CHAPTER IV

RESULTS

Extraction of DNA from paraffin embedded tissue

The formalin fixed paraffin embedded tissue was extracted according to the method described previously in materials and methods. The concentration of the extracted DNA in our preparation ranges from 435 ng/μl to 595 ng/μl and the average was 520.6 ng/μl.

Preparation of standard plasmid HPV-DNA

All 5 standard HPV-DNA plasmids were cloned in the *E. coli* strain HB101. To purity the plasmid DNA, the bacteria were grown in LB broth medium and the plasmid were extracted by plasmid DNA "miniprep" technique [99]. The concentration of the plasmid was determined at the OD of 260 nm. The concentration of HPV-6 was 1.52 μg/μl, HPV-11 was 115 ng/μl, HPV-16 was 340 ng/μl, HPV-18 was 935 ng/μl, and HPV-33 was 75 ng/μl.

The sensitivity and specificity of PCR reaction

To determine the PCR sensitivity, 5 purified plasmids HPV-DNA (HPV 6,11,16,18and 33) were diluted and applied to PCR amplification with L1 consensus primers (MY09, MY11) and β-globin primers (PC04, GH20). After that the amplified products were analyzed by GE. The expected band of L1 amplified product was 450 bp.

The results revealed that at least 1 pg of plasmid HPV-DNA type 6,16,18 and 33 could be detected except the plasmid HPV-DNA type-11 showed at least 1 ng to be detected. No band of β-globin gene product at 268 bp was observed. (Figure 8)

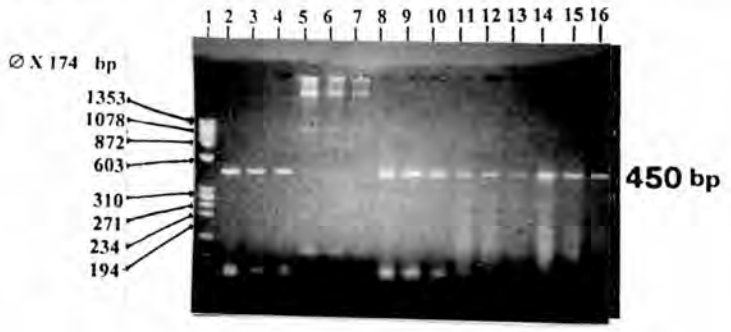

Figure 8. Determination of PCR sensitivity for detection of purified plasmid HPV-DNA (HPV-6,11,16,18 and 33).

Lane 1 marker ∅ x 174

Lanes 2-4 plasmid HPV-DNA type 6 (1μg, 1ng, 1 pg)

Lanes 5-7 plasmid HPV-DNA type 11 (1 μg, 1ng, 1pg)

Lanes 8-10 plasmid HPV-DNA type 16 (1 μg, 1ng, 1pg)

Lanes 11-13 plasmid HPV-DNA type 18 (1 μg, 1ng, 1pg)

Lanes 14-16 plasmid HPV-DNA type 33 (1 μg, 1ng, 1pg)

Detection of HPV-DNA in clinical specimens by PCR, GE and DH

One hundred of CIN-III patients and 100 of chronic cervicitis patients were randomly selected. The age of the CIN-III patients ranges from 24 to 63 years (mean age 39.3 years, SD= 8.6 and median age 38.5 years) and that of the chronic cervicitis ranges from 20 to 75 years (mean age 43.9 years SD= 8.3 and median age 42.9 years). The chronic cervicitis patients served as control group in this present study.

To detect the HPV-DNA, all tissues obtained from those patients were extracted and amplified with L1 consensus primers and the amplified products were analyzed by GE. The result revealed that 64% (64/100) of CIN-III and only 1% (1/100) of the control group were positive for HPV-DNA. The appearance of positive and negative samples were illustrated in Figure 9. To increase the sensitivity of detection method, one μg of all PCR products were dotted on nylon membrane (Hybond^{TM} N^{+} ; Amersham, England) and hybridized with generic probes (GP01, GP02). After hybridization, 72 % (72/100) of CIN-III and 6% (6/100) of the control group were confirmed positive for HPV-DNA (Table 6; Figure 10). Analyzing the data, statistic significance of the association between HPV infection and stage of CIN-III was indicated (χ^2 at d.f.1 = 91.55, $p< 0.05$). Moreover, the CIN-III patients have a chance to detect HPV-DNA greater than control group (OR= 40.28; 95% CI : 19.23-84.35). (Table 6)

