CHAPTER V

RESULTS

1. HPV detection by L1-PCR

In order to detect HPV-DNA, we optimised PCR condition using either set of primers (MY09/11 or L1C1/C2). Firstly, we tested sensitivity of detection by amplifying standard HPV-16 DNA at various concentration (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg). Sensitivity of L1-PCR was higher when the L1 was amplified by MY09/11 primer set ($\geq$ 1 fg) (Fig. 7) than the one that amplified by L1C1/C2 primer set ($\geq$ 1 pg) (Fig. 8). Moreover, MY09/11 primer set could be used to amplify all 7 standard HPV types (HPV-6, 11, 16, 18, 31, 33 and 35), whereas L1C1/C2 primer set could amplify only 6 HPV types (HPV-6, 11, 16, 18, 31 and 33) (Fig. 9 and 10, respectively). Secondly, we tested the efficiency of detection using either primer set on paraffin-embedded tissues. A total of 30 paraffin-embedded tissues was used for this purpose. The human β-globin (268 bp) could be amplified in all samples (Fig. 11). Nine out of 30 (30%) samples were positive with MY09/11 L1-PCR and 30 out of 30 (100%) samples were positive with L1C1/C2 L1-PCR (Table 9). The results described above showed the difficulty in amplify in L1 gene by MY09/11 primers in paraffin-embedded tissues. The formaldehyde fixation might cause DNA fragmentation and hence being difficult to amplify longer PCR product by MY09/11. Therefore, further HPV detection in tissues section by L1-PCR and identification of HPV type was performed using L1C1/C2 primer set.

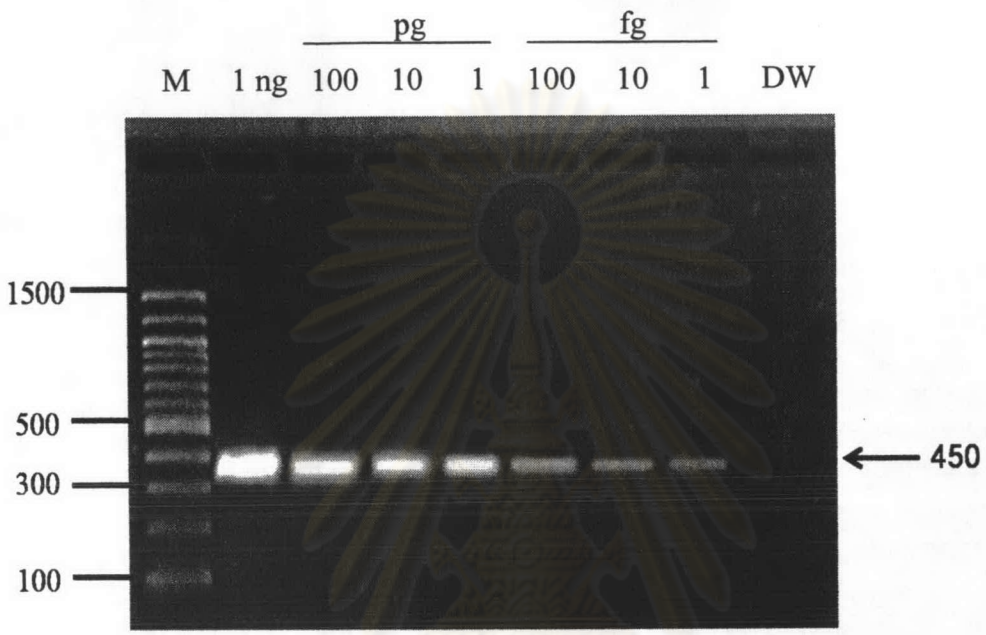


Figure 7. The sensitivity of HPV L1-PCR using MY09/11 primer. The standard HPV-16 DNA was 10-fold serially diluted from 1 ng to 1 fg. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

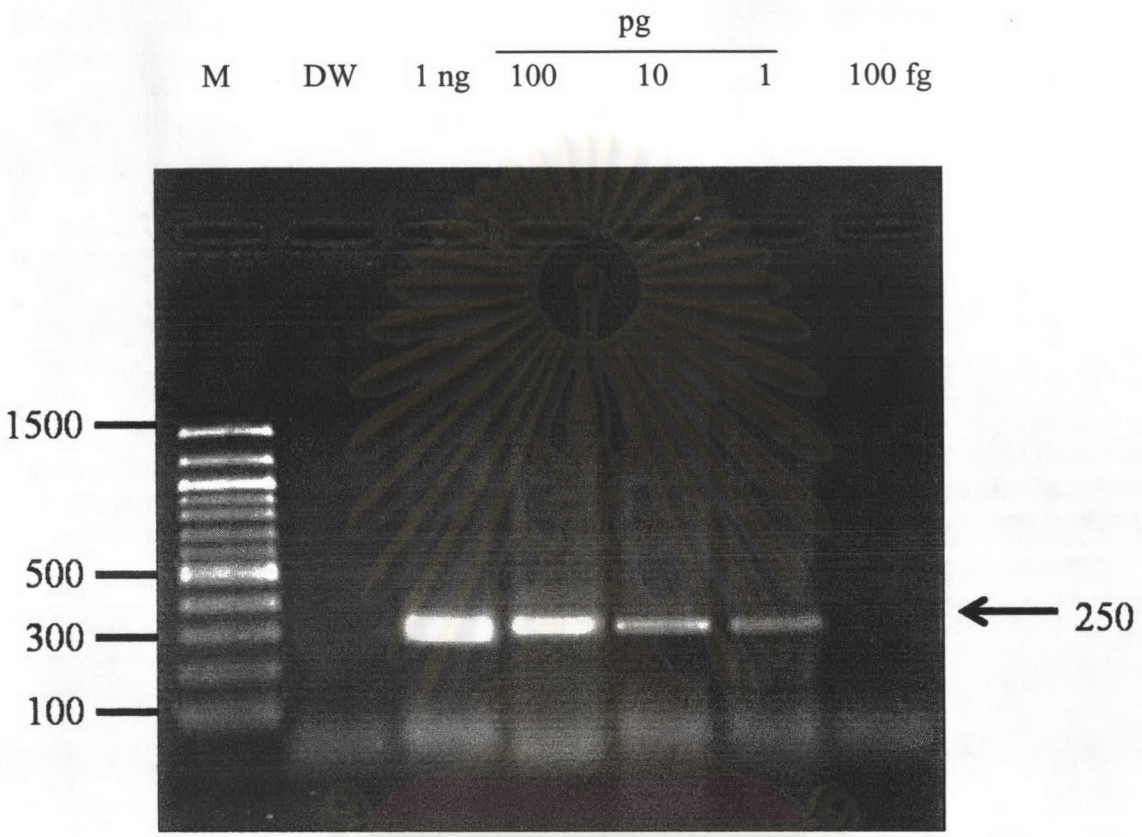


Figure 8. The sensitivity of HPV L1-PCR using L1C1/C2 primer. The standard HPV-16 DNA was 10-fold serially diluted from 1 ng to 100 fg. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

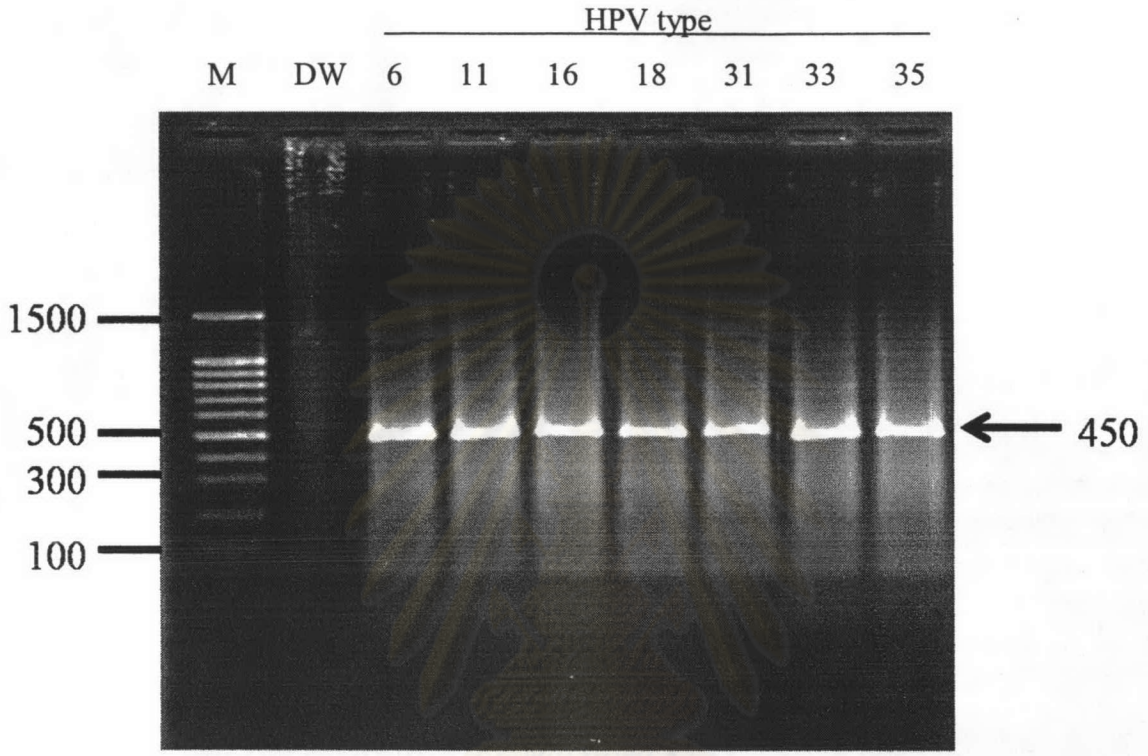


Figure 9. Amplification of L1 gene in 7 standard HPV DNA using MY09/11 primer set. The primers could amplify all 7 HPV types: HPV-6, 11, 16, 18, 31, 33 and 35. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

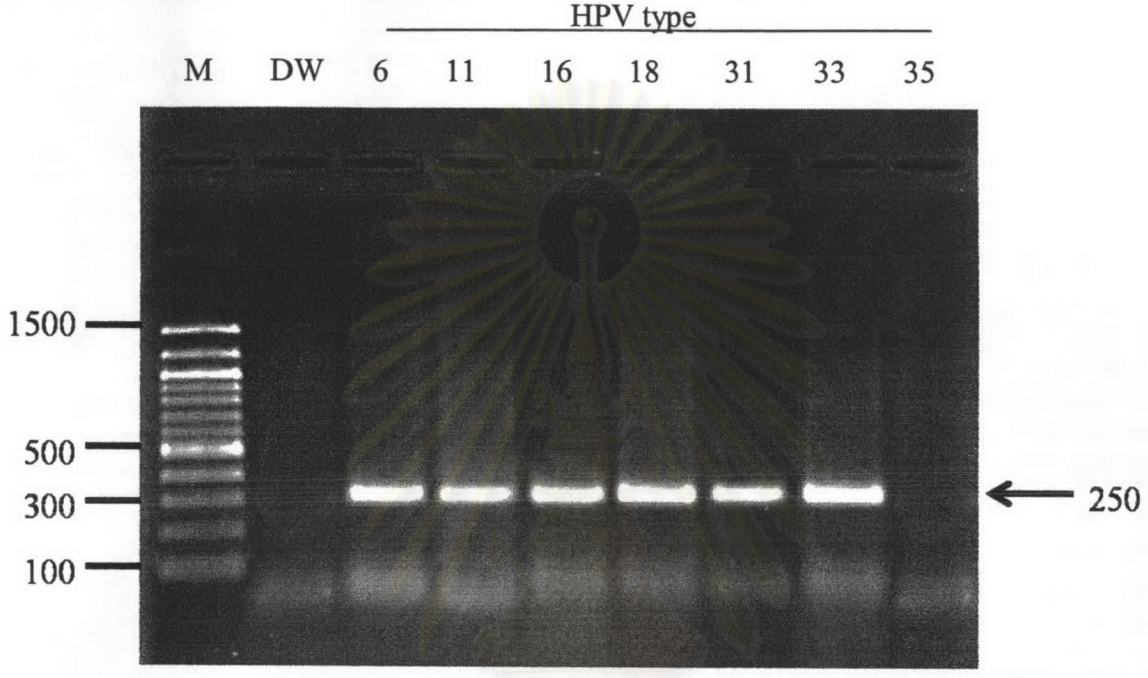


Figure 10. Amplification of L1 gene in 7 standard HPV DNA using L1C1/C2 primer set. The primers could amplify only 6 HPV types: HPV-6, 11, 16, 18, 31 and 33. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

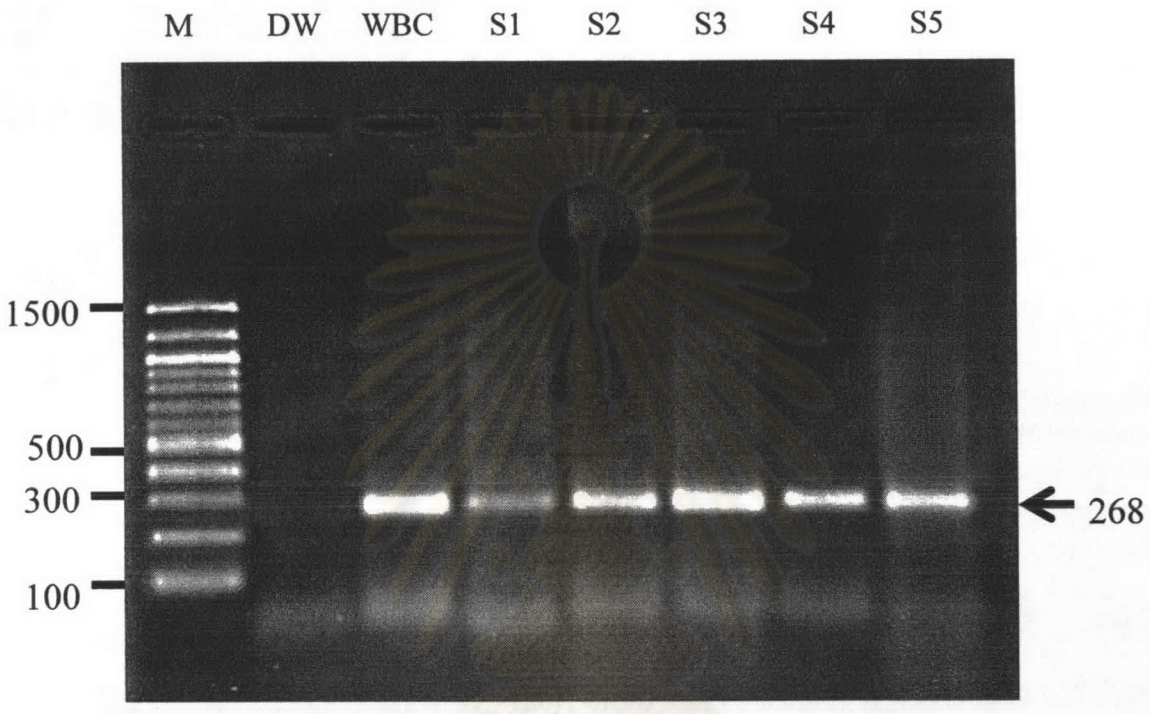


Figure 11. Amplification of human β-globin gene from clinical specimens. The β-globin gene could be amplified from tissue specimens. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.), S1 – S5: paraffin-embedded tissue specimens.

