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A new PCR-based assay amplifies the E6–E7 genes of most mucosal human papillomaviruses (HPV)

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Abstract

We established a new assay to detect the E6–E7 DNA of mucosal human papillomaviruses (HPV) by a PCR-based method using four pairs of degenerate LCR and E7 primers (LCR-E7 PCR). This assay amplifies the full length of E6 and the N-terminal part of E7. HPV typing was performed using restriction-fragment-length polymorphism (RFLP), and by analyzing the sequences of cloned PCR products. We compared this assay with the first generation hybrid captured assay (HCA-I) and the MY09/11-PCR method. LCR-E7 PCR was able to detect more than 34 mucosal HPV types and theoretically should detect two additional types. LCR-157 PCR and HCA-I detected HPV DNA in 70% (69/99) and 55% (54/99) of low-grade cervical intraepithelial lesions (LSIL), 89% (105/118) and 76% (90/118) of high-grade cervical intraepithelial lesions (HSIL), and 90% (56/62) and 79% (49/62) of invasive squamous cell carcinomas (SCC), respectively. LCR-E7 PCR was more sensitive than the HCA-1 test. Discordant results between the LCR-E7 and MY 11/09-PCR tests were observed in one of 185 (0.5%) normal samples, seven of 85 (8.2%) LSIL samples, seven of 82 (8.5%) HSIL samples, and four of 72 (5.6%) SCC samples. The discordant results were mostly observed in samples with a low-copy number of the HPV genome or with multiple HPV infection. The sensitivity of LCR-E7 PCR was equivalent to that of MY 11/09 ECR, and false positives were less frequent in LCR-E7 PCR. LCR-E7 PCR may be useful for determining the biological activity of detected HPV types, since this method amplifies the entire E6 gene. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cervical cancer; E6–E7 genes; Human papillomavirus; Hybrid capture assay; PCR

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1. Introduction

Uterine cervical cancer is the second most common cancer in women worldwide and induced by sexually transmitted agents (NIH Consensus statements, 1996). Southern blot hybridization first revealed that human papillomavirus (HPV) infection is strongly associated with cervical cancer (Lorincz et al., 1992; Matsukura et al., 1995). Recently, the use of the polymerase chain reaction (PCR) to detect HPV DNA in cervical swab samples has proved sensitive and reliable, and has made large-scale epidemiological studies of the role of HPV in cervical neoplasia feasible (de Villiers et al., 1992; Schiffman et al., 1993; Bernard et al., 1994; de Roda Husman et al., 1994). Many such studies have demonstrated a strong association between HPV infection and cervical neoplasia.

More than 80 HPV types have been identified and more than 35 distinct types are known to infect the genital mucosal epithelium. HPV types 16 and 18 are common in cervical cancer and its precursor lesions. Many recent studies have suggested that HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 may also be associated with cervical cancer or premalignant lesions (Bosch et al., 1995; Sasagawa et al., 1996). However, the pathological role of some of these and other unique HPV types in cervical cancer remains to be established. Sensitive and reliable assays for detecting and typing human papillomaviruses (HPV) in clinical samples are essential for resolving this problem, and for managing patients with premalignant cervical lesions associated with HPV infection (Frenczy, 1995). A human HPV vaccine has recently been developed and clinical trials are about to start in some countries (News, Journal National Cancer Institute, 1997). HPV type-specific antigens may be necessary for inducing effective immunization to protect against HPV infection (White et al., 1998). Therefore, a sensitive and reliable HPV-typing test is also required for surveys and for monitoring the vaccine trials.

Many PCR-based HPV assays have already been established. The PCR-based HPV tests using L1 consensus primers (MY9/MY11) (Manos et al., 1989) and (GP5/GP6) (Snijders et al., 1990) are most commonly used worldwide, since the L1 sequence is conserved in different HPV types. Therefore, these assays can detect many HPV types in mucosal epithelium. These PCR tests also contribute to identifying new HPV types in clinical samples. However, it is also true that this causes confusion, since there are no clues as to whether the HPV types newly-identified with these assays are high-risk or not for cancer (Astori et al., 1997). On the other hand, it is known that HPV E6 and E7 are always retained and are expressed in cervical cancer tissues (Schwarz et al., 1985), and that they are essential for the immortalization of human primary keratinocytes (Hawley-Nelson, et al., 1989) and the transformation of rodent cell-lines (Yasumoto et al., 1986; Yutsudo et al., 1988). Although the E6 and E7 gene sequences are not well conserved in different HPV types, regions important for p53 (Werness et al., 1990) or Rb binding (Dyson et al., 1989) or degrading p53 (Crook et al., 1991) are conserved in high-risk HPV types. This evidence suggests that the E6 and E7 sequences of newly isolated HPV types may provide precious information about their malignant potential. Identifying new HPV isolates from cancer tissue from any organ should prove especially interesting. This prompted us to establish a new-PCR based system to detect the E6–E7 genes of many mucosal HPV types.

Four pairs of degenerate LCR and E7 primers were prepared for this PCR, in order to detect as many HPV types as possible. LCR-E7 PCR was able to detect 32 known HPV types and four new types. More than 600 clinical samples were examined for the presence of HPV DNA with this method, in order to demonstrate the clinical usefulness of this assay. It is able to amplify the entire E6 gene and the N-terminal part of E7, including the p53 and Rb binding sites. Therefore, the sequence of the PCR products may provide precious information about identified HPV types. Moreover, the cloned E6–E7 genes may be useful for in vitro experiments to determine the biological activity of the isolated E6 gene.