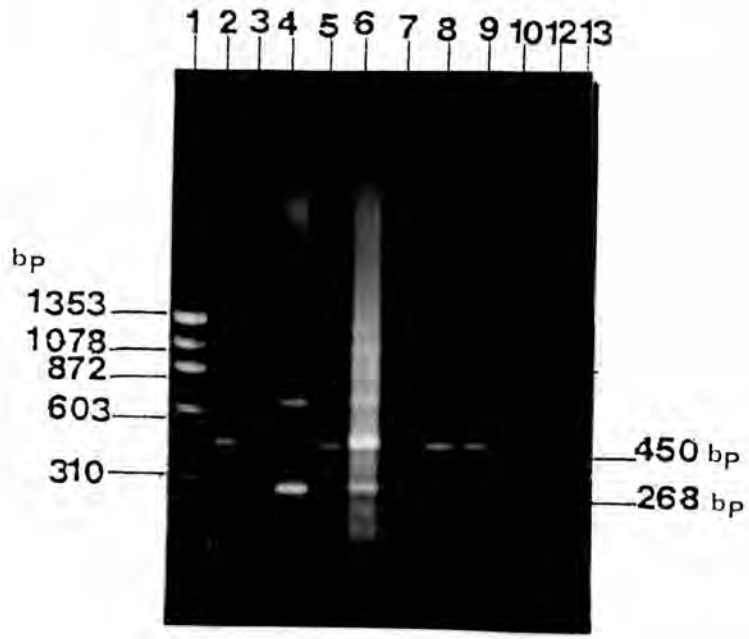

Figure 9. Detection of HPV-DNA in tissue samples by PCR and GE.

Lane 1 marker ØX 174

Lane 2 positive control (HeLa DNA)

Lane 3 negative control (DW)

Lane 4 Human DNA

Lanes 5,6,8,9,11 positive samples

Lanes 7,10,12,13 negative samples

Table 6. Crude ORs for the association between CIN-III and HPV-DNA in CIN-III and control group.

Samples	Total	HPV-DNA		χ^2	Crude OR	95%CI
		+	-			
CIN-III	100	72	28	91.55	40.28	19.23-84.35
Control	100	6	94			

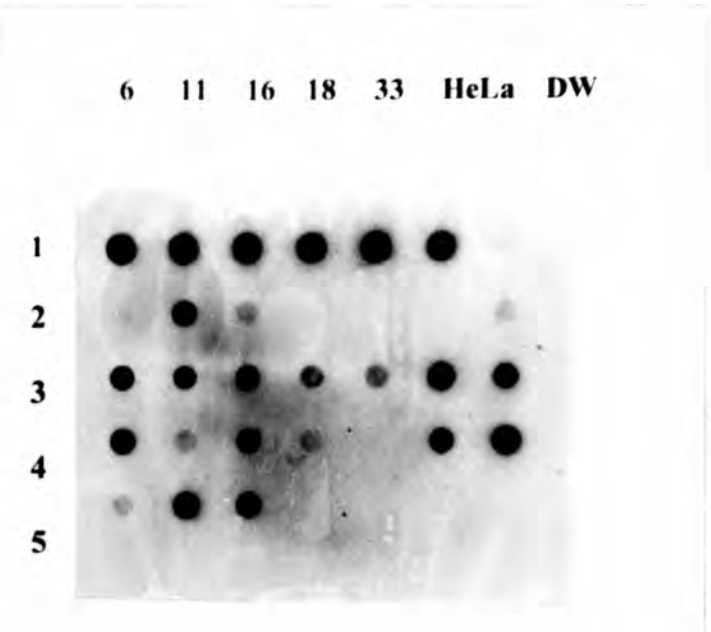

Figure 10. Detection of HPV-DNA by DH with GPs (GP 01 and GP 02).

Row 1. Plasmid HPV-DNA type 6,11,16,18, 33, HeLa DNA and sterile water.

Row 2-5. Amplified products of samples.