Table 9. The efficiency of HPV-L1 PCR detection by MY09/11 primer set and L1C1/C2 primer set on paraffin-embedded tissues.

Primer set / Samples	MY09/11	L1C1/C2
S1	-	+
S2	+	+
S3	-	+
S4	-	+
S5	-	+
S6	-	+
S7	+	+
S8	+	+
S9	-	+
S10	-	+
S11	+	+
S12	-	+
S13	-	+
S14	-	+
S15	-	+
S16	+	+
S17	-	+
S18	+	+
S19	-	+
S20	-	+
S21	-	+
S22	-	+
S23	+	+
S24	-	+
S25	-	+
S26	-	+
S27	-	+
S28	+	+
S29	-	+
S30	+	+

+: L1-PCR positive; - : L1-PCR negative

2. HPV detection and typing in patients

2.1 Demographic and clinical information

Total of 75 women (age 27 – 72, mean: 44.80 years), who attended the Gynecology Clinic at King Chulalongkorn Memorial Hospital during August 2003 – June 2004 were enrolled into this study. Age of patients in CIN group was in a range of 27 – 63 years (mean: 41.80 years). The population in CIN group were 7 patients with CIN I (age 32 – 57, mean: 42.86 years), 4 patients with CIN II (age 27 – 49, mean: 39.50 years) and 28 patients with CIN III (age 29 – 63, mean: 41.80). In CaCx group (age 30 – 72, mean: 47.00 years) were 15 patients with adenocarcinomas (age 37 – 68, mean: 45.60 years) and 21 patients with squamous cell carcinomas (age 30 – 72, mean: 47.81 years). The demographic and clinical information in each group was summarised in Table 10.

2.2 Prevalence of HPV infection in patients with CIN and CaCx

HPV detection and typing in CIN and CaCx patients were performed on fresh or paraffin-embedded tissues section by L1C1/C2-PCR and RFLP as described in materials and methods. Forty seven out of 75 (62.67%) patients were HPV-L1 positive (Table 11 and Fig. 12). The prevalence of HPV infection in CIN group was 41.03% (16/39) and CaCx group was 86.11% (31/36) (Table 11). Within CIN groups, prevalence of HPV infection in CIN I, CIN II and CIN III was 57.14% (4/7), 0% (0/4) and 42.86% (12/28), respectively (Table 11). In CaCx groups, prevalence of HPV infection in adenocarcinoma and squamous cell carcinoma was 93.33% (14/15) and 80.95% (17/21), respectively (Table 11).

2.3 Prevalence of HPV types in patients with CIN and CaCx

HPV typing was performed by RFLP. Six standard HPV types, for example, were typed by RFLP (Table 12 and Fig. 13 – 15). HPV-16 and HPV-18 were found predominantly in CIN group (43.75%), followed by untyped (12.50%) and mixed infection HPV-18/31 (6.25%) (Table 11). On the other hand, HPV-18 was predominant in CaCx group (48.39%), followed by untyped (29.03%), HPV-16 (16.13%), mixed

infection HPV-16/18, 18/6, 18/11 and 18/33 (12.90%), HPV-33 (9.68%), HPV-6 (3.23%) and HPV-31 (3.23%) (Table 11). HPV typing patterns from clinical specimens were shown in Fig. 16 - 17.

Table 10. The demographic and clinical information of CIN and CaCx group.

	CIN				Cancer			Total
	CIN I	CIN II	CIN III	Total of CIN group	Adenocarcinoma	Squamous cell carcinoma	Total of CaCx group	
Age range (yrs)	32 – 57	27 – 49	29 – 63	27 – 63	37 – 68	30 – 72	30 – 72	27 – 72
Mean age (yrs)	42.86	39.50	41.80	41.80	45.60	47.81	47.00	44.80
SD (yrs)	7.45	10.79	9.79	9.30	8.15	10.58	9.60	9.71
Number of case	7	4	28	39	15	21	36	75

Table 11. The results of patients were detected by L1 PCR.

	CIN (39)				Cancer (36)			
	CIN I (7)	**CIN II (4)**	**CIN III (28)**	**Total**	**Adenocarcinoma (15)**	**Squamous cell Carcinoma (21)**	**Total**	**Grand Total (75)**
L1 - pos	4 (57.14%)	0	12 (42.86%)	16 (41.03%)	14 (93.33%)	17 (80.95%)	31 (86.11%)	47 (62.67%)
HPV - 6						1 (4.76%)[a]	1 (3.23%)[a]	1 (2.13%)[a]
HPV - 11						1 (4.76%) 2 (9.52%)[a]	1 (3.23%) 2 (6.45%)[a]	1 (2.13%) 2 (4.26%)[a]
HPV - 16	1 (25.00%)		6 (50.00%)	7 (43.75%)	2 (14.29%)	2 (9.52%) 3 (14.29%)[a]	4 (12.90%) 5 (16.13%)[a]	11 (23.40%) 12 (25.53%)[a]
HPV - 18	2 (50.00%)		4 (33.33%) 5 (41.67%)[a]	6 (37.50%) 7 (43.75%)[a]	7 (50.00%)	4 (19.05%) 8 (38.10%)[a]	11 (35.48%) 15 (48.39%)[a]	17 (36.17%) 22 (46.81%)[a]
HPV - 31			1 (8.33%)[a]	1 (6.25%)[a]			1 (3.23%)[a]	2 (4.26%)[a]
HPV - 33					1 (7.14%)	1 (4.76%) 2 (9.52%)[a]	2 (6.45%) 3 (9.68%)[a]	2 (4.26%) 3 (6.38%)[a]
Mixed				1 (6.25%)		4 (19.05%)	4 (12.90%)	5 (10.64%)
HPV-16/18						1 (4.76%)	1 (3.23%)	1 (2.13%)
HPV-18/6						1 (4.76%)	1 (3.23%)	1 (2.13%)
HPV-18/11						1 (4.76%)	1 (3.23%)	1 (2.13%)
HPV-18/31			1 (8.33%)	1 (6.25%)				1 (2.13%)
HPV-18/33						1 (4.76%)	1 (3.23%)	1 (2.13%)
Untyped	1 (25.00%)		1 (8.33%)	2 (12.50%)	4 (28.57%)	5 (23.81%)	9 (29.03%)	11 (23.40%)

a: The number includes mixed infection

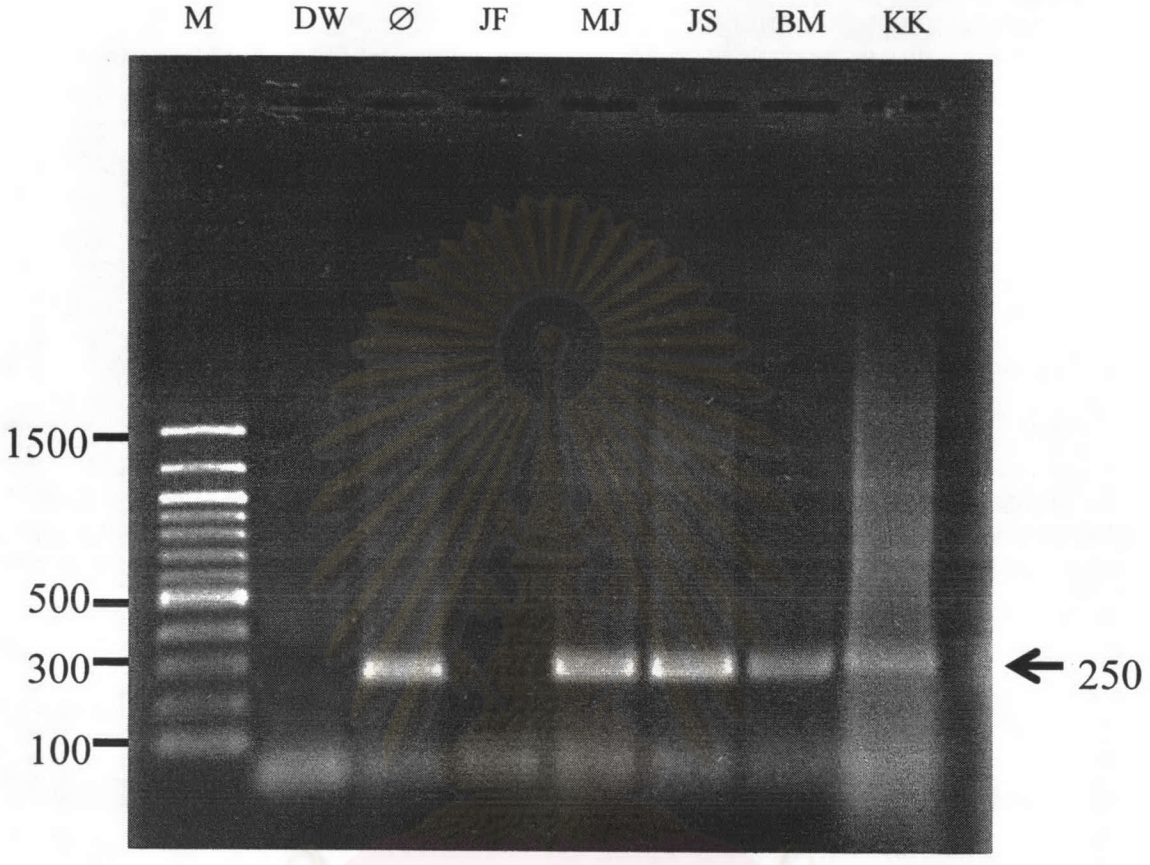


Figure 12. Amplification of HPV-L1 gene from clinical specimens. Five tissue specimens were amplified by L1C1/C2 primer set. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.), ∅: standard HPV-16 1 ng as HPV-DNA positive control. There was one patient (JF) who was HPV-L1 PCR negative. Four patients (MJ, JS, BM and KK) were HPV-L1 PCR positive.

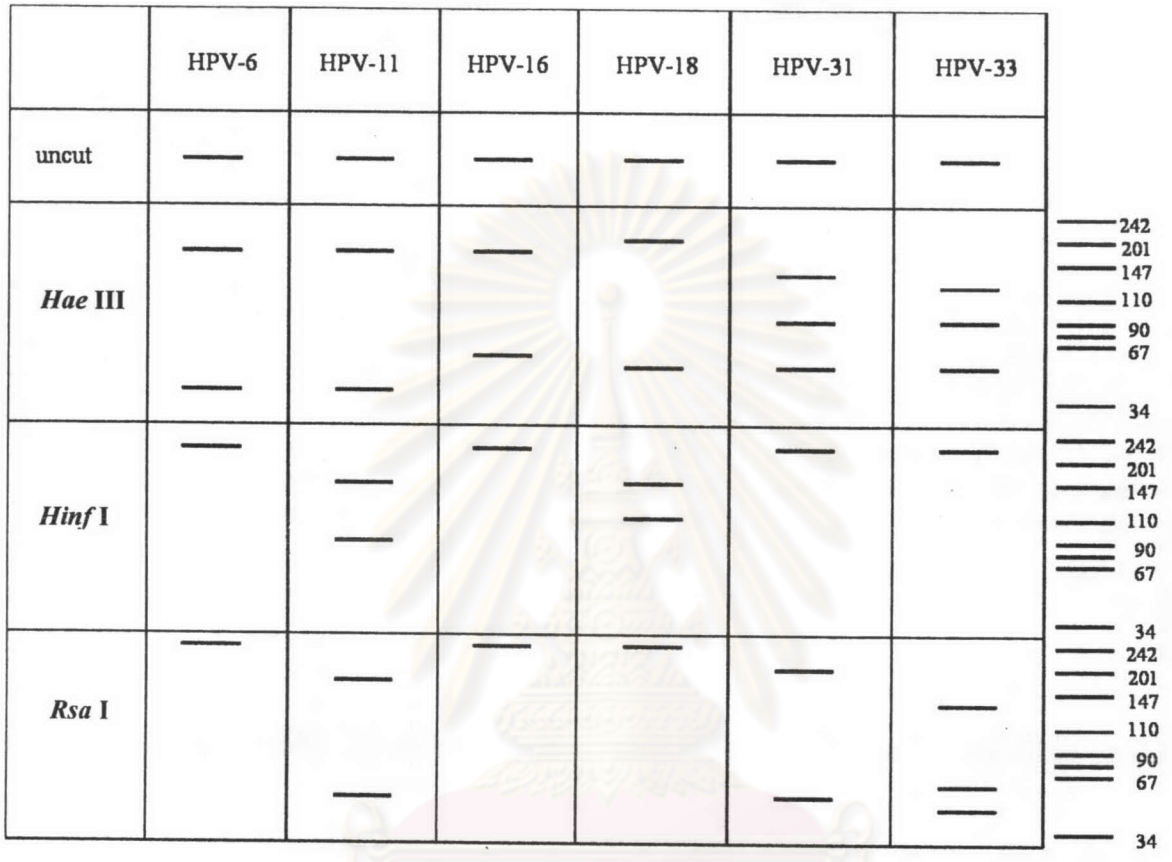


Table 12. Schematic of L1C1/C2 L1-PCR product restriction pattern used to identify HPV types. The 6 standards HPV-L1 PCR products were digested with RE. Row 1: uncut, row 2: *Hae* III, row 3: *Hinf* I and row 4: *Rsa* I. Bars on the right side of each panel are molecular markers [pBR322 DNA-*Msp* I marker (New England BioLabs,U.S.A.)].