2. Materials and methods

2.1. Study population

A random sample of 600 eligible women (16–82 years of age) was drawn from women who had enrolled in the cancer screening program conducted in Fukui, Toyama, and Ishikawa prefectures in Japan, between April 1995 and August 1999. Women with an abnormal Pap smear received a gynecological evaluation, including a pelvic examination with a Pap smear and a colposcopic examination with a colposcopically-directed cervical biopsy, in clinics in the Departments of Obstetrics and Gynecology at Kanazawa University School of Medicine, Turuga Municipal Hospital, Fukui Prefectural Hospital, and Toyama Prefectural Central Hospital.

2.2. Study group

All 289 women in the study group had cytologically abnormal Pap smears. This group included 99 women with LSIL (low-grade intraepithelial lesion), 118 with HSIL (high-grade intraepithelial lesion), 62 with SCC (invasive squamous cell carcinoma), and ten with cervical adenocarcinoma (AdCa). Eleven cervical samples from condyloma patients and their condyloma tissue were also evaluated.

2.3. Control group

We randomly chose 300 women from the eligible women in the same population who had a cytologically normal cervix (NCX) and no past or current evidence of cervical disease on a Pap smear. The controls were matched by age with the cytologically abnormal women (within 5 years).

2.4. Sample collection and pathological evaluation

Cervical cells were collected with a cytobrush from both the endo- and exocervices of all the subjects. Each sample was divided in two: one part for the Pap test and the other to detect HPV DNA. The latter portions were suspended in phosphate-buffered-saline (PBS) and stored at −20°C until DNA purification. Experienced gynecologists performed all the procedures.

The smears were screened by three cytotechnologists. All smears suspected as abnormal and all histologic slides were reviewed independently by two surgical pathologists and the final diagnoses were based on a consensus between the pathologists. The clinical diagnoses of the women with abnormal cytology were made by histopathological evaluation of biopsy samples obtained under colposcopic guidance. The cervical lesions were classified as negative, low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and invasive cervical carcinoma using the WHO classification (Bonfiglio, 1994) and the Bethesda system (Solomon, 1989). HPV detection and the pathological diagnosis were performed independently.

2.5. HPV detection and typing using the polymerase-chain reaction (PCR)

The cervical cells were suspended in 50 mM Tris–HCl (pH 8.0) with 10 mM EDTA containing 200 μg/ml Proteinase K and incubated overnight at 37°C for cell lysis. DNA was extracted from this lysis solution by the phenol–chloroform–isoamylalcohol method. To avoid contamination, we used disposable utensils and discarded them immediately after a single use. A reaction mixture without template DNA was included in every set of PCR runs as a negative control.

Primers for a fragment of the beta-actin gene served as an internal control to assess the quality and quantity of template DNA in each PCR specimen. Four degenerate LCR forward primers (LCRF1, LCRF2, LCRF3, and LCRF4) and four E7 reverse primers (E7RI, E7R2, E7R3, and E7R4) were used to amplify E6–E7 DNA of 32 known mucosal HPV types. Details for each primer and the number of mismatches with each HPV sequence are shown in Table 1. One hundred nanograms of sample DNA was added to a 10 μl PCR solution containing 20 mM Tris–HC1 (pH 7.5), 0.5 μg BSA, 8 mM $MgCl_2$, 200 μM of each dNTP, a mixture containing 0.2 μM of each primer, and 0.25 units of KOD Dash poly-

merase (Toyobo, Tokyo, Japan). The PCR was performed using an ASTEC PCR Thermal Cycler-PC 707-02 (ASTEC, Fukuoka, Japan) with the following conditions. After a 2 min denaturing step at 95°C, the next 30–35 cycles were at 95°C for 45 s, 55°C for 20 s and 74°C for 45 s. There was a final step at 74°C for 5 min. The amplified DNA samples were run on 2% classic type ME agarose (Nakarai, Japan) or 3% NuSieve agarose gels (FMC BioProducts, Rockland, ME) in TBE buffer, and the DNA bands stained with ethidium bromide were viewed with a UV monitor camera (Epi-Light UV FA 1100, AIC, Japan) (Fig. 1).

2.6. Hybridization analysis on LCR-E7 and MY09/11 ECR products

Three micro-liters of the PCR products were loaded on agarose gels and electrophorezed. The electrophorezed DNA samples were transferred onto nylon membrane (Hybond N+, Amersham, Buckinghamshire, England). The membrane was hybridized with FITC labelled-oligoprobe and washed under the stringent condition (Tm = −10°C). Hybridized DNA was detected with non-isotope detection system using ECL DNA detection reagents (Amersham, LIFE SCIENCE). All procedures were according to the instruction manual provided from the company. Mixed MY1019 and MY 18 consensus probes were used for hybridization in detection of HPV6 and 16 L1 DNA in MY09/11 PCR, and new E6–E7 oligonucleotide probes for HPV6b-related types (H6R-AS) and HPV16-related types (H16R-AS) were used in LCR-E7 PCR. Sequences of H6R-AS and H16R-AS were as follows; H6R-AS: 5′CAATG-

Table 1
The primers and their related HPV sequences in LCR E7-PCR[a]

