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Additional Human Papillomavirus Types Detected by the Hybrid Capture Tube Test among Samples from Women with Cytological and Colposcopical Atypia

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The type specificity of the human papillomavirus (HPV) Hybrid Capture Tube (HCT) test was evaluated by using typing with PCR (MY09-MY11)-restriction fragment length polymorphism (RFLP) and sequencing. All samples HCT test positive for only low-risk HPV (n = 15) or only high-risk HPV (n = 102) were confirmed, whereas 9 of 12 HCT test double-positive samples contained only high-risk HPV types as determined by PCR-RFLP. Several high-risk HPV types (HPV-53, -58, -62, -66, -CP8304, and -MM4) not included in the HCT test were indeed detected, indicating a broader detection range with retained distinction between low-risk and high-risk HPV types.

Infection of the female genitalia by oncogenic human papillomavirus (HPV) types is the major cause of precancerous squamous intraepithelial lesions (SIL) of the cervix uteri (10). Invasive cancer is generally preventable by organized cervical screening programs (17). Persistent infection with oncogenic HPV types and a high viral load confer an increased risk for persistent or progressing SIL (9). Infections in women above 30 years of age and infections with cancer-associated HPV types tend to persist longer, and women with persistent HPV infections are at particularly increased risk for cervical cancer (8). Thus, additional testing for concomitant HPV infections may improve screening efficacy (4, 5, 15).

The commercially available Hybrid Capture Tube (HCT) test (Digene Diagnostics, Inc., Beltsville, Md.) is designed to detect the genomes of 14 HPV types by hybridization to type-specific RNA probes (11). The probes are routinely used in two cocktails, the first of which (designated A) contains probes specific to low-risk HPV types (types 6, 11, 42, 43, and 44) causing benign proliferation of the genitalia and the second of which (designated B) consists of probes specific to high-risk, oncogenic types (types 16, 18, 31, 33, 35, 45, 51, 52, and 56) associated with high-grade SIL and invasive cervical cancer. The clinical utility of the HCT test is well established; it can diagnose cervical dysplasia cases with good sensitivity and specificity (3, 6, 7, 13, 14, 20). However, the number of newly identified HPV types is still growing and the hybridization method of the HCT test raises the possibility of cross-hybridization to other types. Also, the instructions included with the HCT test mention cross-reaction with two essentially extragenital types (HPV-13 and -30). We evaluated whether cross-hybridization may influence the specific distinction between low-risk and high-risk types.

Clinical samples. Clinical samples were taken from women referred to gynecologic outpatient clinics because of cytological or colposcopical abnormalities (cervical screening in Hungary routinely includes colposcopy). Exfoliated cells from the cervix uteri were collected with a Digene specimen collection kit and stored at −20°C until processed. Papillomavirus DNA was detected with the Digene HCT test. In the first step of the HCT test protocol, the specimen is alkali denatured. A 150-μl aliquot of the denatured specimen is hybridized to each probe, while the remaining part can be stored at −20°C and retested later. Storage and tests were performed in accordance with the manufacturer's instructions.

PCR amplification. Of 570 consecutive cervical samples, 145 were positive by the HCT test and were further processed for PCR amplification. DNA was recovered from a 300-μl aliquot of the frozen, stored, alkali-denatured specimen. After thawing, 750 μl of ethanol (96%) and 60 μl of glacial acetic acid were added. The precipitate was centrifuged for 15 min at 4°C and 12,000 × g and redissolved in 200 μl of Tris (10 mM, pH 7.5)-EDTA (0.1 mM) buffer. After the DNA recovery step, 16 specimens were excluded from HPV-specific PCR testing because they could not be amplified with the human β-globin primers pCO3 and pCO4 (18). Ten microliters of each of the remaining 129 samples was subjected to PCR amplification with the consensus primers MY09 and MY11 with 40 cycles in 50-μl volumes (12). A 2-μl aliquot of each PCR mixture was visualized by silver staining after nondenaturing polyacrylamide (5%; acrylamide/bisacrylamide ratio, 50:1) gel electrophoresis (PAGE).