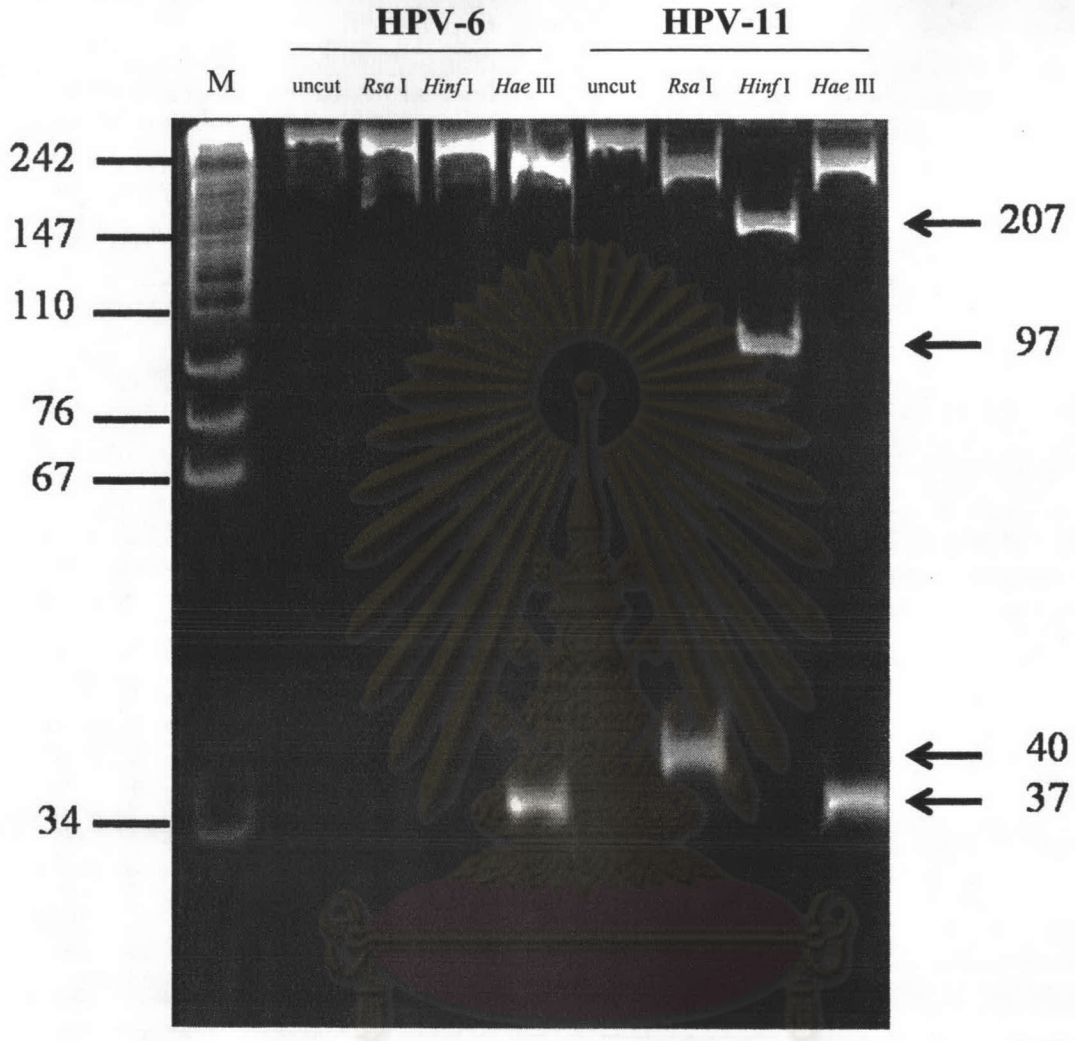


Figure 13. Restriction patterns of HPV-6 and HPV-11 L1-PCR products. The DNA was digested with *Rsa* I, *Hae* III and *Hinf* I. After electrophoresis, the gel (10% acrylamide) was stained and photographed. M: pBR322 DNA-*Msp* I marker (New England Bio Labs, U.S.A.).

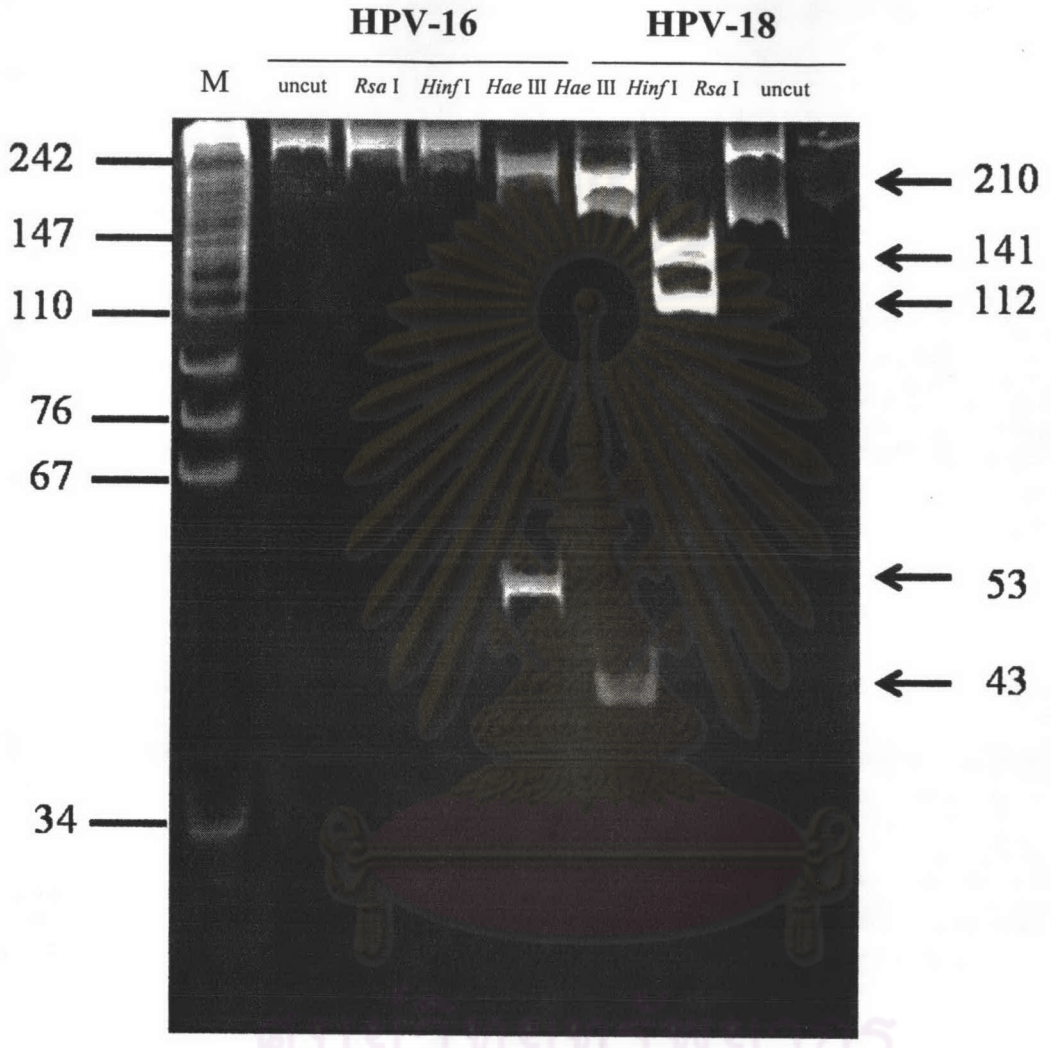


Figure 14. Restriction patterns of HPV-16 and HPV-18 L1-PCR products. The DNA was digested with *Rsa* I, *Hae* III and *Hinf* I. After electrophoresis, the gel (10% acrylamide) was stained and photographed. M: pBR322 DNA-*Msp* I marker (New England Bio Labs, U.S.A.).

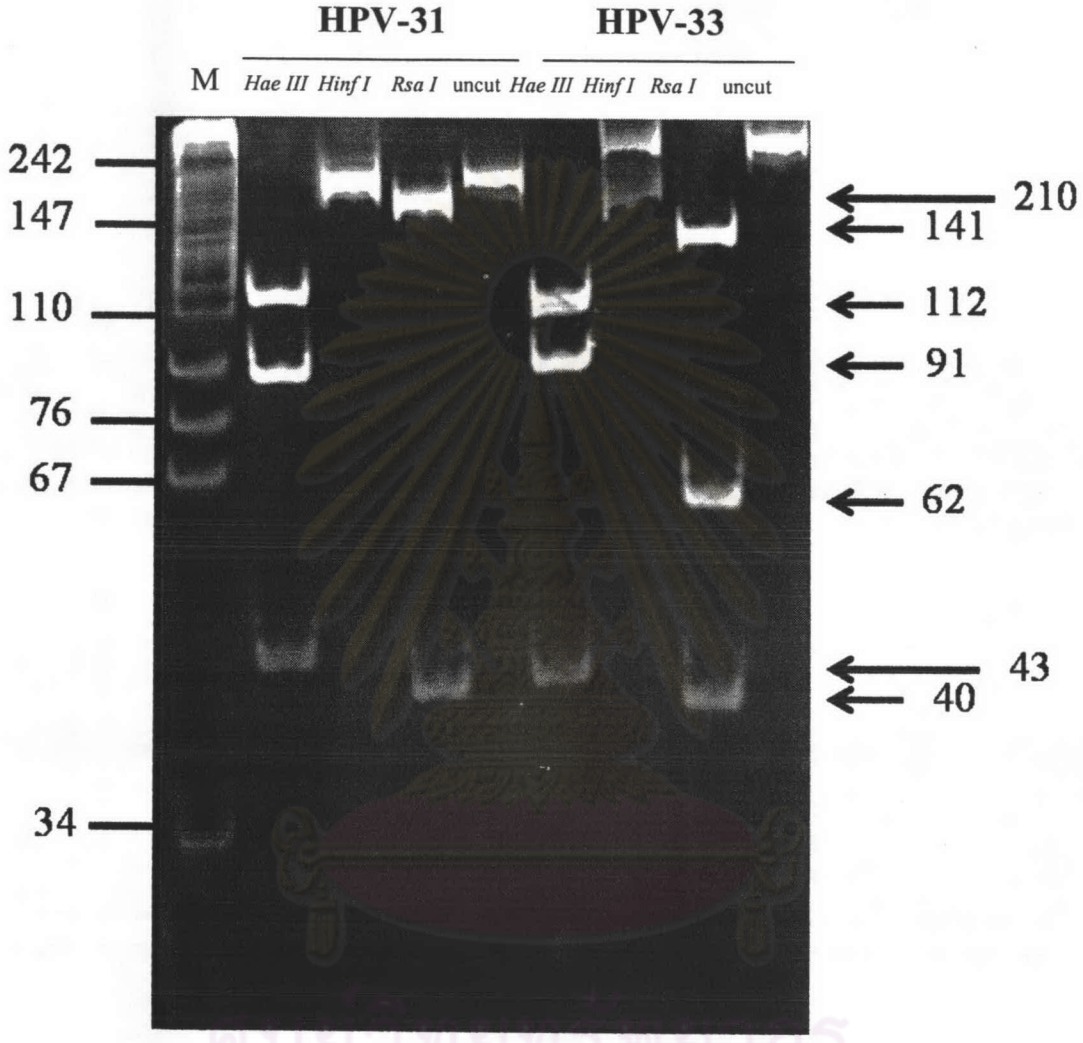


Figure 15. Restriction patterns of HPV-31 and HPV-33 L1-PCR products. The DNA was digested with *Rsa* I, *Hae* III and *Hinf* I. After electrophoresis, the gel (10% acrylamide) was stained and photographed. M: pBR322 DNA-*Msp* I marker (New England Bio Labs, U.S.A.).

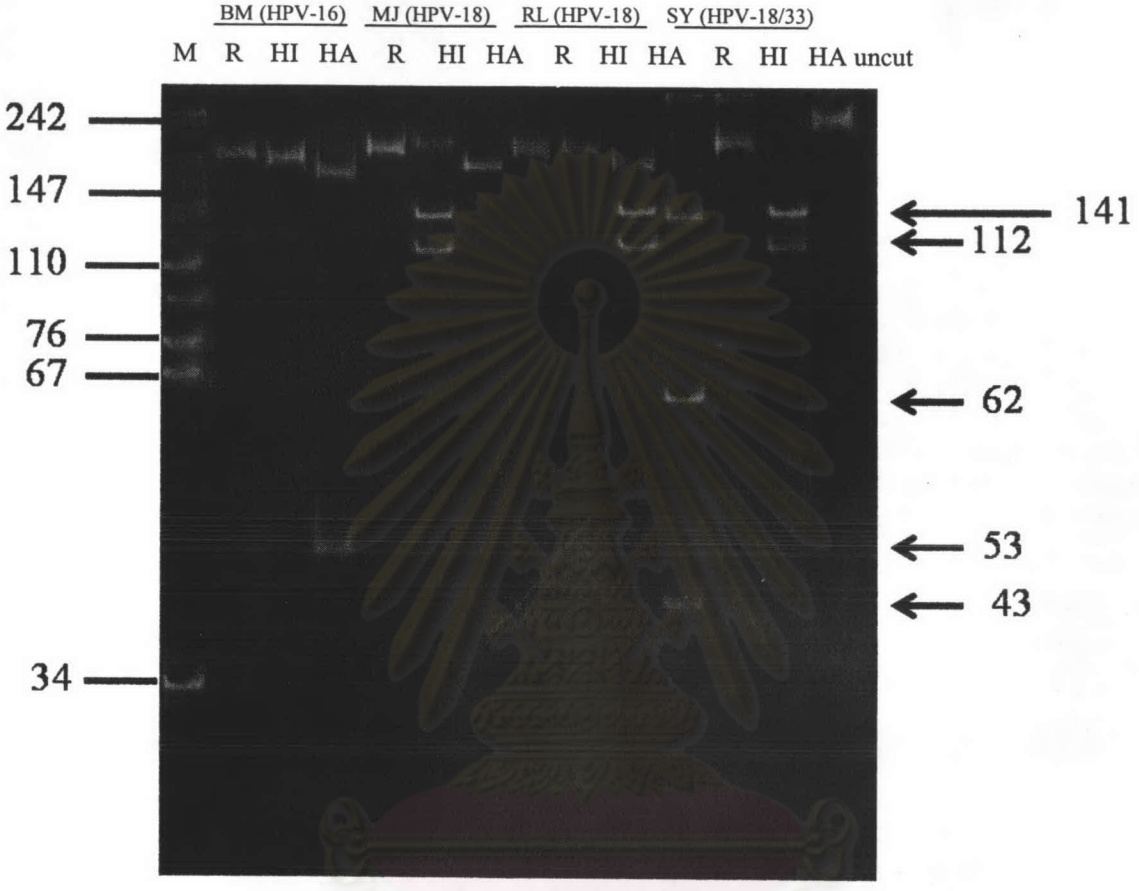


Figure 16. RFLP patterns of the L1-PCR product from clinical specimens (BM, MJ, RL and SV). Each sample was digested with *Rsa* I, *Hae* III and *Hinf* I. After electrophoresis, the gel (10% acrylamide) was stained and photographed. M: pBR322 DNA-*Msp* I marker (New England Bio Labs, U.S.A.), R: *Rsa* I, HI: *Hinf* I, HA: *Hae* III.