LCRF-primers

No.	HPV Type	HPV Group	5′position	LCRF-1: 5′-W W A R G G W G T R A C C G A A A A C G G-3′
1	6b	A10	25	A A T A G G A G G G A C C G A A A A C G G
2	11	A10	25	A A G A G G A G G G A C C G A A A A C G G
3	13	A10	26	A A T A G G T G G G A C C G A A A A C G G
4	16	A9	25	T A A G G G C G T A A C C G A A A T C G G
5	18	A7	32	A A A G G G A G T A A C C G A A A A C G G
6	26	A5	20	A A A G G G T G T A A C C G A A A A C G G
7	34	A11	22	A T A G G G T G T A A C C G A A A A C G G
8	35	A9	14	A C A G G G A G T G A C C G A A A A C G G
9	39	A7	33	A A A G G G A G T A A C C G A A A A C G G
10	40	A8	24	A A A G G G T G T A A C C G A A A A C G G
11	42	A1	34	T A A G G G A G T A A C C G A A T T C G G
12	45	A7	29	A A A G G G T G T A A C C G A A A A C G G
13	51	A5	36	A A G G G T T A T G A C C G A A A A C G G
14	52	A9	28	A T A G G G T G T A A C C G A A A A C G G
15	53	A6	42	A T A G G T T A G G A C C G A A A A C G G
16	57	A4	32	A T A G G G C G T A A C C G A A A A C G G
17	58	A9	30	C T A G G G T G T A A C C G A A A A C G G
18	59	A7	-13	A A A G G G T G T A A C C G A A A A C G G
19	72	A3	28	G T A A G A C A A G A C C G A A A A C G G
20	73	A11	22	A A A G G G T G T A A C C G A A A A C G G

No.	HPV Type	HPV Group	5′position	LCRF-2: 5′-W W W G G G T C S A A C C G A A A A C G G-3′
	6	A10	25	A A A C G G T T C A A C C G A A A A C G G
	11	A10	25	A A A C G G T T C A A C C G A A A A C G G
	13	A10	26	A A A C G G T T T A A C C G A A A A C G G
21	31	A9	42	A A A G T G G T G A A C C G A A A A C G G
22	32	A1	29	T A T C G G T T T A A C C G A A A A C G G
	42	A1	49	A T T C G G T T T A A C C G A A A A C G G
23	44	A10	43	T T C G G T T C C A A C C G A A A A C G G
24	54	A?	25	T A A G G G A G G G A C C G A A A A C G G
25	55	A10	40	T T C G G T T C C A A C C G A A A A C G G
26	70	A7	31	A A A G G G A G G C A C C G A A A A C G G

No.	HPV Type	HPV Group	5′position	LCRF-3: 5′-W R G G K T T A G G A C C G A A A A C G G-3′
27	30	A6	42	T A G G T T T T C A A C C G A A A A C G G
	51	A5	36	A A G G G T T A T G A C C G A A A A C G G
	53	A6	42	A T A G G T T A G G A C C G A A A A C G G
28	56	A6	43	A G G G T T T A A G A C C G A A A A C G G
29	66	A6	43	T G G G T T T A G G A C C G A A A A C G G
30	68(ME180)	A7	3889	A A C G G T C A T G A C C G A A A A C G G

No.	HPV Type	HPV Group	5′position	LCRF-4: 5′-G T A R G G Y R A G A C C G A A A R C G G-3′
31	33	A9	44	G T A G G G T G T A A C C G A A A G C G G
32	61	A3	22	G T A G G T T G G C A C C G A A A A C G G
	72	A3	28	G T A A G A C A A G A C C G A A A A C G G

E7R-primers

No.	HPV Type	HPV Group	E7R-1: 5′-T C M K C C T C T K C Y T C T G A G Y T G T-3′	3′position
1	6b	A10	T C C A C C T C A T C T T C T G A G C T G T	642
2	11	A10	T C C A C C T C A T C T T C T G A G C T G T	642
3	13	A10	T C C A C C T C G T C T T C T G A G C T G T	638
4	34	A11	T C A T C C T C A T C C T C T G A G T T G T	660
5	40	A8	T C A T C T T C T G A G T C T G A G C T G T	633
6	44	A10	T C C A C C T C A T C T T C T G A G C T G T	642
7	55	A10	T C C A C C T C A T C C T C T G A G C T G T	639
8	57	A4	T T A G T A T C T T C T T C T G A G T T G T	639
9	61	A3	T C C A C C T C T G A C T C T G A G C T G T	627
10	72	A3	T C T G A C T C T G A C T C T G A G C T G T	633
11	73	A11	T C A T C C T C A T C C T C T G A G T T G T	661

No.	HPV Type	HPV Group	E7R-2: 5′-T C W T C M T C H T C R T C T G A G C T G T-3′	3′position
12	16	A9	T C A T C C T C C T C C T C T G A G C T G T	671
13	26	A5	G T T T C A T C C T C A T C T G A G C T G T	680
14	30	A6	T C A T C C T C T T C C T C T G A G C T G T	674
15	31	A9	A C A T C C T C C T C A T C T G A G C T G T	669
16	32	A1	T C A T C A T C T T C A T C T G A G G T G T	682
17	33	A9	C C T T C A T C C T C A T C T G A G C T G T	682
	34	A11	T C A T C C T C A T C A T C T G A G T T G T	660
18	35	A9	T C T T C C T C C T C C T C T G A G C T G T	671
19	42	A1	T G G T C A T C T T C A T C T G A G C T G T	657
20	51	A5	T C A T C C T C C T C C T C T G A G C T G T	669
21	52	A9	G T A T C C T C C T C A T C T G A G C T G T	662
22	53	A6	T C A T C C T C A T C C T C T G A G C T G T	678
23	56	A6	T C A T C C T C A T C C T C T G A G C T G T	681
24	58	A9	A T T T C A T C C T C G T C T G A G C T G T	683
25	66	A6	T C A T C C T C A T C C T C T G A G C T G T	681
	73	A11	T C A T C C T C A T C C T C T G A G T T G T	661

E7R-3

No.	HPV Type	HPV Group	5′-C A C W A W A T T K T G T G A C G C T G T G-3′	3′position
18		A7	C A C A A C A T T G T G T G A C G T T G T G	777
59		A7	C A C T G T A T T G T G T G A C G C T G T G	788
45		A7	C A C A A A A T T T T G T G A C G C T G T G	777
59		A7	C A C A C A A T G T T G T G A C G C T G T G	741
(ME180)		A7	C A C T G A A T T G T G T G A C G C T G T T	4629
70		A7	C A C T G T A T T T T G T G A C G C T G T G	788