Optimization of the temperature for type specific probes hybridization

The hybridizing and washing condition were optimized by varying temperatures. All 5 HPV type-specific plasmids (HPV-6,11,16,18 and 33) including HeLa-DNA were used as standard HPV-DNA types. Human-DNA and distilled water were used as negative control. The optimized temperature of hybridizing and washing of TS-6,11 and 33 were at 40° C, and 40° C, TS-16 : 50° C and 55°C, TS-18 : 59° C and 65° C, respectively (Figure11)

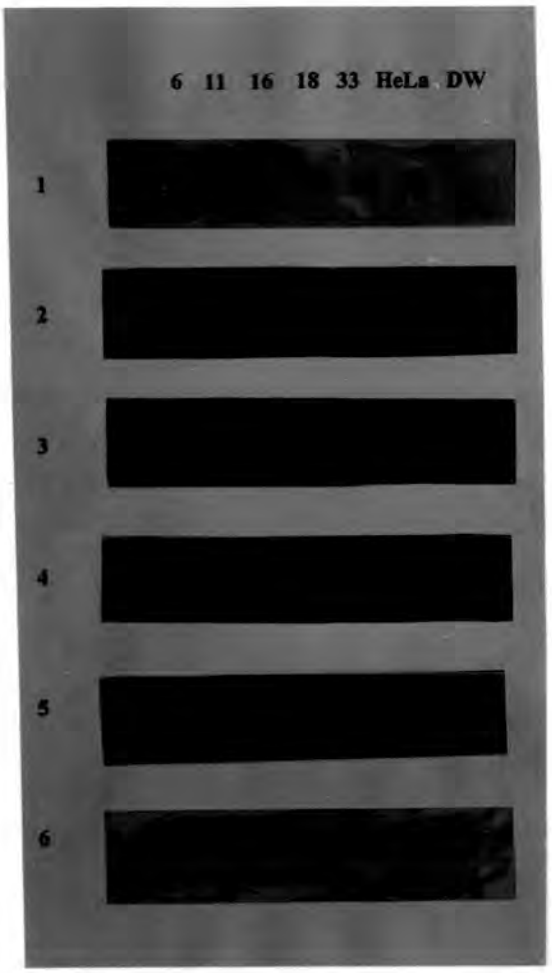

Figure 11. Optimization of temperature for type specific probes hybridization.

1. Purified plasmid HPV-DNA with generic probes
2. TS- 6 at 40°C for hybridization and 40°C for washing
3. TS-11 at 40°C for hybridization and 40°C for washing
4. TS-16 at 50°C for hybridization and 55°C for washing
5. TS-18 at 59°C for hybridization and 65°C for washing
6. TS-33 at 40°C for hybridization and 40°C for washing

Typing of HPV-DNA by type specific probes hybridization

To identify HPV type, all positive samples were applied on nylon membrane (Hybond^{TM} N^{+} ; Amersham, England) and hybridized with type specific probes (Figure 12). HPV-16 is the most prevalence type among CIN-III HPV-DNA positive patients (48.61%; 35/72), followed by HPV-18 (15.27%; 11/72), HPV-6 and 33 were detected in equal percentage (5.56%; 4/72) and HPV-11 was 2.7% (2/72). There were 10 CIN-III cases have mixed infection between types (HPV-6/16, 16/18, 16/33 and 16/18/33) and 27 (37.5%) cases could not be typed (Table7).

In contrast, two HPV-6 (33.3%) and one (16.6%) of HPV-18 were typed among control group and the other 3 cases (50%) were untyped (Table 7).