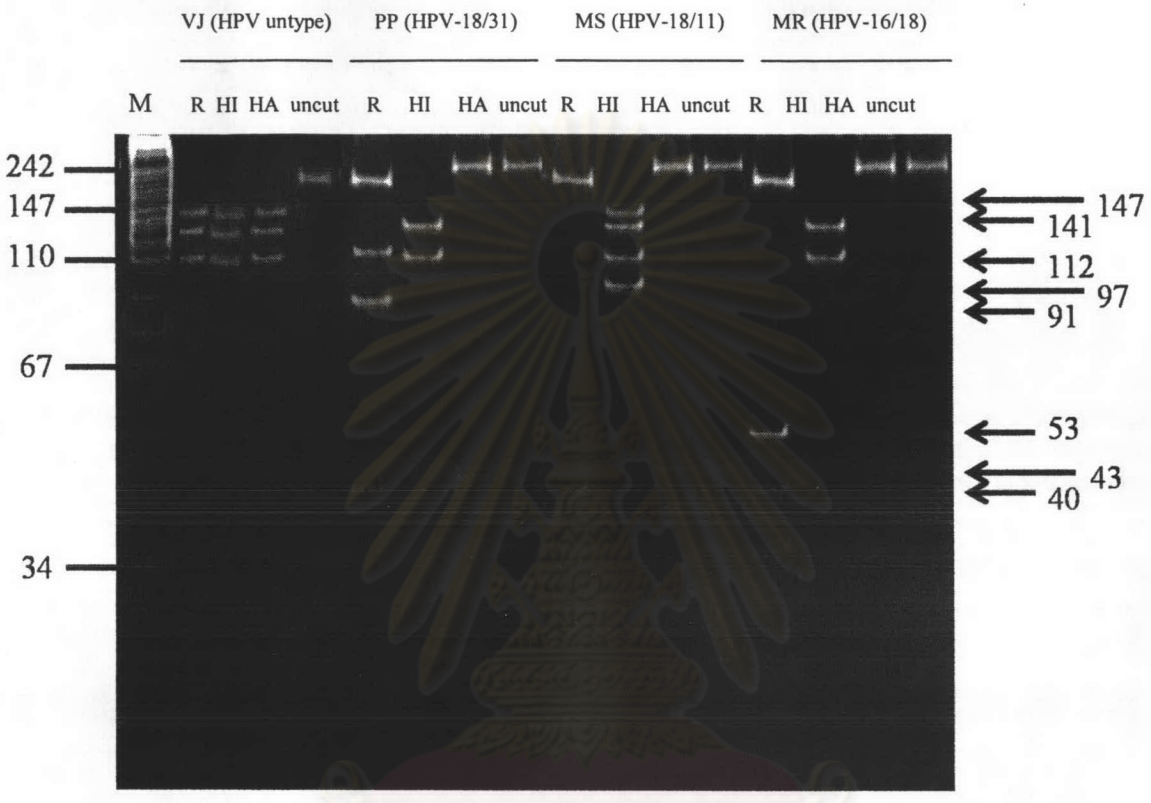


Figure 17. RFLP patterns of the L1-PCR product from clinical specimens (VJ, PP, MS and MR). Each sample was digested with *Rsa* I, *Hae* III and *Hinf* I. After electrophoresis, the gel (10% acrylamide) was stained and photographed. M: pBR322 DNA-*Msp* I marker (New England Bio Labs, U.S.A.), R: *Rsa* I, HI: *Hinf* I, HA: *Hae* III.

3. Analysis of HPV-E7-specific CD8+ T cell responses

3.1 Design of HPV-E7 overlapping peptide

3.1.1 Nucleotide and amino acid sequence of HPV-E7

The sensitivity, the 10-fold serially diluted standard HPV-16 DNA was amplified by using the HPV-16-E7 PCR (E7-PCR). At least 1 pg of standard HPV-16 DNA could be amplified (Fig. 18). The standard DNA of other HPV types (HPV-6, 11, 18, 31, 33 and 35) was included to analyse the specificity. Only E7 gene of standard HPV-16 could be amplified by this detection system (Fig. 19).

We used E7-PCR system for sequencing of HPV-16 E7 gene. This information was used for overlapping peptide design. We amplified HPV-16 E7 gene from 20 cases of HPV-16 infected patients (Fig. 20). The alignment of HPV-16 E7 gene sequence from database at Los Alamos (http://hpv-web.lanl.gov) revealed 100% nucleotide identity and 100% amino acid sequence homology to our control standard HPV-16 E7 gene. Nucleotide and amino acid sequence of standard HPV-16 E7 gene showed in Figure 21. In the present study, most samples had conserved E7 gene. Only 4 out of 20 (20%) samples contained variations. Among these variations, only one non-synonymous was identified at nt 647 (A to G) (Fig. 22A) resulting in a change of HPV-16-E7 amino acid at position 29 from asparagine to serine (N29S) (Fig. 22B).

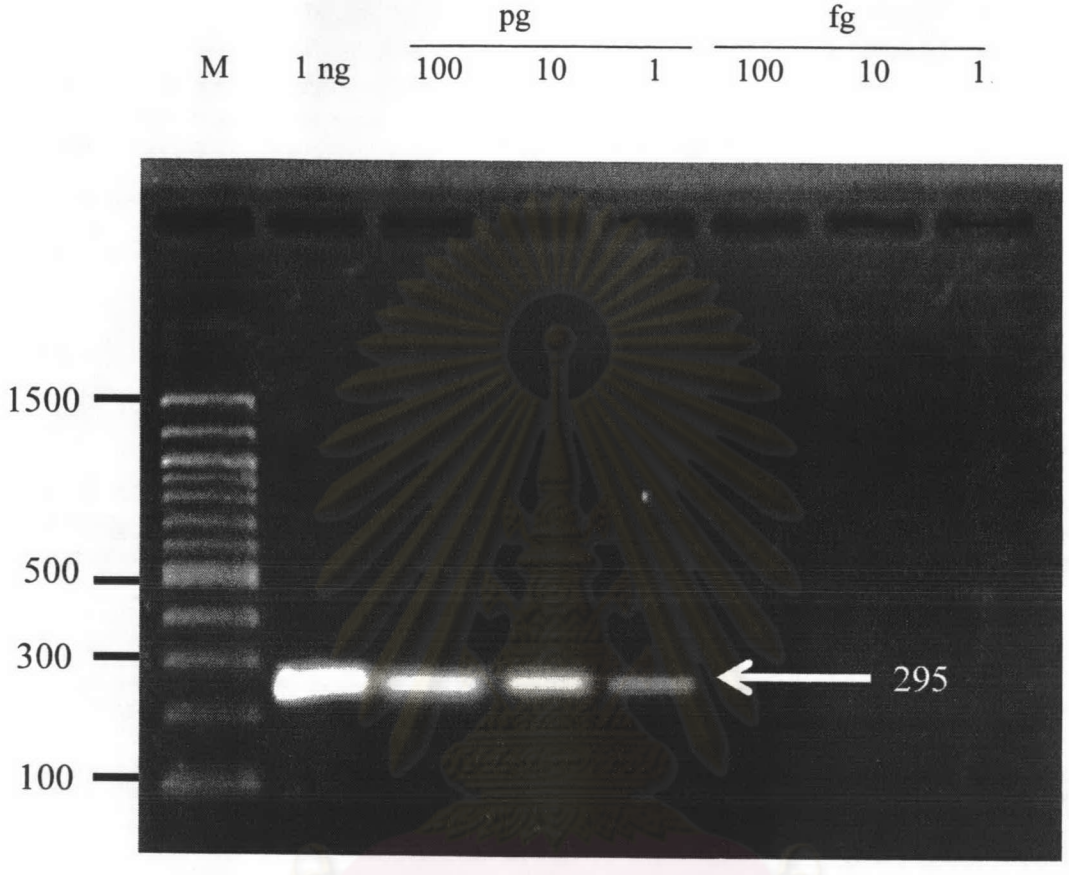


Figure 18. The sensitivity of E7-PCR. The standard HPV-16 DNA was 10-fold serially diluted from 1 ng to 1 fg. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

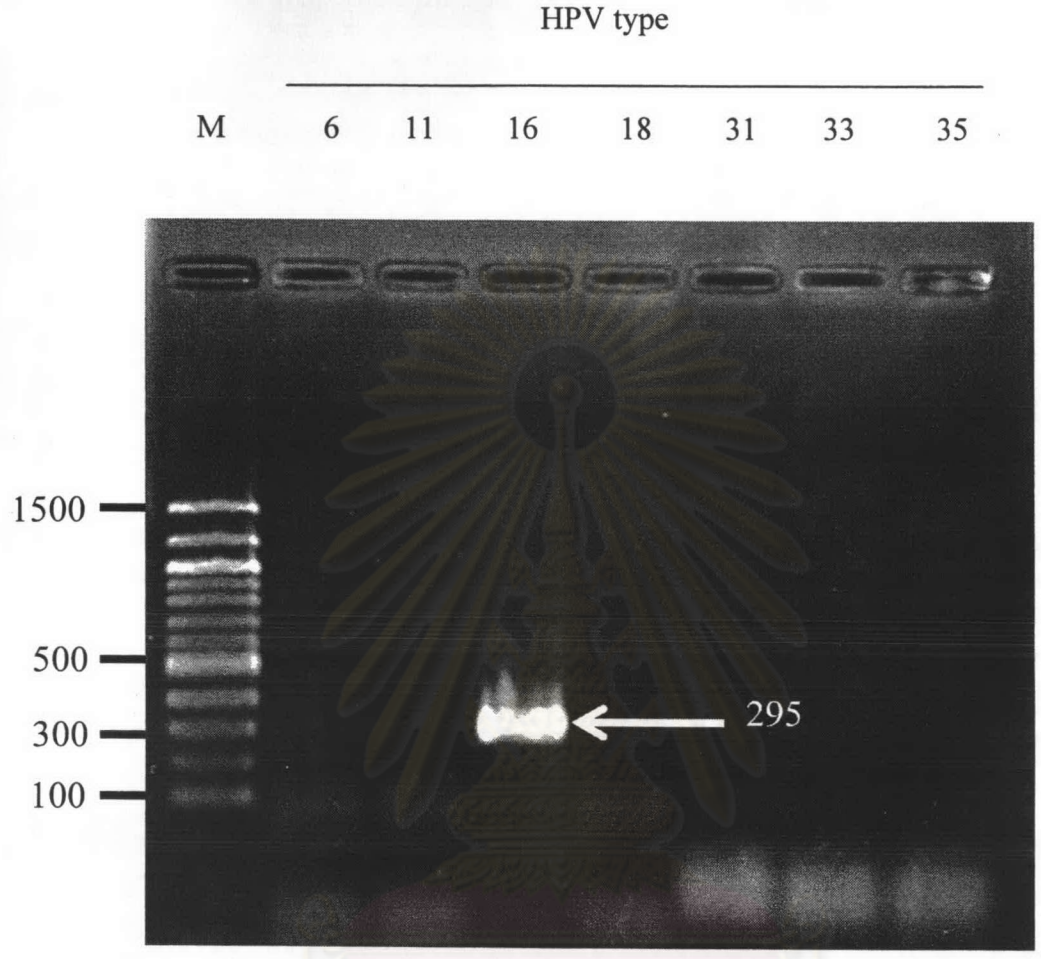


Figure 19. The specificity of E7-PCR. Only HPV-16 E7 gene was amplified by this detection system. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.).