HPV Type	HPV Group	5′position	Sequence
73	A11	22	A A A G G G T G T A A C C G A A A A C G G

R-4

No.	HPV Group	5′-C A T C A G A G T C T T C T A A T T G C T C-3′	3′position
54	A?	C A T C A G A G T C T T C T A A T T G C T C	632
(ME180)	A7	C G T C T G A A T C T C C T A A T T G C T C	4538
70	A7	T G T C T G A A T C T T C T A A T T G C T C	706
42	A1	C A T C T G A G C T G T C C A A T T G T T C	647

▒▒▒ : mismatch sequence

[a] R, A/G; W, A/T;, Y, C/T; K, G/T;S, G/C; V, G/A/C; H, A/T/C; B, G/T/C; N, A/G/C/T.

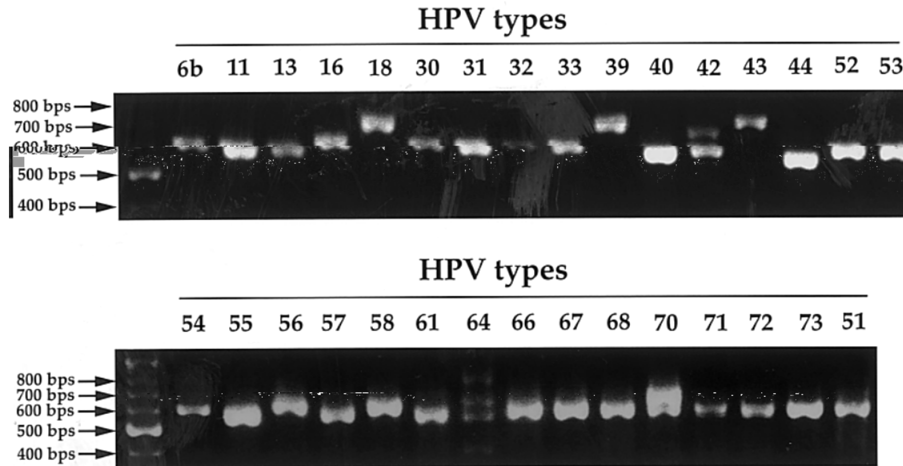

Fig. 1. Detection of mucosal HPV types with LCR-W PCR. Four picograms of cloned HPV DNA (all types except for HPV51) and 100 ng of cellular DNA from a clinical sample which was infected with HPV51 were used for amplification with PCR. Three microliters; of PCR products were run on agarose gels and viewed with a UV-monitor camera after ethidium bromide staining.

DAARCAGCGACCCTTCCA-3′, H16R-AS 5′-AATTGCTCATARCAGTAKAGRTCAGTTG-3′ (D: A or G or T, R: A or G, K: G or T.).

2.7. Hybrid capture assay

One microgram of purified DNA was tested for the presence of high and low-risk HPV types using the hybrid capture assay, generation 1 (DIGENE) (Schiffman et al., 1995) in the MBC (Mitsubishi BCL) central laboratory (Tokyo, Japan).

2.8. Statistical analyses

We used the Chi-square test or Fisher's exact probability test to determine the statistical difference between the results of the LCR-E7 PCR and HCA-I assays.

3. Results

3.1. The LCR-E7 PCR test

At present, more than 35 mucosal HPV types have been identified in cervical epithelium. To study the association between HPV infection and cervical lesions, we designed four sets of primers to amplify the E6–E7 DNA of all of these mucosal HPV types. These primers and the target sequences of various HPV types are shown in Table 1. The primers LCRF- 1, 2, 3, 4 and E7R-1, 2, 3, 4, were designed to amplify known 32 mucosal HPV types. Each E7R primer was specific to certain HPV groups determined in the phylogenetic analysis (Bernard et al. 1994). E7R1 was specific to groups A3 (HPV61, 72) and A10 (HPV6b, 11, 44 and 55); E7R2 was specific to groups A1 (HPV32 and 42), A5 (HPV26, 51), A6 (HPV30, 53, 56, 66), A9 (HPV16, 31, 33, 35, 52 and 58), and A11 (HPV34, 73), and E7R3 was specific to group A7 (HPV18, 39, 45, 59, 68 and 70). E7R4 was specific for HPV54. Such association between primer and HPV group was not apparent in LCRF primers, (Table 1). A mixture of these primers was able to amplify 27 cloned HPV DNA which are known their sequences such as HPV6b, 11, 13, 16, 18, 30, 31, 32, 33, 39, 40, 42, 43, 44,52, 53, 54,55, 56,57,58, 61, 66, 68, 70, 72, and 73 (Fig. 1A). The E6–E7genes of HPV64, 67, and 71 were also amplified, although their nucleotide sequences have not yet been reported (These clones were kindly provided by Dr T. Matsukura, NIH of Japan, Tokyo, Japan). This

method is able to detected HPV34 and 45, since their genes have only one mismatched nucleotide with our primers (Table 1). In addition, HPV26, 35, 51 (shown in Fig. 1), and 59 were detected in some clinical samples, although these clones were not available for developing this assay. In total, LCR-E7 PCR test was able to detect 36 HPV types.