A

HPV 6	HPV 11	HPV 16	HPV 18	HPV 33	wbc -DNA
sample 1	2	3	--	--	--

B

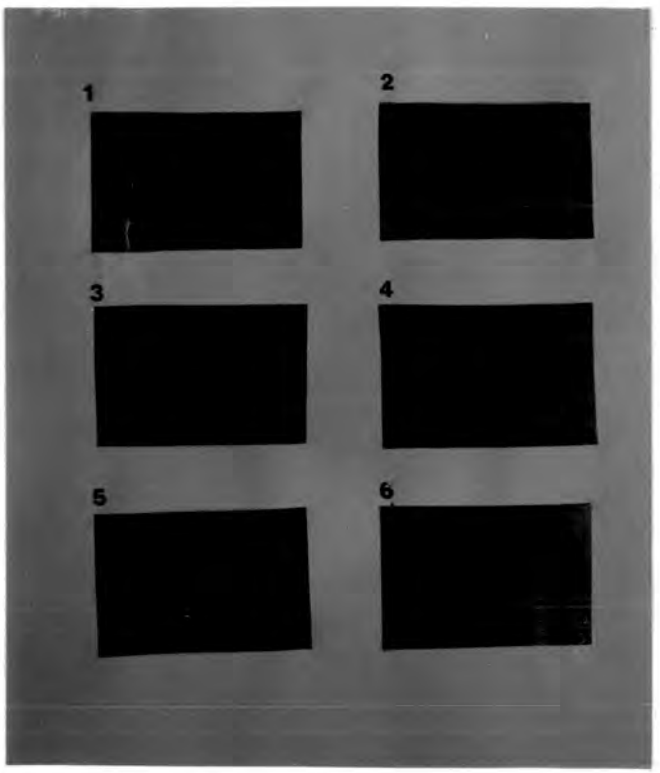

Figure 12. Detection and typing of amplified product by DH. A : scheme represent pattern of the experimental design. B: GPs (1) and TS probes for HPV-6(2), HPV-11(3), HPV-16 (4), HPV-18 (5) and HPV-33 (6).

Table 7. Distribution of HPV-type(s) in CIN-III and control group.

Samples	PCR- GE		PCR- DH (GP)		HPV-type(s)									Untype	Total
	+	-	+	-	6	11	16	18	33	6/16	16/18	16/33	16/18/33		
CIN-III	64	36	72	28	2	2	25	4	2	2	6	1	1	27	100
(%)	(64)	(36)	(72)	(28)	(2.7)	(2.7)	(34)	(5.5)	(2.7)	(2.7)	(8.3)	(1.3)	(1.3)	(37.5)	
Control	1	99	6	94	2	-	-	1	-	-	-	-	-	3	100
(%)	(1)	(99)	(6)	(94)	(33.3)			(16.6)						(50)	

Southern blot hybridization

Because 8 cases of CIN-III and 5 cases of control showed negative result by GE but, they generated weak signal when analyzed by DH with generic probes. However, 1 case of CIN-III group and 1 case of control group could be classified by type specific probes hybridization. Therefore, the remaining of 11 cases were repeated the amplified process and the amplification products were confirmed by Southern blot hybridization using generic probes. The result revealed that all 11 cases contained HPV-DNA (Figure13).

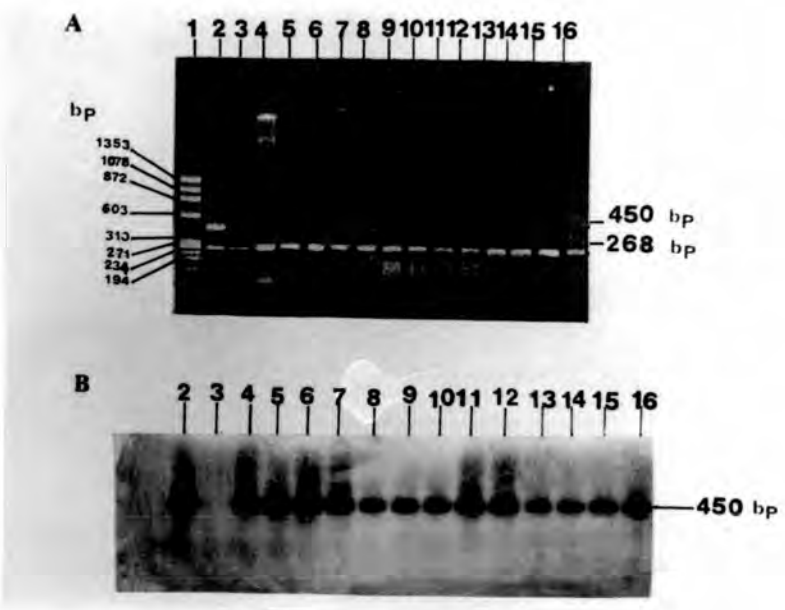

Figure 13. Detection of HPV-DNA by Southern blot hybridization in samples which PCR negative but DH positive.

A. Amplified product analysed by GE, lane 1 ∅ X 174, lane 2 HeLa DNA, lane3 Human DNA, lane 4-16 amplified products.

B. Southern blot hybridization with generic probes.