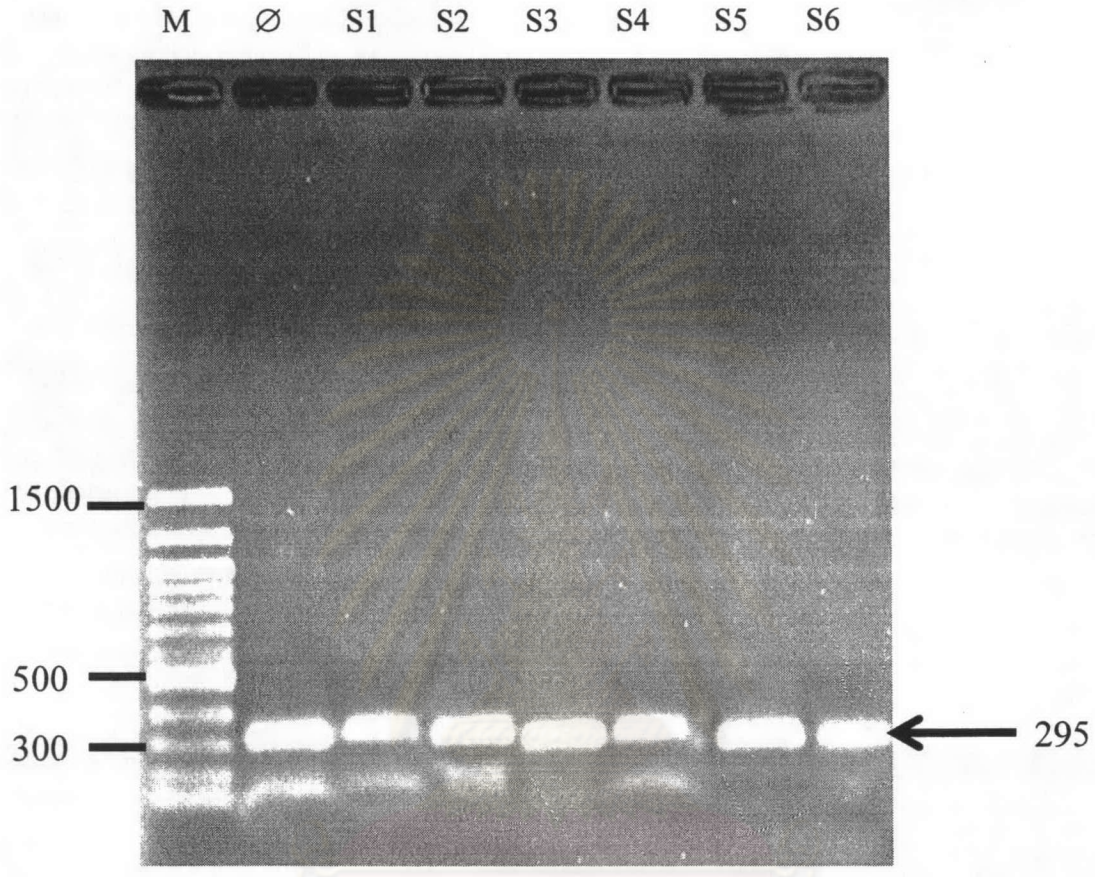
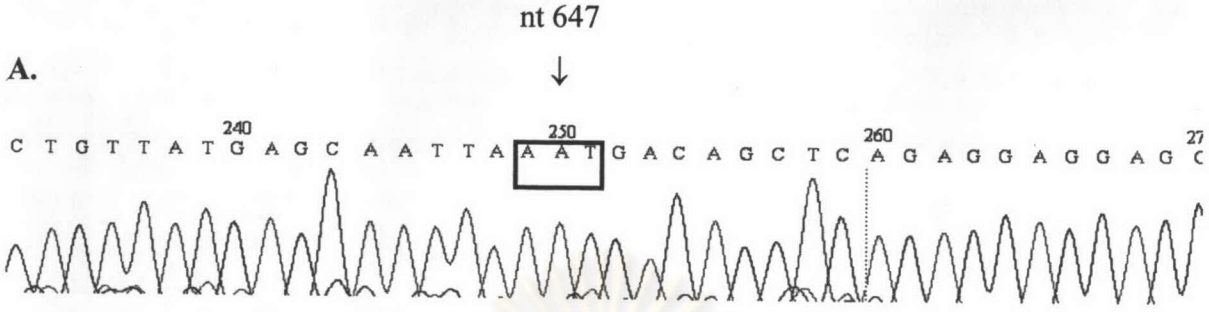


Figure 20. Amplification of the E7-PCR product from clinical specimens. The extracted HPV-16 DNA samples were amplified by HPV-16 E7 primer. M: 100-bp DNA ladder (New England Bio Labs, U.S.A.), ∅: standard HPV-16 1 ng as PCR positive control. S1 – S6: clinical specimens.

A.



B.

1	ATG	CAT	GGA	GAT	ACA	CCT	ACA	TTG	CAT	GAA	TAT	ATG	TTA	GAT	TTG	45
1	M	H	G	D	T	P	T	L	H	E	Y	M	L	D	L	15
46	CAA	CCA	GAG	ACA	ACT	GAT	CTC	TAC	TGT	TAT	GAG	CAA	TTA	AAT	GAC	90
16	Q	P	E	T	T	D	L	Y	C	Y	E	Q	L	N	D	30
91	AGC	TCA	GAG	GAG	GAG	GAT	GAA	ATA	GAT	GGT	CCA	GCT	GGA	CAA	GCA	135
31	S	S	E	E	E	D	E	I	D	G	P	A	G	Q	A	45
136	GAA	CCG	GAC	AGA	GCC	CAT	TAC	AAT	ATT	GTA	ACC	TTT	TGT	TGC	AAG	180
46	E	P	D	R	A	H	Y	N	I	V	T	F	C	C	K	60
181	TGT	GAC	TCT	ACG	CTT	CGG	TTG	TGC	GTA	CAA	AGC	ACA	CAC	GTA	GAC	225
61	C	D	S	T	L	R	L	C	V	Q	S	T	H	V	D	75
226	ATT	CGT	ACT	TTG	GAA	GAC	CTG	TTA	ATG	GGC	ACA	CTA	GGA	ATT	GTG	270
76	I	R	T	L	E	D	L	L	M	G	T	L	G	I	V	90
271	TGC	CCC	ATC	TGT	TCT	CAG	AAA	CCA	294							
91	C	P	I	C	S	Q	K	P								

Figure 21. Nucleotide and amino acid sequence of standard HPV-16 E7 gene **A)** Nucleotide sequence of standard HPV-16 E7 gene **B)** Amino acid sequence of standard HPV-16 E7 protein.

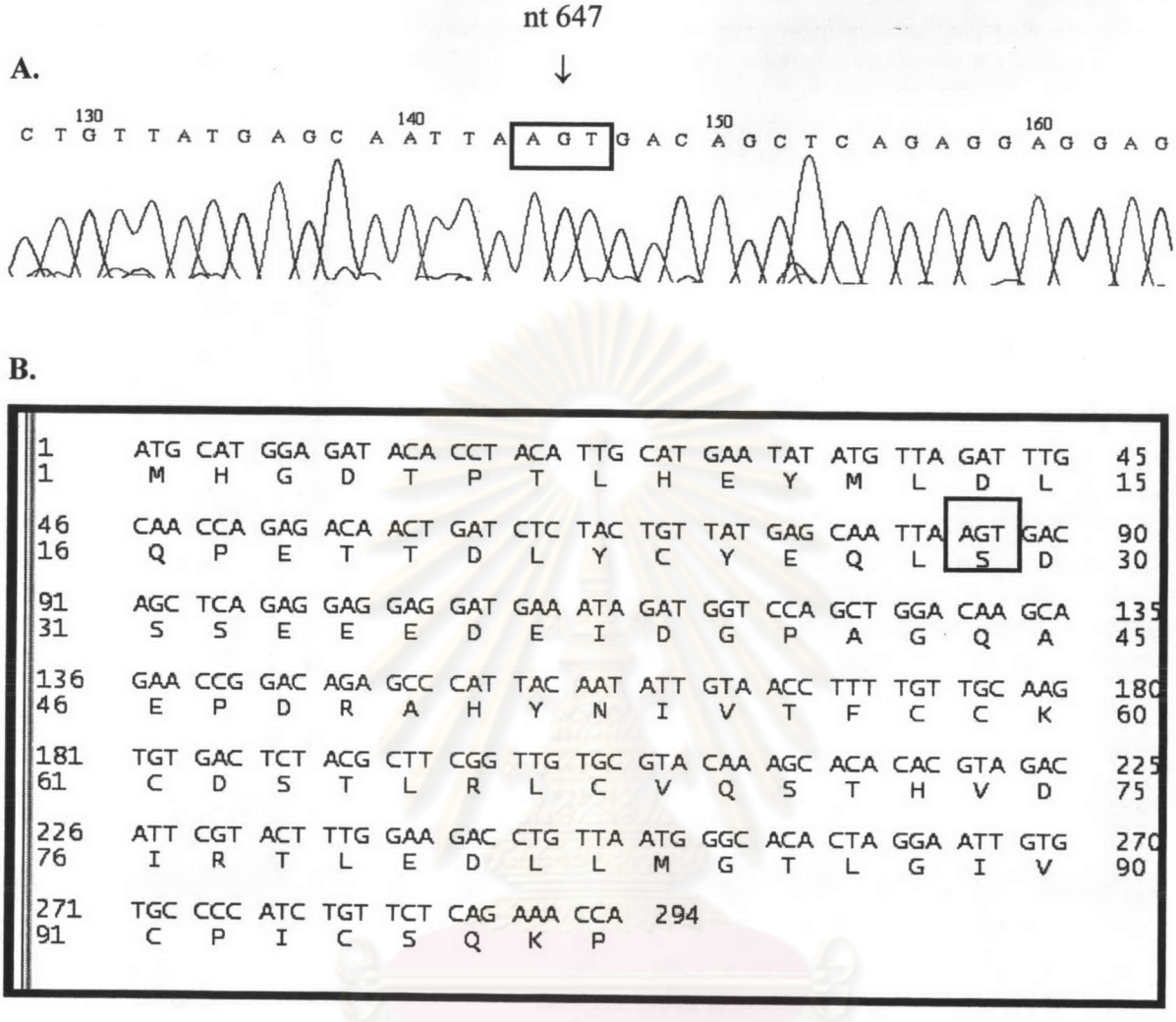


Figure 22. Nucleotide and amino acid sequence of HPV-16 E7 gene from a clinical specimen. **A)** Nucleotide variation of HPV-16 E7 DNA from clinical specimen was identified at nt 647 (A → G). **B)** Amino acid variation of HPV-16 E7 from clinical specimen was identified at position 29 (N29S).

3.1.2 Overlapping peptide

A total of 20 HPV-16-E7 nucleotide sequences was aligned by Clustal X program and translated into amino acid sequence by Bioedit program. Overlapping peptides with length of 20 aa overlapping by 10 aa was designed by PeptGen program on Los Alamos server (http://hiv-web.lanl.gov). A total of 11 overlapping peptides were designed, two of which contained N29S mutation (Table 13). The overlapping peptide was synthesized at Mimotope, Australia.

Table 13. The 20 amino acid overlapping peptides spanning HPV-16-E7 protein.

Overlapping peptides	Position	Abbreviation
MHGDTPTLHEYMLDLQPETT	1 - 20	MHGD
YMLDLQPETTDLYCYEQLND	11 - 30	YMLD
DLYCYEQLNDSSEEEDEIDG	21 - 40	DLYC
SSEEEDEIDGPAGQAEPDRA	31 - 50	SSEE
PAGQAEPDRAHYNIVTFCCK	41 - 60	PAGQ
HYNIVTFCCKCDSTLRLCVQ	51 - 70	HYNI
CDSTLRLCVQSTHVDIRTLE	61 - 80	CDST
STHVDIRTLEDLLMGTLGIV	71 - 90	STHV
DLLMGTLGIVCPICSQKP	81 - 98	DLLM
YMLDLQPETTDLYCYEQLS*D	11 - 30	YMLDv*
DLYCYEQLS*DSSEEEDEIDG	21 - 40	DLYCv*

* Variation in position 29

3.2 Enumeration of HPV-E7-specific CD8+ T cells by IFN-γ ELISpot assay

3.2.1 HPV-E7-specific CD8+ T cell responses in CIN patients

Immune responses to HPV may play an important role in protection and controlling HPV infection as mentioned above. However, little is known about HPV-E7-specific immune responses in HPV-infected Thai women. We have used overlapping peptides spanning HPV-E7 protein to analyse the HPV-E7-specific CD8+ T cell responses by ELISpot assay.

Eleven CIN patients (age 27 – 62, mean: 34 years) were enrolled to analyse the CD8+ T cell responses by ELISpot assay. They were 2 patients with CIN I, 2 patients with CIN II and 7 patients with CIN III (Table 14). Only 4 out of 11 patients had HPV infection: 1 with HPV-16 infection, 2 with HPV-18 infection, and 1 with untyped HPV infection (Table 14). While a total of 7 out of 11 patients had evidence of CIN III about 4 months before the assay was performed, 4 out of 11 patients had evidence of CIN I/II one week before the assay was performed. Interestingly, one CIN II patient (KW) with HIV infection had no evidence of HPV infection (Table 14).

Unexpectedly, no *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in PBMC of CIN patients (Table 15 and Fig. 23). These results might be interpreted as "false negative" since the frequency of *ex vivo* HPV-specific CD8+ T cell responses in peripheral blood might be too low to detect by ELISpot assay. We tried to increase the frequency of the T cell response by culturing the PBMC with pooled overlapping peptides for 14 days before the assay was performed as previously described (158). HPV-E7-specific CD8+ T cell responses were detected in 3 out of 11 (27.27%) of CIN patients (Table 16 and Fig. 24). Whilst 2 patients (AA and KK) recognised only one peptide, one patient (YP) broadly recognised peptides (Table 16). The frequency of peptides responses in these patients to E7 ranged from one to eight with a median of 4 peptides. Two peptides (HYNI and DLYCv) were detected at high frequency in this "cultured ELISpot assay" (66.67%) (Fig. 25). The magnitudes of the responses to HPV-E7 peptides ranged from 88 SFU/10^6 cells to 1,640 SFU/10^6 cells with a median of 1,340 SFU/10^6 cells.

In the present study, we had only one patient (KK) who had HPV-16 infection. This patient had detectable HPV-specific T cell responses to HYNI peptide (Table 16 and Fig. 26). Interestingly, two patients (AA and YP) had HPV-specific T cell responses despite the fact that both of them had no detectable HPV infection. Whilst the patient AA recognised only DLYCv peptide (Table 16 and Fig. 27), the patient YP broadly recognised 8 peptides: MHGD, DLYC, SSEE, HYNI, CDST, STHV, DLLM, YMLDv, and DLYCv (Table 16 and Fig. 28).

Table 14. Demographic information of CIN patients who enrolled in the study of HPV-E7-specific CD8+ T cell response.