3.2. The sensitivity of the LCR-E7 PCR assay and HPV typing

We tested the sensitivity of the LCR-E7 PCR assay using cloned HPV6b and 16 DNA, Four picograms of HPV DNA was serially diluted in 10 μl of solution containing 100 ng of placental DNA and subjected to LCR-E7 PCR. In ethidium bromide staining of LCR-E7 PCR products, HPV DNA bands were detected in the samples containing more than 4×10^{-3} pg (diluted at 1×10^{-3}) of HPV6b and 16 DNA, but not when there was only 4×10^{-4} pg of HPV DNA (Fig. 2). This sensitivity is equivalent to detecting 0.01 copy of HPV genome per cell (about 6.7 copies of virus genome). Hybridization analysis using, the non-isotope detection system increased this sensitivity at ten times (about 6.7 copies of virus genome) (Fig. 2) MY11/09-PCR is one of the most reliable PCR-based assays for detecting the HPV L1 region, since it has been used in many epidemiological studies worldwide (Bosch et al., 1995). In our assay condition- LCR-E7 was a little more sensitive than MY09/11 -PCR, However, the difference appears to be within the limit of inter-assay variation (Fig. 2).

The smallest PCR products ranged in size from 600 to 618 base pairs (bps) (Fig. 1). These were the products of HPV6b, 11, 13, 44, and 55 (mostly benign types). The longest products ranged from 741 to 758 bps and were the products of types

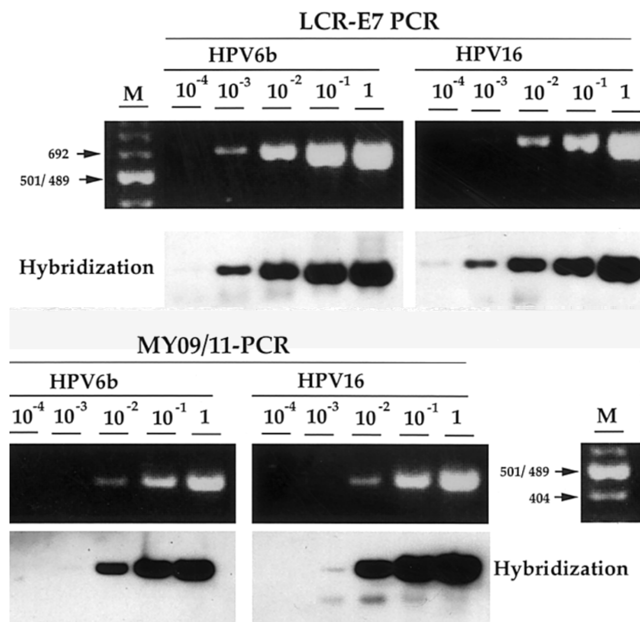

Fig. 2. Sensitivities of the LCR-E7 and MY09/11 PCR assays. Serially diluted HPV plasmid DNA (4 pg, 0.4pg, 0.04 pg, 4×10^{-3} pg, 4×10^{-4} pg, 4×10^{-5} pg) with 100 ng of placental DNA solution was used for amplification with LCR E7-PCR and MY09/11-PCR. Three microliters of the PCR products were electrophorezed on agarose gels. The upper figure shows electrophorezed samples on agarose gels stained with ethidium bromide, and the lower one shows hybridization analysis of the same PCR products. Hybridization was performed in the condition as described in Section 2.

Table 2
HW typing by restriction-fragment-length polymorphism (RFLP)

Type	Size	*Ava*II	*Rsa*I	*Dde*I	*ACC*I	*Bam*HI
Cut pattern-1[a]						
HPV26	661[b]	661	325/176/122/38	419//225/17	661	575/86
HPV58	654	654	654	367/199/71/17	512/142	654
HPV51	650	650	3321171/96/51	633/17	650	650
HPV51	628	628	255/243/130	426/109/43/33/17	4701158	628
HPV44	600	600	375/179146	583/17	600	600
HPV11	618	60919	540178	323/278/17	618	618
HPV55	600	600	600	583/17	600	535/65
HPV13	613	604/9	613	279/169/148/17	569/44	613
Cut pattern-2						
HPV32	655	598/57	378/170/83124	344/275/36	655	655
HPV42	624	56/163	233/133/127/52/42/37	607/17	339/285	624
HPV35	658	553/105	633/25	641/17	470/188	658
HPV52	635	532/103	635	332/286/17	436/199	552/83
HPV30	633	463/99/71	337/112/63/43/23/22/17	320/185/111/17	633	633
Cut pattern-3						
HPV45	748	564/184	472/276	607/141	426/322	748
HPV18	746	540/181/25	746	425/144/89/88	496/250	658/88
HPV39	756	474/190/51/25/16	756	756	488/268	756
HPV70	670	501/102/51/16	670	758	529/103/38	670
HPV59	755	499/187/69	504/251	516/239	755	666/89
HPV68	650	482/102/50/16	414/236	241/223/109/77	612/38	650
HPV73	638	478/160	296/195/147	419/202/17	638	638
HPV56	639	463/176	521/96/22	435/144/43/17	639	639
HPV34	639	478/161	296/148/137/58	525/97/17	639	639
HPV33	639	487/152	585/54	438/105/79/17	639	639
HPV66	639	454/176/9	278/243/96/22	478/111/33/17	340/160/139	639
HPV57	608	465/143	547/61	287/231/73/17	608	608
Cut pattern-4						
HPV54	608	381/126/92/15	381/84/76/37/30	608	491/117	608
HPV16	647	386/173/88	474/173	630/17	647	647
HPV6b	618	385/224/9	470/148	368/233/17	594/24	618
HPV40	610	331/96/87/76/20	278/1661/29/37	396/197/11/6	486/124	610
Cut pattern-5						
HPV61	606	282/224/60/24/16	390/179/37	327/219/43/17	404/202	606
HPV72	606	282/190/114/20	569/37	339/190/60/17	606	606
HPV53	637	278/179/170/10	220/219/96/60/23/19	363/220/37/17	637	637

[a] Classification of cut patterns by digestion with *Ava*11.
[b] Size of DNA.

related to HPV18 (HPV18, 39, 45, 59 and 70) and HPV43 (the E7 sequence is not reported). These two groups were easily distinguished from each other by size (Table 1 and Fig. 1). However, other types, such as those related to HPV16, 51, and 56, produced intermediate-sized products from 628 to 661 bps, which were difficult to distinguish from the shortest and longest products. Two bands were visible for HPV types 42 and 70 and four bands were for HPV64 (HPV64 not reported). We tried to identify most of these HPV types using restriction fragment length polymorphism (RFLP) (Table 2). The PCR products were digested with three restriction enzymes: *Ava*ll, *Rsa*l, and *Dde*I.