RFLP typing of PCR products. The enzymes *Alu*I, *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, and *Pst*I were used for restriction fragment length polymorphism (RFLP) typing. The RFLP patterns of the MY09-MY11 fragments of the different HPV types are reported elsewhere (2), with the exception of *Alu*I digestion. *Alu*I digests the MY09-MY11 fragments of the different types as follows: HPV30, 281, 86, and 73 bp; HPV32, 271, 109, and 69 bp; HPV39, 277 and 178 bp; HPV42, 328, 82, and 39 bp; HPV51, 289, 117, and 39 bp; HPV52, 244 and 206 bp; HPV53, 290, 124, and 34 bp; HPV54, 172, 167, and 113 bp; HPV56, 325, 73, and 51 bp; HPV58, 266, 184, and 61 bp; HPV61, 198, 131, and 127 bp; HPV66, 196, 94, and 88 bp; HPV67, 266 and 183 bp; HPV68, 349 and 107 bp; HPV69, 331 and 124 bp; HPV70, 296 and 159 bp; HPV72, 331, 86, and 39 bp; HPV73, 280, 110, and 68 bp; HPV-CP141, 296, 124, and 35 bp; HPV-CP4173, 331, 86, and 39 bp; HPV-CP8061, 247, 166, and 39 bp; HPV-

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TABLE 1. RFLP typing scores of MY09-MY11 fragments

1-2-4 code		HPV type(s)	Differentiation of HPV types with the same score	
A[a]	B[b]		Enzyme	Distinctive restriction fragment lengths (bp)
0	4	16		
0	5	59		
0	7	62		
1	1	61		
1	5	CP8304		
2	7	11, (43)[c]	*Hae*III	HPV11: 217, 124, 108 vs (HPV43: 331, 124)[c]
3	1	69		
3	2	68		
3	3	54, 70	*Alu*I	HPV54: 172, 167, 113 vs HPV70: 296, 159
3	5	LVX82, MM7	—[d]	
3	7	CP141, L1AE1, LVX160	—[d]	
4	1	44		
4	3	6, 55, MM8	*Dde*I	HPV6: 382 vs HPV55: 112, 111, 101, 85 vs HPVMM8: 220, 142, 90
4	4	57		
4	5	34, 35	*Pst*I	HPV34: 253, 179 vs HPV35: 426
4	6	33		
4	7	31		
5	1	72, CP4173, LVX100	—[d]	
5	2	IS039, MM4, L1AE2	*Dde*I	HPV-IS039: 243, 212 vs HPV-MM4: 288, 167 vs HPV-L1AE2: 223, 192
5	3	53		
5	4	73, MM9	—[d]	
5	5	52, 56	*Pst*I	HPV52: 419 vs HPV56: 246, 203
5	6	39, 58	*Pst*I	HPV39: 330, 125 vs HPV58: 216, 207
5	7	67, CP8061	*Hin*fI	HPV67: 234, 215 vs HPV-CP8061: 346, 106
6	0	40		
6	1	CP6108		
6	4	18, 45	*Dde*I	HPV18: 432 vs HPV45: 324, 131
6	7	13, 64	*Dde*I	HPV13: 326 vs HPV64: 211, 151, 87
7	1	(51)[c]		
7	2	42		
7	3	32		
7	4	66		

[a] A, 1-2-4 code based on *Alu*I, *Bam*HI, and *Dde*I.
[b] B, 1-2-4 code based on *Hae*III, *Hin*fI, and *Pst*I.
[c] Probably not amplified with the unmodified MY09-MY11 primer pair.
[d] —, Cannot be distinguished with any of the six restriction enzymes due to identical or very similar restriction fragment lengths.

CP8304, 358 and 94 bp; HPV-IS039, 253, 163, and 39 bp; HPV-L1AE1, 276, 104, and 35 bp; HPV-L1AE2, 253, 143, and 19 bp; HPV-LVX100, 331, 86, and 39 bp; HPV-LVX160, 296, 124, and 35 bp; HPV-LVX82, 361 and 91 bp; HPV-MM4, 253, 163, and 39 bp; HPV-MM7, 361 and 91 bp; HPV-MM9, 280, 110, and 68 bp.

The restriction fragments of single PCR products were electrophoresed in 2% agarose gels, whereas those of multiple infections were separated by PAGE (see above). We constructed a score table for rapid evaluation of RFLP. The RFLP patterns of the different HPV types were coded by a 1-2-4 system (Table 1). Coding was based on whether the typed MY09-MY11 fragment was cleaved by the different restriction enzymes or not. For instance, HPV33 was cleaved by *Dde*I, *Hin*fI, and *Pst*I but not by *Alu*I, *Bam*HI, and *Hae*III. Its score was therefore 46 (i.e., $1_{(AluI)} \times 0 + 2_{(BamHI)} \times 0 + 4_{(DdeI)} \times 1 = 4$ and $1_{(HaeIII)} \times 0 + 2_{(HinfI)} \times 1 + 4_{(PstI)} \times 1 = 6$). Most HPV types had unique scores, while for the HPV types with the same scores, we selected the restriction enzymes that produced the largest difference in fragment size. For instance, HPV-18 and HPV-45 both have a score of 64 but can be distinguished due to the different sizes of their *Dde*I fragments (Table 1).