Patient	Age (years)	HPV infection	HPV type	Diagnosis
VJ	43	+	Untyped	CIN I
YT	32	-	N.A.	CIN I
KW*	34	-	N.A.	CIN II
PD	27	-	N.A.	CIN II
AA	34	-	N.A.	CIN III
DC	31	-	N.A.	CIN III
ER	62	-	N.A.	CIN III
OT	51	+	18	CIN III
KK	39	+	16	CIN III
KO	44	+	18	CIN III
YP	29	-	N.A.	CIN III

* HIV-infection; N.A.: non-applicable

Table 15. *ex vivo* HPV-E7-specific CD8+ T cell responses in 11 patients with CIN

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
VJ	0	0	0	0	0	0	0	0	0	0	0
YT	0	0	4	7	4	0	0	8	3	4	0
KW	0	0	0	0	0	0	0	0	0	0	0
PD	0	0	0	0	0	0	0	0	0	0	0
AA	0	0	0	0	0	0	0	0	0	0	0
DC	0	0	7	0	6	0	0	0	0	0	0
ER	4	6	7	0	11	8	5	5	4	13	5
OT	0	0	0	0	0	0	0	0	0	0	0
KK	0	0	0	0	0	0	0	0	0	0	0
KO	0	0	0	0	0	0	0	0	0	0	0
YP	0	0	0	0	0	0	0	0	0	0	0
Average	0.36	0.55	1.64	0.64	1.91	0.73	0.45	1.18	0.64	1.55	0.45

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in CIN patients

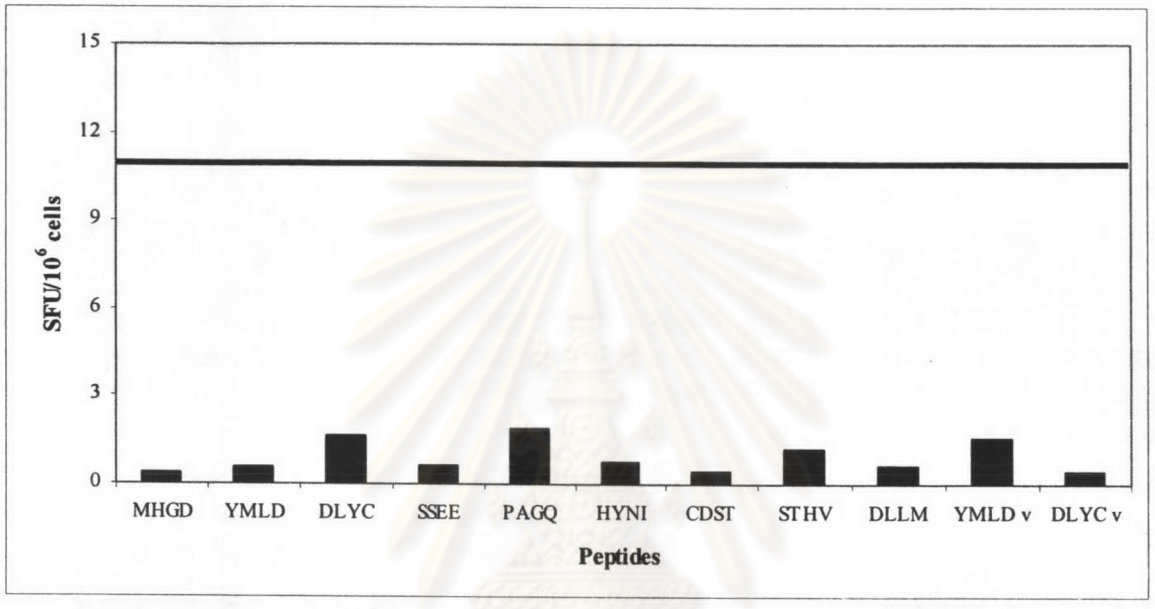


Figure 23. *ex vivo* HPV-E7-specific CD8+ T cell responses in 11 patients with CIN. No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in all patients with CIN. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide. Horizontal line represents positive cut-off value of the T cell responses.

Table 16. HPV-E7-specific CD8+ T cell responses in 11 patients with CIN by cultured ELISpot assay

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
VJ	0	0	0	0	0	0	0	0	0	0	0
YT	0	0	0	0	0	0	0	0	0	0	0
KW	0	0	0	0	0	0	0	0	0	0	0
PD	0	0	0	0	0	0	0	0	0	0	0
AA	0	0	0	0	0	0	0	0	0	0	88
DC	0	0	0	0	0	0	0	0	0	0	0
ER	0	0	0	0	0	0	0	0	0	0	0
OT	0	0	0	0	0	0	0	0	0	0	0
KK	0	0	0	0	0	135	0	0	0	0	0
KO	0	0	0	0	0	0	0	0	0	0	0
YP	1,200	0	1,040	0	0	1,400	1,520	1,560	1,400	1,640	1,280
Average	109.09	0	94.55	0	0	139.55	138.18	141.82	127.27	149.09	124.36

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

HPV-E7-specific CD8+ T cell responses were detected in CIN patients by cultured ELISpot assay

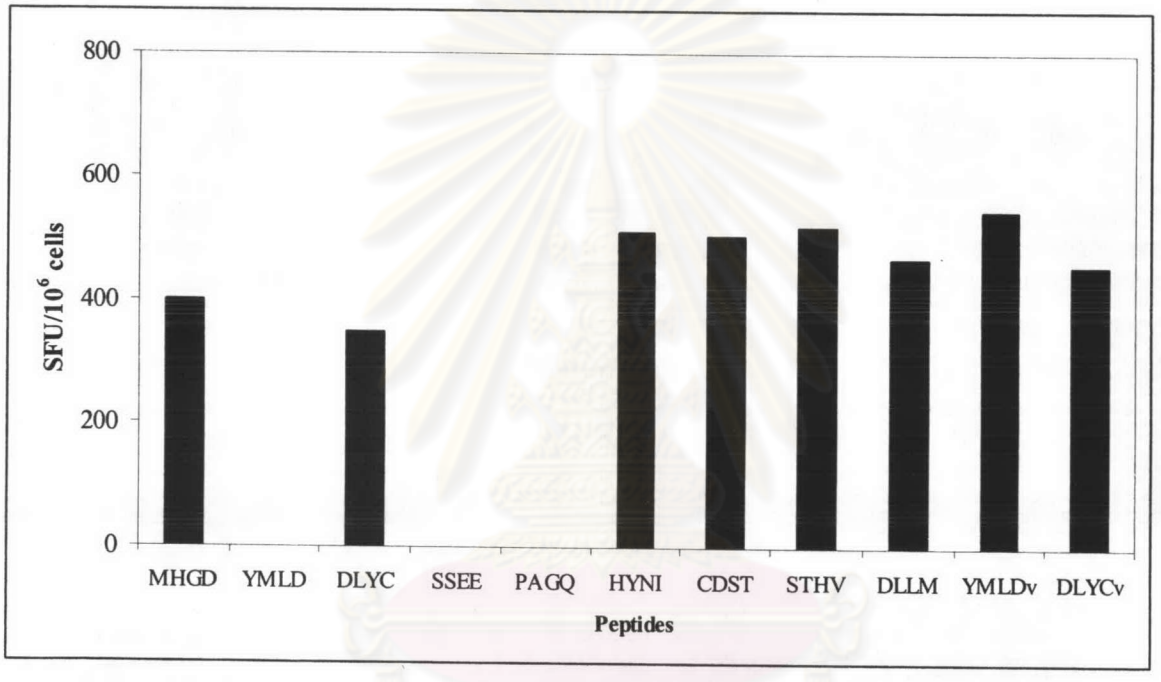


Figure 24. Mean of HPV-E7-specific CD8+ T cell responses in 3 patients with CIN by "cultured ELISpot assay". HPV-E7-specific CD8+ T cell responses were detected in patients with CIN, after 14 days culture with overlapping peptide. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide after background subtraction.

High frequency of T cell responses against HYNI and DLYCv were detected in CIN patients by cultured ELISpot assay

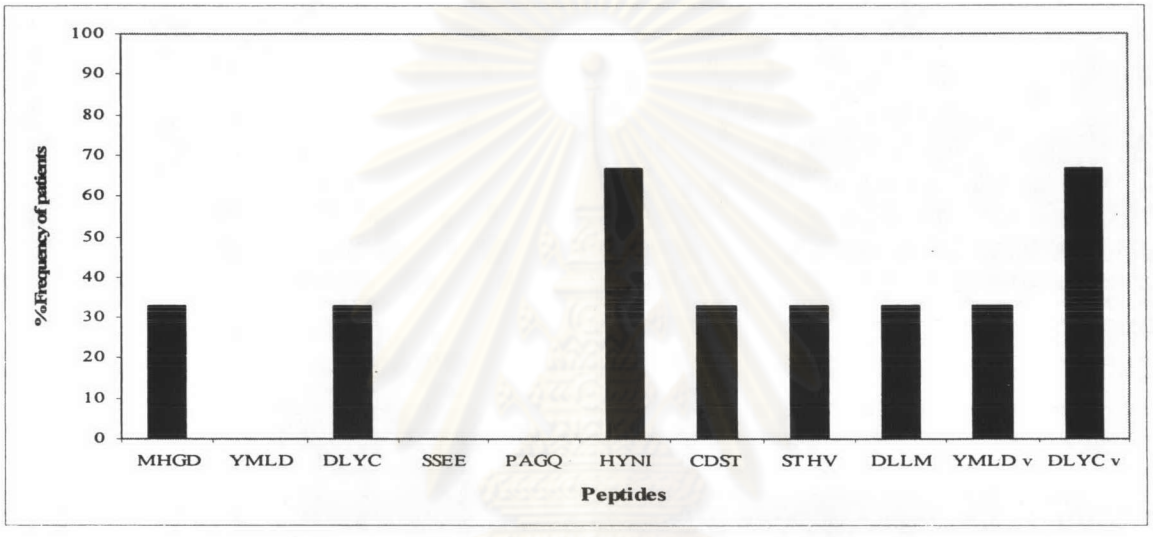


Figure 25. Frequency of overlapping peptides responses in CIN patients. The frequency was in a range of overlapping peptides 1 to 8 (median: 4 peptides). HYNI and DLYCv peptides were detected at high frequency by cultured ELISpot assays. Each bar represents the percentage of the frequency of E7 overlapping peptides.

Only one CIN patient with HPV-16 infection recognised HPV-16 E7 peptide (HYNI) by cultured ELISpot assay

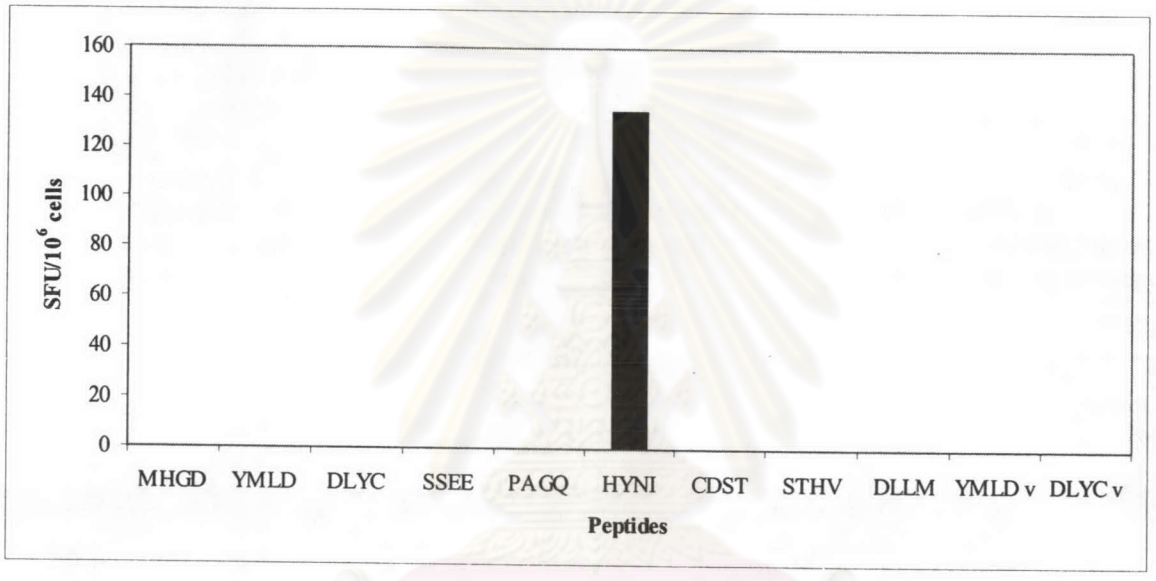


Figure 26. Only one patient (KK) with HPV-16 infection had detectable HPV-E7-specific T cell responses to HYNI peptide by cultured ELISpot assay. The bar represents the magnitude of response to the peptide HYNI after background subtraction.

One patient without detectable HPV infection recognised DLYCv peptide by cultured ELISpot assay

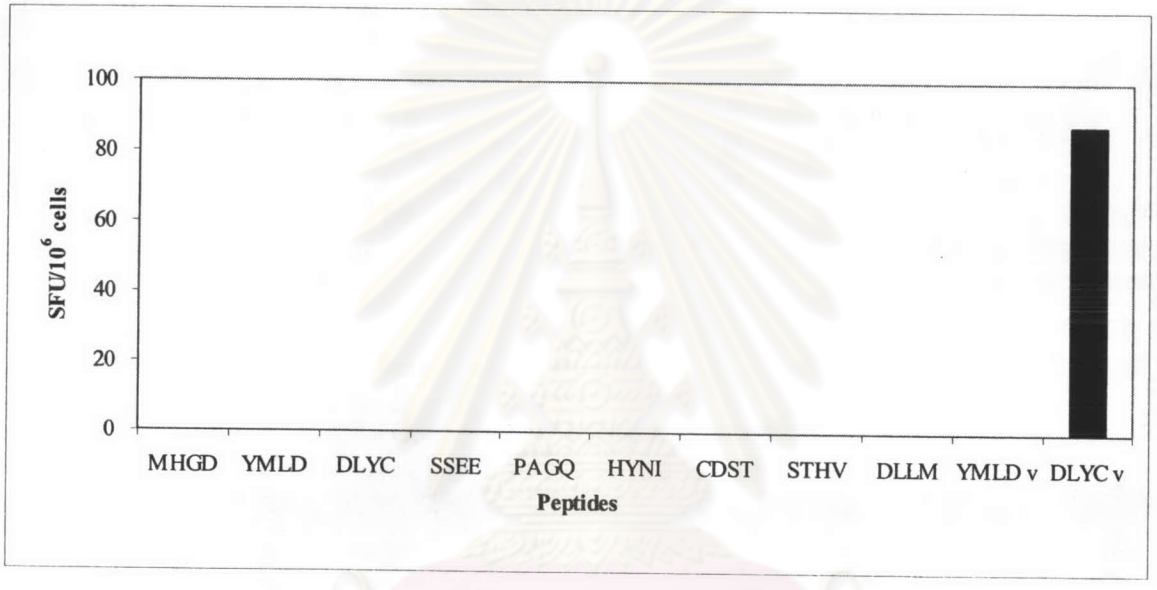


Figure 27. HPV-specific CD8+ T cell responses to DLYCv peptide were detected in the patient AA who had no detectable HPV infection by cultured ELISpot assay. The bar represents the magnitude of response to the peptide DLYCv after background subtraction.