The RFLP analysis consistently determined the HPV types. Typical examples are shown in Fig. 3.

3.3. Detecting and typing HPV DNA in clinical samples using LCR-E7 PCR and hybrid capture assays

We examined 611 clinical samples for the presence of HPV DNA using LCR-E7 PCR and first generation hybrid capture (HCA-I) assays. HCA-I detects low-risk HPV types using mixed probes for HPV6, 11, 42, 44, and 55, and high-risk types with mixed probes for HPV16, 18, 31, 33, 35, 52, and 56. In the HCA-I test, 4% (13/300), 55% (54/99), 76% (90/118), 79% (49/62), and 70% (7/10) of samples were positive for either low or high-risk HPV types in normal services (NCX), low-grade squamous, intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), squamous cell carcinoma (SCC), and adenocarcinoma (AdCa), respectively. In the LCR-E7 PCR, 7% (22/300), 70% (69/99), 89% (105/118), 90% (56/62) and 80% (8/10) were positive in NCX, LSIL, HSIL, SCC, and AdCa, respectively (Table 3). In vulvar or vaginal condyloma patients, 100% (11/11) of the condyloma tissues were positive with both the HCA-I and LCR-E7 PCR assays, whereas 73% (8/11) and 91% (10/11) of their cervical samples were positive using the HCA-I and LCR-E7 PCR assays, respectively. LCR-E7 PCR assay was marginally more sensitive in SCC ($0.1 > P > 0.05$) and signifi-

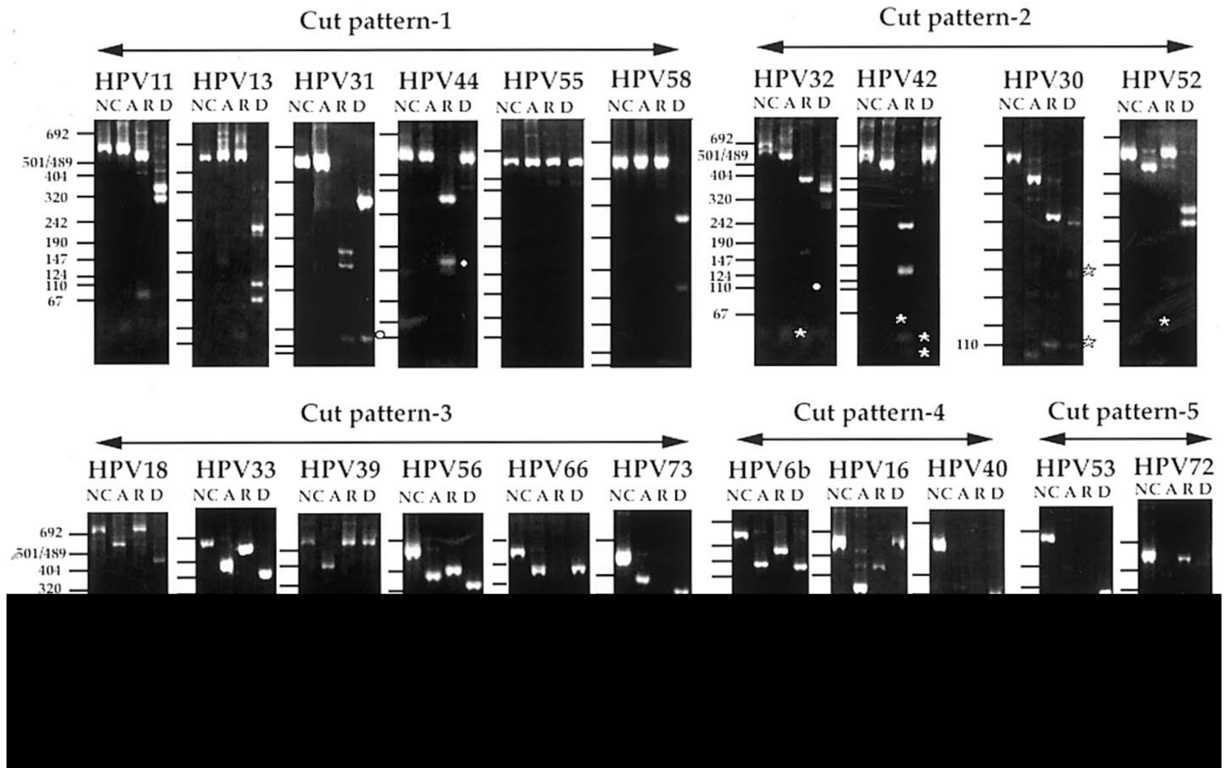

Fig. 3. RFLP analysis for HPV typing in LCR-W PCR NC: No cut PCR product, (A) digested with *Ava*II, R, digested with *Rsa*1, D, digested with *Dde*1. A white asterisk indicates a band faintly observed and a white circle indicates a band incompletely digested. Black bars on the left of each figure show positions of the molecular weight markers. Cut pattern 1–5 are five different cut patterns determined by the *Ava*11 digestion as indicated in Table 3.