DNA sequencing. PCR amplimers of rare, unexpected HPV types were purified with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany), cloned in a Promega T-Easy vector (Promega Corp., Madison, Wis.), and sequenced with a Pharmacia T7 sequencing kit (Pharmacia Biotech AB, Uppsala, Sweden) using primers MY09 and MY11 and direct [^{35}S]dATP incorporation.

Results and discussion. The HCT test results of the samples subjected to PCR-RFLP typing were the following: 15 low-risk HPV positive (A), 102 high-risk HPV positive (B), and 12 HCT test double positive (AB); i.e., the latter samples hybridized to both the low-risk and high-risk probes. The PCR detection method suited two requirements. (i) It did not alter the HCT test protocol, since the DNA for PCR amplification was recovered from the specimens after the HCT test results had been obtained. (ii) Hybridization and PCR were performed on the same specimens, allowing reliable comparison of the methods (3, 16). Separate sampling for each method might have biased the intermethod comparisons because different amounts of infected cells, virus, etc. might have been obtained.

By nondenaturing PAGE, PCR products with very similar lengths (449 to 458 bp) have different electrophoretic mobilities due to the difference between their nucleotide sequences. Thus, multiple infections could be detected as well as single infections (Fig. 1a). For typing of the PCR products, RFLP (2) was the method selected because it is independent of hybridization. Its combination with the 1-2-4 evaluation system (Table 1) eliminated the need for precise restriction fragment length determination, while its combination with nondenatur-

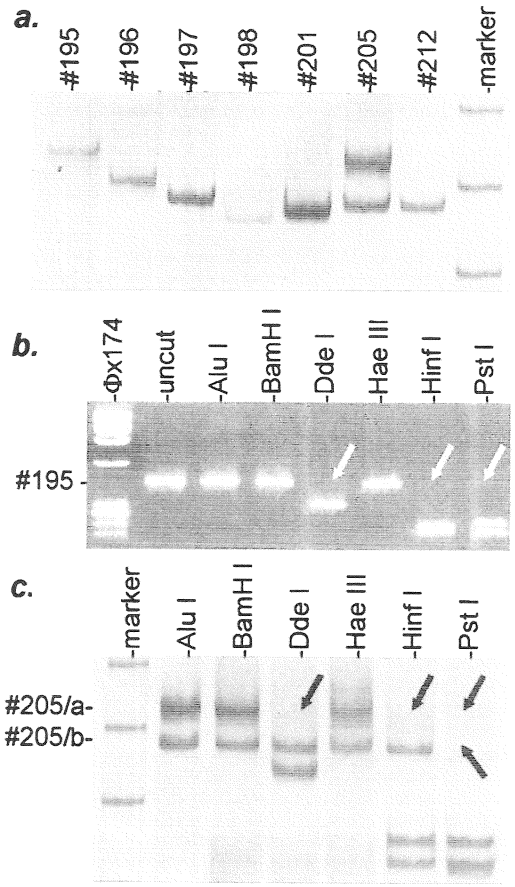

FIG. 1. (a) Different PAGE mobilities of MY09-MY11 fragments. Marker bands are 500, 400, and 330 bp long. (b) RFLP of a single infection (#195) in 2% agarose gel with an *Hae*III-digested ϕX174 marker. (c) RFLP of a double infection (#205) revealed by PAGE and silver staining. (b and c) Arrows indicate restriction enzyme cleavage. Identified types: HPV-33 (#195), HPV-52 (#196), HPV-16 (#197), HPV-56 (#198), HPV-6 (#201), HPV-16/33 (#205), and HPV-18 (#212).

ing PAGE enabled us to identify all of the coinfecting HPV types in multiple infections (Fig. 1b and c). With this method, we identified a triple infection as well, which was confirmed 1 month later in another sample from the same patient.

The HCT test results of the samples that reacted exclusively with either the low-risk (A) or the high-risk (B) probe mixture were completely concordant with the PCR-RFLP typing results (Table 2). Among the 102 high-risk (B)-positive HCT test samples, 13 had multiple infections with all of the coinfecting types belonging to the high-risk group. In addition, we detected single infections with HPV-53 (two samples), HPV-58 (two samples), HPV-66 (three samples), HPV-MM4, and HPV-CP8304, which also belong to the high-risk group but are not included in the high-risk HCT test probe mixture. These types probably cross-hybridized with some of the high-risk probes. Among the additional types, there was HPV-58, which has a type-specific probe in the new-generation Hybrid Capture II plate test. Although we do not know the detection limit of the HCT test for additional types, profound infections by them are probably not missed even by the first-generation test. Detection of additional types, HPV-53, -66, -67, -73, -CP6108, and -CP8061, by Hybrid Capture II test has recently been reported (16). At least two of these types, HPV-53 and -66, also hybridized with the HCT test high-risk probes.