The patient YP broadly recognised E7 overlapping peptides by cultured ELISpot assay

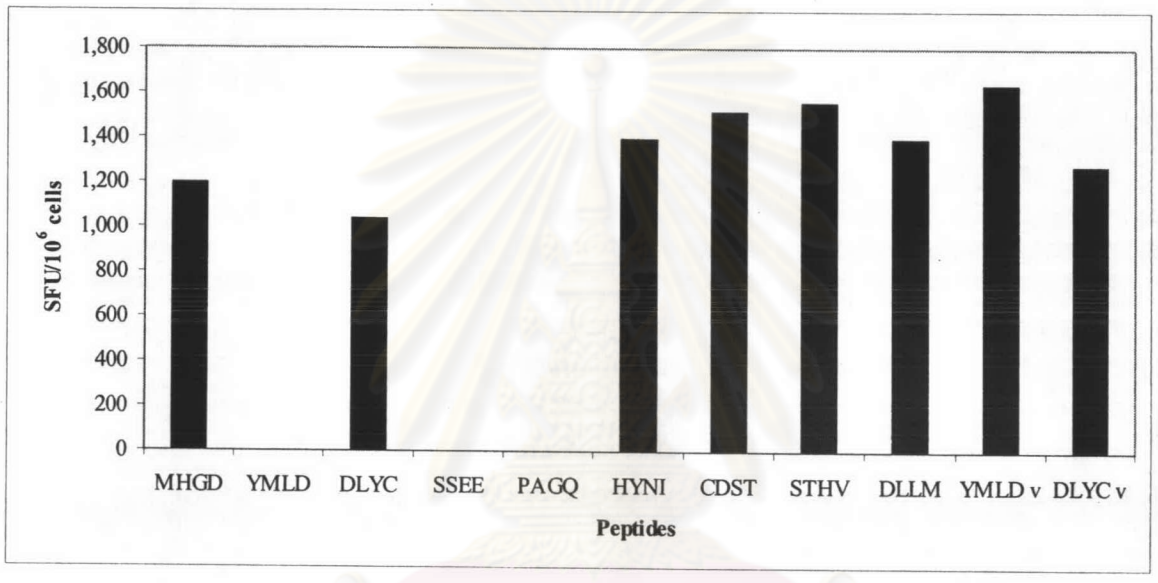


Figure 28. HPV-specific CD8+ T cell responses were detected in the patient YP who had no detectable HPV infection by cultured ELISpot assay. This patient broadly recognised 8 peptides: MHGD, DLYC, HYNI, CDST, STHV, DLLM, YMLDv, and DLYCv. Each bar represents the magnitude of response to individual E7 overlapping peptide after background subtraction.

3.2.2 HPV-E7-specific CD8+ T cell responses in CaCx patients

If HPV-specific T cell responses are important in protection against CaCx as we hypothesised, the investigation into the different responses between patients with precancerous lesion and patients with cancer may reveal the role of HPV-specific T cells.

Eleven CaCx patients (age 37 – 67, mean: 47 years) were enrolled to study CD8+ T cell responses. They were 6 patients with adenocarcinoma and 5 patients with squamous cell carcinoma. Nine out of 11 patients had HPV infection, 2 with HPV-18 infection, 1 with HPV-11 infection, 1 with HPV-33 infection, 1 with mixed HPV-18/6 infection, and 4 with untyped HPV infection (Table 17). All 11 patients had operations to remove malignant tumours approximately 3 to 4 days before the ELISpot assays were performed. Only 2 patients (NN and PC) were on chemotherapy (Table 17).

Similarly to what we observed in CIN patients, no *ex vivo* HPV-16-E7-specific CD8+ T cell responses were detected in CaCx patients (Table 18 and Fig. 29). So we stimulated PBMC of CaCx patients with pooled peptide before analysis of augmented HPV-specific T cell responses by "cultured ELISpot assay". As previously hypothesis, the responses detected in CaCx patients were lower (Table 19 and Fig. 30) than those of CIN patients. Only one (NK) of 10 patients (10%) had detectable HPV-specific T cell responses to STHV peptide (Table 19 and Fig. 31). The magnitude of response to this peptide was 188 SFU/10^6 cells.

Table 17. Demographic information of CaCx patients who enrolled into this study.

Patient	Age (years)	HPV infection	HPV type	Diagnosis
MJ	45	+	18	Squamous cell carcinoma
SK	67	+	11	Squamous cell carcinoma
PL	48	-	N.A.	Squamous cell carcinoma
NN*	39	+	Untyped	Squamous cell carcinoma
ST	64	+	18/6	Squamous cell cacinoma
JS	54	+	33	Adenocarcinoma
PP	41	+	Untyped	Adenocarcinoma
SV	42	+	18	Adenocacinoma
PC*	49	+	Untyped	Adenocarcinoma
NK	47	-	N.A.	Adenocarcinima
JV	37	+	Untyped	Adenocarcinoma

* On chemotherapy; N.A.: non-applicable

Table 18. *ex vivo* HPV-E7-specific CD8+ T cell responses in 11 patients with CaCx

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
MJ	0	0	0	0	0	0	0	0	0	0	0
SK	0	5	0	0	0	0	0	0	0	0	0
PL	0	10	0	0	0	0	0	0	0	0	0
NN	0	0	0	0	0	0	0	0	0	0	0
ST	2	4	1	5	3	1	2	2	0	1	3
JS	0	4	0	0	4	7	4	3	0	0	5
PP	0	0	0	0	0	0	0	0	0	0	0
SV	11	0	11	26	0	0	0	11	0	0	0
PC	0	0	0	0	0	0	0	0	0	0	0
NK	0	0	0	0	0	0	0	0	0	10	0
JV	0	0	0	0	0	0	0	0	0	0	1
Average	1.18	2.09	1.09	2.82	0.64	0.73	0.55	1.45	0.00	1.00	0.82

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in CaCx patients

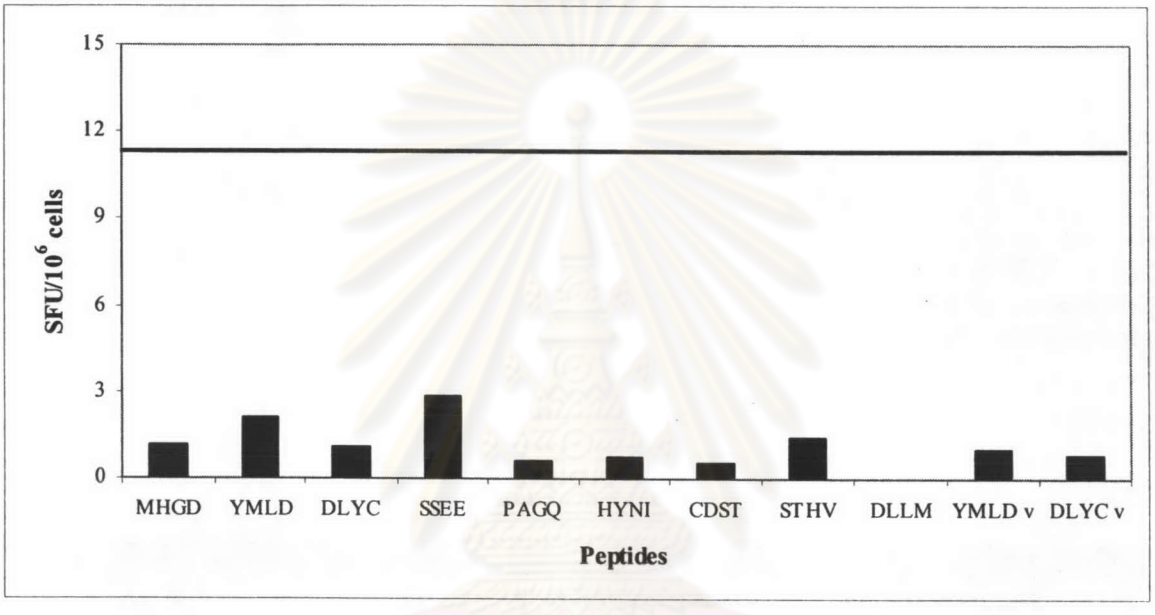


Figure 29. *ex vivo* HPV-E7-specific CD8+ T cell responses in 11 patients with CaCx. No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in all patients with CaCx. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide. Horizontal line represents positive cut-off value of the T cell responses.

Table 19. HPV-E7-specific CD8+ T cell responses in 10 patients with CaCx by cultured ELISpot assay

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
MJ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SK	0	0	0	0	0	0	0	0	0	0	0
PL	0	0	0	0	0	0	0	0	0	0	0
NN	0	0	0	0	0	0	0	0	0	0	0
ST	0	0	0	0	0	0	0	0	0	0	0
JS	0	0	0	0	0	0	0	0	0	0	0
PP	0	0	0	0	0	0	0	0	0	0	0
SV	0	0	0	0	0	0	0	0	0	0	0
PC	0	0	0	0	0	0	0	0	0	0	0
NK	0	0	0	0	0	0	0	188	0	0	0
JV	0	0	0	0	0	0	0	0	0	0	0
Average	0	0	0	0	0	0	0	18.8	0	0	0

ND: Not done

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

No HPV-E7-specific CD8+ T cell responses were detected in CaCx patients by cultured ELISpot assays

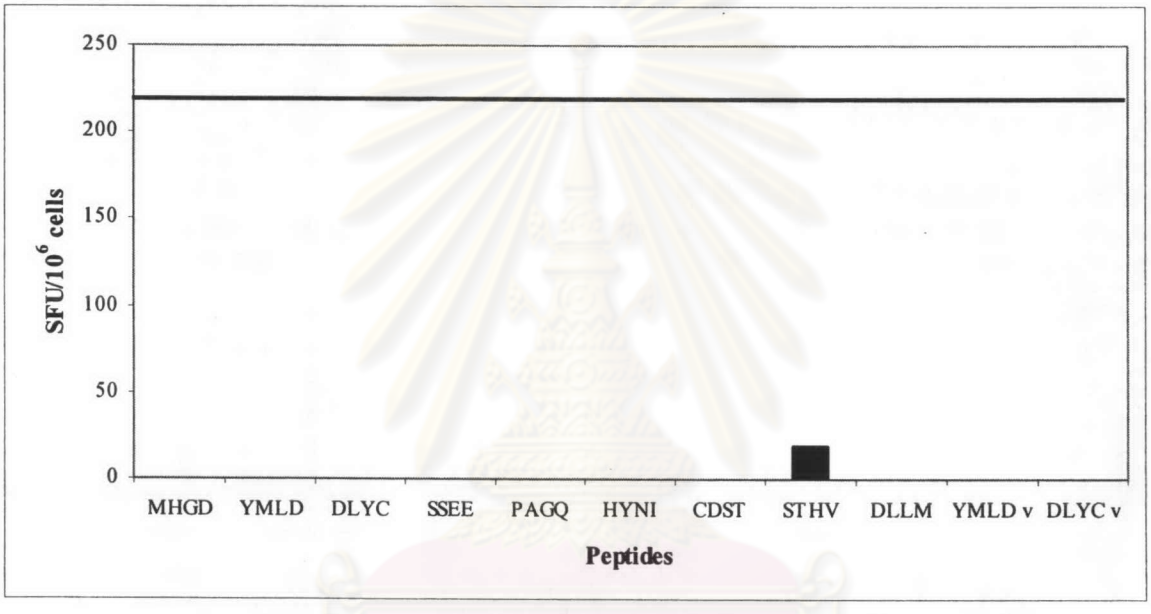


Figure 30. HPV-E7-specific CD8+ T cell responses in 10 patients with CaCx by cultured ELISpot assay. HPV-E7-specific CD8+ T cell responses were not detected in CaCx patients after 14 days culture with overlapping peptide. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide. Horizontal line represents positive cut-off value of the T cell responses.

Only one patient (NK) with cervical cancer had detectable HPV-E7 specific CD8+ T cell responses

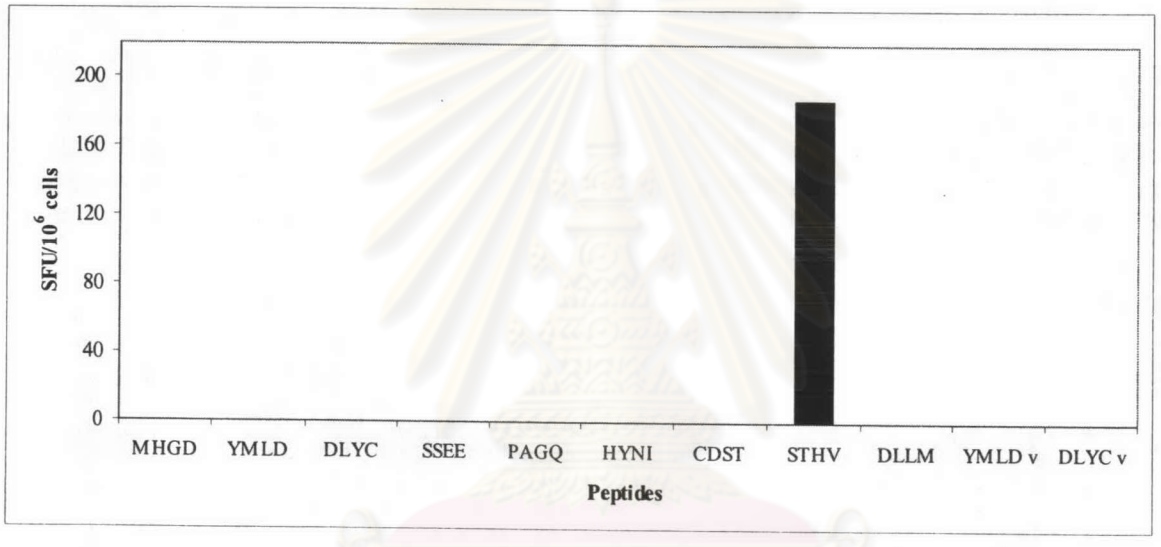


Figure 31. HPV-specific CD8+ T responses to peptide STHV were demonstrated in the patient NK who had no detectable HPV infection by cultured ELISpot assay. The bar represents the magnitude of response to the peptide STHV after background subtraction.