Table 3
Comparison of the results in HCA-I and LCR E7-PCR

Patients	Materials	N	Hybrid capture assay (HCA-I)						LCR-E7 PCR	
			Low-risk[a] Positive	%	High-risk[b] Positive	%	All types Positive	%	All types Positive	%
Condyloma	Tissue	11	11	100	8	73%	11	100%	11	100%
	Cervical cells	11	6	55%	6	55%	8	73%	10	91%
Normal	Cervical cells	300	2	1%	12	4%	13	4%	22	7%
LSIL	Cervical cells	99	13	13%	53	54%	54	55%	69*	70%
HSIL	Cervical cells	118	4	3%	90	76%	90	76%	105**	89%
SCC	Cervical cells	62	4	7%	49	79%	49	79%	56***	90%
Ad Ca	Cervical cells	10	0	0%	7	70%	7	70%	8	80%

[a] Low-risk HW types including HPV6, 11, 42, 43, 44.
[b] High-risk HW types including WV1 6, 18, 31, 33, 35, 45, 51, 52, 56.
* $0.05 < P < 0.1$, marginally higher positivity with that of HCA-I test.
** $P < 0.05$, significantly higher positivity with that of HCA-I test.
*** P, $0.05 < P < 0.1$, marginally higher positivity with that of LCR E7-PCR test.

cantly more sensitive in LSIL and HSIL ($P < 0.05$) than the HCA-I assay, while the sensitivities of both assays did not differ with the NCX, condyloma, and AdCa samples. Infected HPV types was identified by the restriction fragment length polymorphism (RFLP) on PCR products. Sequence analysis were performed in some of these samples in which HPV types were not determined by RFLP analysis because of faint DNA bands or multiple HPV infection. HPV typing demonstrated that 25 HPV types, including HPV6b, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 56, 58, 59, 61, 66, 68, 70, 72 and 73, were identified in our clinical samples.

3.4. Comparison with L1 PCR test in clinical samples

It is well known that HPV genomes are integrated into the host genome during the process of carcinogenesis and that certain HPV gene loci are interrupted by the integration. In this context, it may be reasonable to target the E6–E7 genes for detecting HPV, since these loci are rarely interrupted by the integration event. The sequences targeted for PCR may produce differences in the detection rate of the HPV genome in cancer tissues. Thus, we compared the rate of detecting HPV DNA in clinical samples between our LCR-IE7 PCR (for E6–E7) method and the MY 11/09-PCR (for L1) method.

Four hundred and twenty-four clinical samples were tested with both the LCR-E7 and MY09/11-PCR methods. These samples were a subset of the samples examined with the HCA-I test and LCR-E7 PCR described above. The PCR reaction for either assay was considered positive when an appropriately sized band ($\approx 600–750$ bps for LCR E7 and about 450 bps for MY09/11-PCR) was visualized on an agarose gel stained with ethidium bromide using a monitor camera. The HPV types were determined using RFLP analysis with both assays. We performed sequence analysis of the cloned PCR products if the HPV type was not determined by the RFLP analysis as the same reasons described above. In LCR-E7 PCR test 7% (13/185) of NCX, 61% (52/85) of LSIL, 80% (66/82) HSIL and 92% (66/72) of invasive cervical cancer (ICCA: SCC + AdCa) showed positive for HPV, while 7% (13/185) of NCX, 65% (55/85) of LSIL 85% (70/82) HSIL and 90% (65/72) of ICCA were positive for HPV in MY09/11-PCR. The overall sensitivities of LCR-E7 PCR and MY09/11-PCR were not statistically different for the clinical samples.

Some LSIL and HSIL samples that were negative by LCR-E7 PCR were positive by MY09/11-PCR (Table 4). However, we could not determine

Table 4
Discordant results between LCR-E7 and MY09/11-PCR

CASE	AGE	HCA-I Low-risk index (RLU)	HCA-I High-risk index (RLU)	LCR-E7 PCR HPV type RFLP	LCR-E7 PCR HPV type SQ	LCR-E7 PCR False positive/negative	MY11/09 PCR HPV type RFLP	MY11/09 PCR HPV type SQ	MY11/09 PCR False positive/negative
NORMAL									
1	28	0.07	0.13	UD			CP6108		
2	17	0.1	5.09	N			UD	Human gene-1[b]	False positive
LSIL									
1	56	0.59	8.5	N		False negative	UD	39, UD	
2	34	0.35	4.2	UD	6[a]		UD	56	
3	25	9.28	0.1	UD	44, 51		N		False negative
4	46	0.08	0.8	54			CP8304		
5	21	0.51	0.6	N		False negative	11		
6	51	0.05	0.5	N		False negative	53	53	
7	48	0.07	0.1	N		False negative	MM4	MM4	
8	46	0.39	0.6	N			UD	Human gene-1	False positive
9	40	0.44	0.6	N			UD	Human gene-1	False positive
10	45	0.26	0.6	N			UD	Human gene-1	False pos
11	38	0.39	0.6	N			UD	Human gene-1	False pos
12	50	0.47	0.5	N			UD	Human gene-1	False pos
13	24	0.36	0.5	N			UD	Human gene-1	False pos
14	57	0.45	0.4	N			UD	Human gene-2	False pos
15	43	0.49	0.4	N			UD	Human gene-1	False pos
16	20	0.39	0.3	N			UD	Human gene-1	False pos
17	57	0.46	0.3	N			UD	Human gene-1	False pos
18	62	0.43	0.3	N			UD	Human gene-1	False pos
HSIL									
1	55	0.68	469.6	UD	16		52	52	
2	35	0.06	90.1	UD	42, 51, 56		UD	56	
3	45	0.57	35.3	31			UD	58	
4	49	0.4	16.9	UD	56		UD	31	
5	39	0.3	2.0	N		False negative	66	66	
6	34	0.37	1.9	UD	31		UD	16	
7	42	0.06	0.8	N		False negative	58		
8	42	0.47	1.0	N			UD	Human gene-2	False pos
ICCA									
1	35	0.48	1822.6	11, UD	11, 51		UD	35	

