	246		
Negative	1	59	60		
	63	243	306	98.4	75.7

CIN, cervical intraepithelial neoplasia.

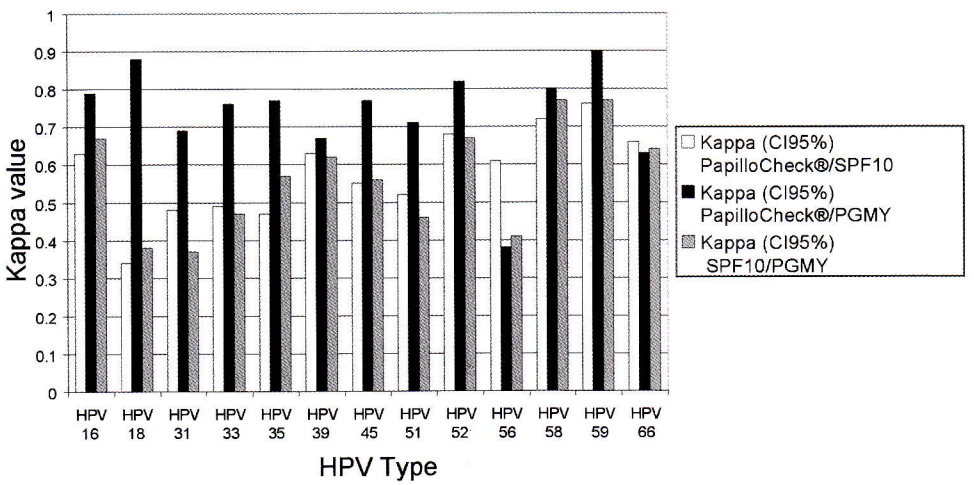

Fig. 2. Pairwise comparison of the kappa values obtained by comparing two genotyping tests for the detection of the types HPV 16, 31, 52, 51, and HPV 39 most frequently observed in the patient sample collection; CI, confidence interval.

In summary this analysis provides data showing that the use of different genotyping tests applied to the same group of cervical smears leads to largely comparable results especially with regard to the detection of cervical intraepithelial neoplasias of grade 2 or higher and the type specific detection rates of the 13 class I carcinogenic HPV types. The PGMY09/11 and PapilloCheck® genotyping tests were more similar in performance amongst each other and with the HC2 assay that has a clinically defined cut-off. The LiPav1 test is extremely sensitive and therefore seems to be of lower value in clinical practice as a stand-alone test than tests with a sensitivity similar to HC2 as it will detect more clinically irrelevant infections. Such highly sensitive tests seem to be more appropriate for studies aiming to define the exact HPV type prevalence in different populations.

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