3.2.3 Comparison of HPV-E7-specific CD8+ T cell responses between CIN and CaCx patients

The aim of this analysis was to compare HPV-E7-specific CD8+ T cell activity against the HPV-16-E7 proteins in women with CIN and CaCx. HPV-E7-specific CD8+ T cell responses were more commonly detected in CIN patients than in CaCx patients (Fig. 32). In CIN patients had shown broader and higher magnitude of HPV-specific T cell response, whereas the magnitude of T cell response was least detectable in CaCx patients (Fig. 32). Moreover, CaCx patients recognised only STHV peptide and did not recognised YMLDv and DLYCv peptide whilst CIN patients recognised almost all peptides, including YMLDv and DLYCv peptide (Fig. 32).

HPV-E7-specific CD8+ T cell responses were higher and broader in CIN than in CaCx patients

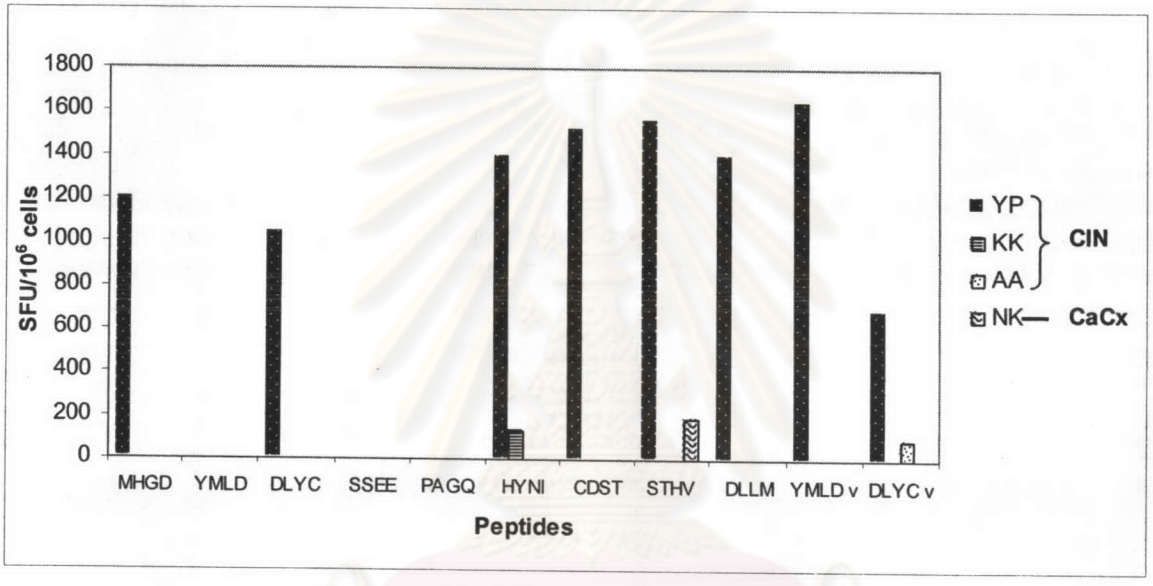


Figure 32. Comparison of HPV-E7-specific CD8+ T cell responses between CIN and CaCx patients. Magnitude of HPV-E7-specific CD8+ T cell responses detected in CIN were greater than those in CaCx patients. CIN patients had broader T cell response against E7 epitopes. YMLDv and DLYCv were recognised in CIN patients but not by CaCx patients. Only one peptide, STHV, was recognised by both groups. Each bar represents the magnitude of response to individual E7 overlapping peptide after background subtraction.

3.3 HPV-E7-specific CD8+ T cell depletion

We confirmed that the T cell responses were mediated by CD8+ T cells by performing CD8+ T cell depletion experiment as described in materials and methods. We randomly selected 3 patients whose T cells were positive for E7 overlapping peptides by ELISpot assay. As expected, the decreases of T cell responses were demonstrated when CD8+ T cells were depleted by immuno-magnetic beads. The numbers of spot were indeed decreased more than 50% in all 3 patients after CD8-depletion (Table 20).

Table 20. HPV-E7-specific CD8+ T cell depletion in 3 patients

Patient	Peptide	Magnitude of HPV-E7-specific CD8+T cell responses (SFU/10^6 cells)		% Reduction
		Before CD8 depletion	After CD8 depletion	
YP	MHGD	1,200	0	100.00
	DLYC	1,040	0	100.00
	HYNI	1,400	31	97.79
	CDST	1,520	1	99.40
	YMLDv	1,640	101	93.87
	DLYCv	1,280	31	97.58
KK	HYNI	135	12	91.12
NK	STHV	188	0	100.00

Each row represents the magnitude of response to individual E7 overlapping peptide after background subtraction.

3.4 Detection of HPV-E7-specific CD8+ T cell responses in control group

In our study, we investigated the role of HPV-E7-specific T cell responses in CIN and CaCx patients. In order to show specificity of this ELISpot, we screened 5 cord blood samples with ELISpot as the control. No HPV-16-E7-specific CD8+ T cell responses were detected in either *ex vivo* (Table 21 and Fig. 33) or cultured ELISpot assay (Table 22 and Fig. 34). Moreover, we calculated the positive cut-off value from negative control well in control group. The 1.25 time had shown sensitivity of the ELISpot assay (Fig. 35).

Table 21. *ex vivo* HPV-E7-specific CD8+ T cell responses in control group.

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
C1	0	1	1	0	-3	1	1	-4	-3	-1	-2
C2	1	0	0	-1	0	0	-2	-1	-1	-1	0
C3	2	0	-2	0	-1	3	-1	2	-2	-3	-1
C4	-1	0	-1	0	0	0	0	-1	-1	1	0
C5	0	-1	0	-2	-2	-1	0	-3	-3	-2	1
Average	0.4	0	-0.4	-0.6	-1.2	0.6	-0.4	-1.4	-2	-1.2	-0.4

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in control group

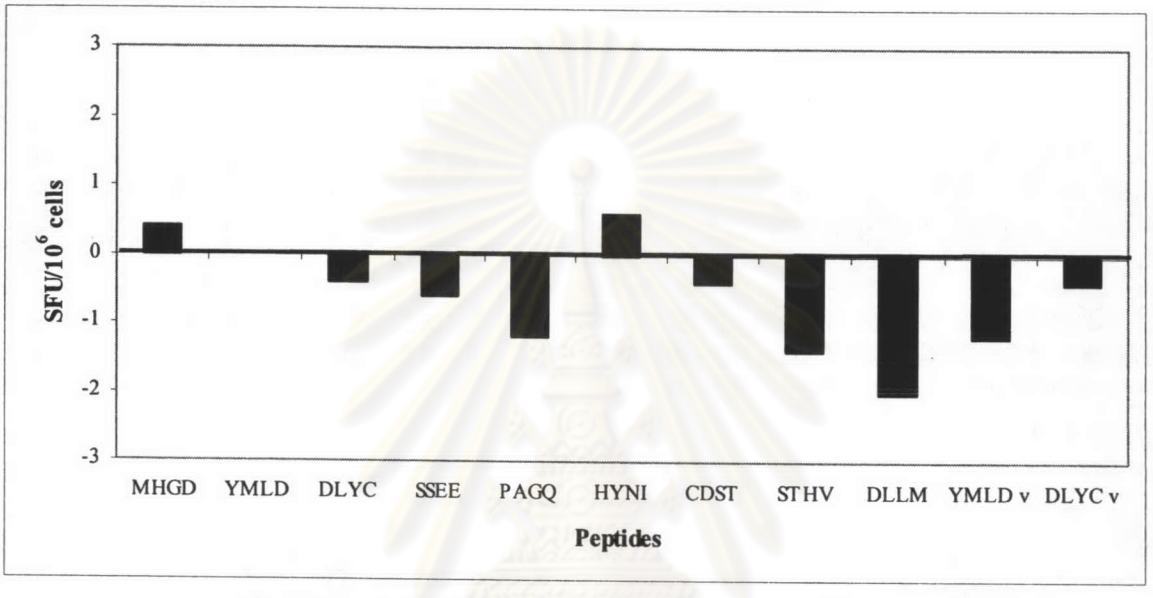


Figure 33. *ex vivo* HPV-E7-specific CD8+ T cell responses in 5 cord blood samples by IFN-γ ELISpot assay. No *ex vivo* HPV-E7-specific CD8+ T cell responses were detected in control group. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide after subtraction from the background.

Table 22. HPV-E7-specific CD8+ T cell responses in control group by cultured ELISpot assay

Patient	Peptides (SFU/10 ^{6} cells)										
	MHGD	YMLD	DLYC	SSEE	PAGQ	HYNI	CDST	STHV	DLLM	YMLDv	DLYCv
C1	155	157	157	183	-79	126	169	-73	-73	238	203
C2	-12	-213	-213	153	53	62	166	9	9	183	-135
C3	-148	183	183	178	3	173	253	188	188	150	260
C4	15	180	180	185	-23	-3	270	200	200	10	26
C5	48	158	158	228	98	146	92	248	248	26	117
Average	11.6	93	93	185.4	10.4	100.8	190	114.4	114.4	121.4	94.2

Each column represents the magnitude of response to individual E7 overalpping peptide after background subtraction.

No HPV-E7-specific CD8+ T cell responses were detected in control group by cultured ELISpot assay

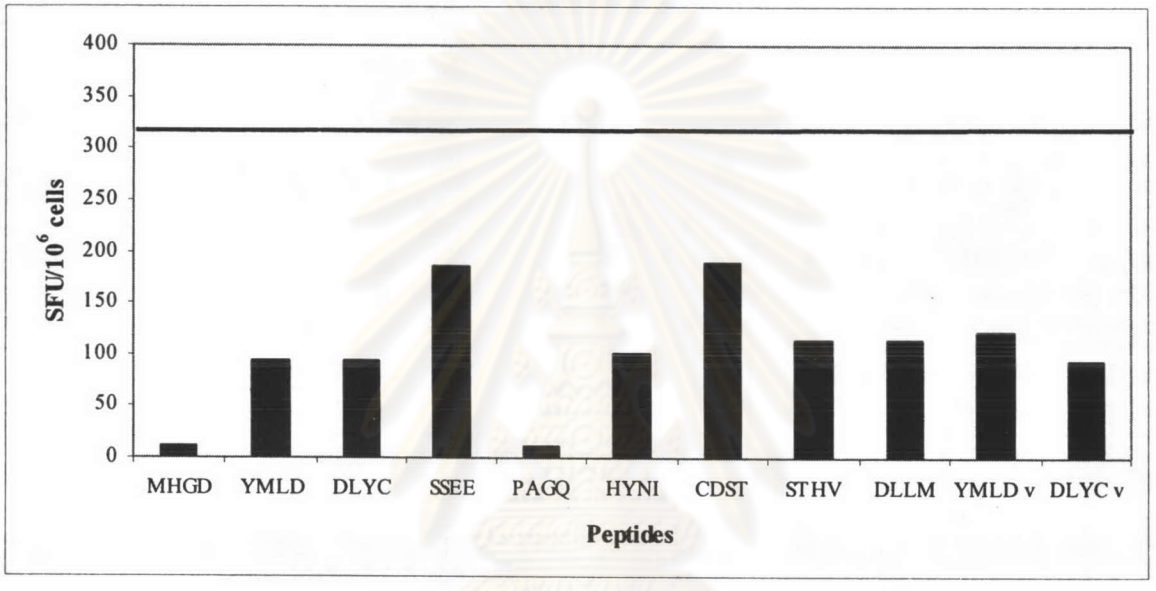


Figure 34. HPV-E7-specific CD8+ T cell responses in 5 cord blood samples by cultured ELISpot assay. HPV-E7-specific CD8+ T cell responses were not detected in control group, after 14 days culture with overlapping peptide. Each bar represents the mean of the magnitude of response to individual E7 overlapping peptide after background subtraction. Horizontal line represents positive cut-off value of the T cell responses.

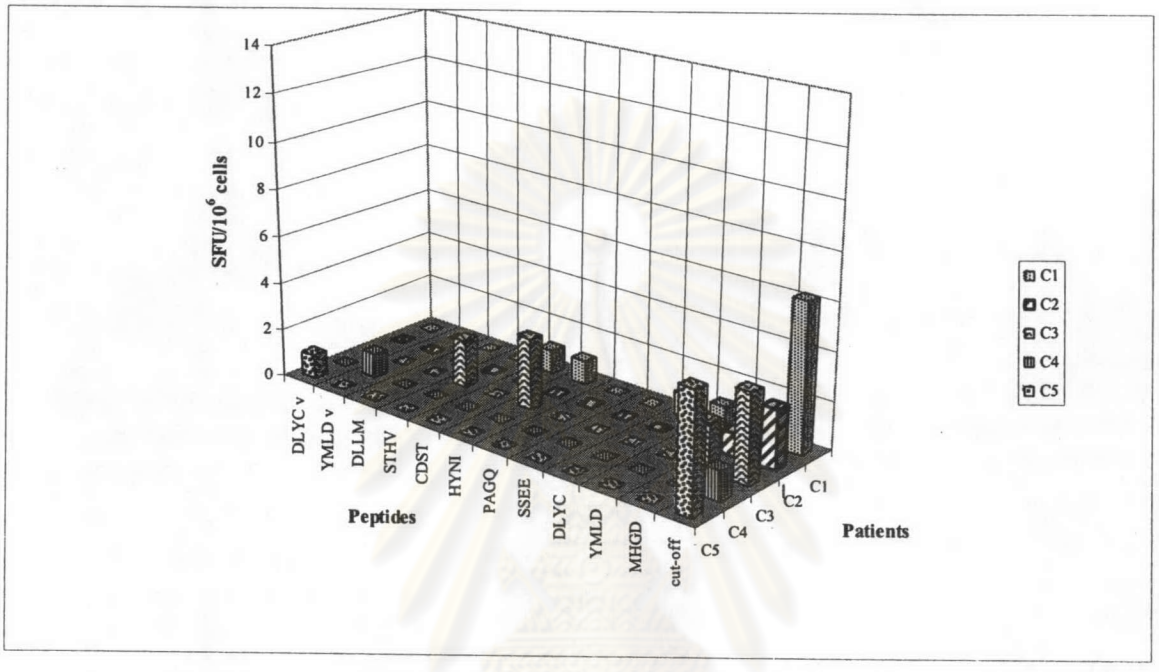


Figure 35. The positive cut-off value of HPV-E7-specific CD8+ T cell responses. The value was calculated from 5 cord blood samples. Cut-off bar was 1.25 time negative control well. Each bar represents the magnitude of response to individual E7 overlapping peptide after subtraction from the background.