the HPV types in some of these samples, because the RFLP analysis produced an unknown restriction pattern in MY09/11-PCR test. Sequence analysis of the cloned PCR products from these samples revealed that some of them had no open reading frame and had a sequence homologous to human genes. No human sequences were identified in the 600–750 bps products produced in the LCR-E7 PCR, although some shorter products of the LCR-E7 PCR were homologous to human genes (data not shown). The false positives were more frequent in MY09/11-PCR than in LCR-E7 PCR test. On the other hand. the false negatives in LCR-W PCR were more frequent than in MY09/11-PCR although it was not statistically significant. The discordant results between LCR-E7 and MY09/11-PCR were resulted mainly from infection with multiple HPV types, a low-copy number of the HPV genome (less than 5 luciferase units in the HCA-I test), or infection with unknown or unique HPV types (MM4 and CP8304) (Table 4).

4. Discussion

The hybrid capture assay is a sensitive, reliable assay for detecting both low and high-risk HPV types (19). In our study, the LCR-E7 PCR assay was more sensitive than the first generation hybrid capture assay (HCA-I). One possible explanation about the difference in the sensitivities of the two assays is that the HCA-I test fails to detect low-copy numbers of the HPV genome in malignant lesions in which the HPV DNA is integrated. But this is not a reason, since there were no statistical differences between the sensitivity of HCA-I and LCR-E7 PCR in invasive cervical cancer (SCC and AdCa). The difference in the sensitivities of LCR-E7 PCR and the HCA-I test may be mostly due to differences in the spectrum of detectable HPV types. Probes for some HPV types, such as HPV58, are not included in the HCA-I test although this has been amended in the second-generation assay (HCA-II). In our study, HPV58 was the third most prevalent type in cervices of Japanese women (HPV16, 31% (84/270), HPV52, 13% (34/270), HPV58, 10% (28/270)).

Many HPV tests utilizing PCR have already been established. Of these, PCR assays using MY09/MY11 degenerate primers (Manos et al., 1989) and GP5/GP6 consensus primers (Snijders et al., 1990) are the most commonly used worldwide. There are some PCR-based HPV assays for the E6–E7 region that use consensus primers (Fujinaga et al., 1991; Yoshikawa et al., 1991). However, these E6–E7 assays may not be optimal, since the number of detectable HPV types is limited or HPV typing by RFLP is sometimes difficult in these tests for small PCR products. Restriction fragments shorter than 60 bases are difficult to distinguish from the bands of primer dimers or the unknown artifacts that are occasionally seen when PCR is applied to clinical samples. For this reason, the PCR products amplified with these E6–E7 PCR methods may be too short (≈ 250 bps) for reliable typing by RFLP analysis. The PCR products of our LCR-E7 PCR method are more than 600 bases long, which is a suitable size for HPV typing by RFLP analysis. In addition, our PCR produces different sized PCR products for different groups of HPV types as seen in HPV6b-related and HPV18-related types (Fig. 1). This maybe useful for predicting the presence of multiple HPV infection in the first screening. However, this prediction may not be true for HPV42, 64 and 70 infection. since different sized-bands were amplified with LCR-E7 PCR for these HPV types (Fig. 1)

When 424 samples were examined with both the LCR-E7 and MY09/11-PCR methods, the results of HPV typing concurred in 96% (405/424) of the samples examined. The samples showing discordant results included three false negatives in MY09/11-PCR and seven false negatives in LCR-E7-PCR. Seven of these false negatives showed low relative luciferase unit (RLU) (lower than five) in hybrid capture assay (Table 4). This suggests that low viral load may produce such discordant results. The other discordant results may be due to infection with multiple HPV types as observed in number 3 in LSIL. Number 2 in HSIL and number 1 and number 3 in ICCA or infection with unique HPV types (CP 8304 and MM4 in LSIL (Table 4), The MY09/11 and GP5/GP6 PCR assays for the L1 gene can detect 33 and 27

mucosal HPV types, respectively. The LCR-E7 PCR assay is able to detect 36 HPV types. This suggests that there is no difference in the spectrum of detectable HPV types of these assays. However, we have to be careful in evaluating the PCR products from these PCR assays, especially if the PCR products show an unknown restriction pattern in the RFLP analysis. These unknown patterns might be accidentally amplified human gene sequences, as we showed occurred with MY09/11-PCR. To avoid false positives, hybridization analysis of the PCR-products may be necessary when screening for HPV infection with LCR-E7, as is done in MY09/11-PCR.

A clinicopathological classification of HPV types has not yet been established, although more than 35 distinct mucosal HPV types have been identified by recently established PCR-based HPV tests. The availability of HPV typing and the lack of a clinicopathological classification may confuse many clinicians, who will have to decide how to treat patients with a cytologically normal cervix, but a positive HPV test. Establishing an easy, reliable HPV typing test to use with clinical samples for collecting a large amount of data from different countries is a very important step for establishing a clinicopathological classification of HPV in the future. The LCR-E7 PCR assay may be one of the best assays for these purposes. As described in the introduction, the sequences of HPV E6–E7 genes amplified with LCR-E7 PCR might provide precious information on the biological activity of the HPV, since the E6 and E7 genes contribute to the malignant progression of host cells, and high-risk HPV types conserve the sequences essential for p53 or Rb binding and p53 degradation within these genes. Furthermore, cloned E6–E7 DNA from LCR-E7 PCR can be used for in vitro experiments to determine the biological activity of the E6 gene, since the PCR product includes the entire E6 gene.

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