TABLE 2. Correlation of PCR-RFLP and HCT test results

PCR-RFLP result	No. of samples with HCT test result of:		
	Low-risk positive	Double positive	High-risk positive
Single low-risk types			
HPV-6	11	1	
HPV-11	3		
HPV-44	1		
Single high-risk types			
HPV-16		3	46
HPV-18		1	6
HPV-31		2	10
HPV-33			5
HPV-35			2
HPV-45			2
HPV-52			6
HPV-56			3
Other types		1[a]	9[b]
Multiple types		4[c]	13[d]
Total	15	12	102

[a] HPV-62.
[b] HPV-53, -58, -66, -MM4, and -CP8304.
[c] HPV-6/16, -6/45, -16/31, and -16/52.
[d] HPV-18/33, -18/58, -31/52, -33/35, -52/56, -16/31 (detected in three specimens), -16/33 (detected in three specimens), -31/56/CPLVL1 (detected in two consecutive samples collected 1 month apart from the same patient).

In the HCT test double-positive samples (AB), PCR-RFLP could detect both low-risk and high-risk types only in two specimens, while all but one of the remaining samples contained high-risk types (Table 3). The lack of simultaneous detection of low- and high-risk HPV types in these specimens is unlikely due to methodological failure, since the sensitive silver staining could readily detect the multiple PCR bands even if there was a 100-fold difference between the copy numbers of the competing target sequences (data not shown). Thus, we considered the PCR-RFLP typing method reliable and retested these samples with the HCT test, which is useful for exclusion of false-positive HCT test signals (19). As 12 of 13 specimens consistently hybridized to both low- and high-risk

TABLE 3. Correlation of PCR-RFLP results and hybridization signals for HCT test double-positive samples

Sample no.	HPV type(s) determined by PCR-RFLP	Hybridization signals[a]
294	6	10.0 > 1.8
61	6, 16	3.7 > 1.3
191	6, 45	159.6 > 1.8
488	62	24.4 > 4.1
17	16	6.7 < 7.0
116	16	2.4 < 22.4
257	16	38.8 < 201.9
34	16, 31	26.9 < 192.2
492	16, 52	61.3 < 81.4
411	18	12.8 < 83.5
271	31	1.7 < 37.1
368	31	2.5 < 180.4

[a] Relative luminescence of the samples divided by the mean relative luminescence of three positive control replicates. The values are for the low-risk probe mixture followed by the high-risk probe mixture.

probes, we investigated the significance of the amplitude of the hybridization signal, which was expressed as the ratio of sample luminescence to the mean luminescence of three positive controls run in each test series (6). A high-risk (B) signal exceeding the low-risk (A) signal indicated an infection dominated by high-risk types, whereas in all three samples containing low-risk HPV, the A signal was stronger (Table 3). Interestingly, an additional HPV type (HPV-62) was present in one of the specimens with a dominant low-risk (A) signal.

For the additional high-risk types (HPV-53, -58, -62, -66, -CP8304, and -MM4), the RFLP typing results were confirmed by sequencing. The MY09-MY11 amplimers were cloned, sequenced, and aligned (1) with the corresponding reference HPV types. The sequencing of the first four clones was performed in two directions. Since the major parts of the sequences obtained from the two directions overlapped, we performed unidirectional sequencing of the rest of the clones. The extent of homology to the reference sequences was 96 to 100%. Since the amplifications were done with *Taq* polymerase, a few nucleotide differences between the clones and the reference sequences might have been due to amplification errors. This did not influence the typing and confirmation of the PCR-RFLP results, however.

The HCT test provides a sufficient diagnostic aid by distinguishing between lesions with low-risk and high-risk HPV types. From this point, HCT test double-positive patients should be grouped together with high-risk HPV positives. Indeed, there is a strong association between SIL and high-risk HPV types whether or not low-risk types are also present (19). According to this interpretation, we found a contradictory PCR-RFLP result only in one HCT double-positive sample, where the high-risk type was not detected by PCR-RFLP. The overall type specificity of the HCT test was thus 128 of 129 (99.2%). In conclusion, the HCT test has a broader detection range than assigned, which increases its diagnostic value without influencing the specific distinction between low-risk and high-risk HPV types.

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