

CHAPTER 4

RESULTS

4.1 Detection of HPV DNA by PCR and Southern blot hybridization.

4.1.1 PCR amplification of HPV DNA extracted from cervical carcinoma biopsy tissues

DNA from 42 invasive cervical carcinoma biopsies was extracted using phenol chloroform. Each total cellular DNA was first evaluated for integrity by PCR amplification of the β -globin gene. Eight microliters of each amplified product was subjected to agarose gel electrophoresis and stained with ethidium bromide. Forty DNA samples demonstrated the 268 bp β -globin amplified fragment. The two DNA samples which did not amplify on repeat extraction for the β -globin gene were regarded as inadequate and were excluded from further analysis.

The presence of HPV DNA was determined by PCR analysis using consensus degenerate primers MY09 and MY11 (Perkin Elmer, USA), designed to amplify a 450 bp segment of the L1 region. Amplification was performed on up to 0.1 μ g of genomic DNA. Amplified DNA products were subjected to agarose gel electrophoresis (Figures 4.1 and 4.3). Table 4.1 shows that of the 40 cervical carcinoma samples, 32 (80%) were found to be positive for HPV DNA sequences. The remaining 8 (20%) did not exhibit the characteristic 450 bp fragment on the 2% agarose gel. Repeated PCR runs yielded the same results.

4.1.2 Southern blot hybridization typing of HPV DNA from cervical carcinoma biopsy tissues

The use of consensus degenerate primers MY09 and MY11 allowed the detection of a broad spectrum of genital HPVs. Typing of PCR amplified HPV L1 products was performed by Southern blot hybridization using type-specific probes. Digoxigenin-labelled, type-specific probes for the L1 PCR products of HPV16 and HPV18 were generated by PCR using primers MY09 and MY11 on plasmids containing inserts of HPV 16 and 18, respectively. The resulting digoxigenin-labelled, double-stranded, type specific probes for HPV 16 and HPV 18 were 450 bp each and correspond to sequences of the L1 ORF of HPV 16 and HPV 18, respectively (Figures 4.2 and 4.4).

After Southern transfer and hybridization with HPV 16 and HPV 18 probes separately, positive signals were detected in a total of 32 fresh frozen cervical cancer biopsies (Table 4.1). HPV 16 were detected in 22 of the 32 PCR-positive samples and in one of the eight PCR-negative samples. HPV 18 were detected in 22 of the 32 PCR-positive samples and in one PCR-negative samples. Two PCR-positive and six PCR-negative samples were negative for HPV 16 and/or HPV 18 DNA.

4.1.3 PCR amplification of HPV DNA from normal cervical scrapes

Thirty cervical scrapes from women without any cervical cytological abnormality were collected and DNA obtained. All of the 30 DNA samples amplified for β -globin genes indicating integrity of DNA. Of the 30, only 10 (33%) amplified for HPV DNA (Table 4.1). The remaining 20 (66%) which did not, were regarded as HPV-negatives. Repeated PCR runs yielded the same results.

4.1.4 Southern blot hybridization typing of HPV DNA from normal cervical scrapes

Of the 30 DNA samples from normal cervical scrapes, only eight samples hybridized with HPV 16 or HPV 18 probes. All eight samples were positive by PCR analysis. HPV 16 DNA was detected in eight samples while HPV 18 DNA was detected in only two. The two remaining PCR-positive samples and all 20 PCR-negative samples were negative for HPV 16 and/or HPV 18 DNA.

4.1.5 PCR amplification of HPV DNA from non-cervical specimens

Cellular DNA was extracted from 30 non-cervical specimens comprising of 20 leukemia, 5 ovarian carcinoma and 5 normal oral tissues. These DNA samples were examined by PCR for β -globin gene and amplifications on all 30 samples were observed. Further PCR analysis was carried out using primers MY09 and MY11 as described earlier. All 30 failed to show amplification for HPV DNA (Table 4.1). Repeated PCRs yielded the same results and the 30 non-cervical specimens were scored negative for HPV.

4.1.6 Southern blot hybridization typing of HPV DNA from non-cervical specimens

Despite being tested negative for HPV DNA by PCR, DNA samples from the non-cervical samples were subjected to further analysis by Southern blot hybridization. All 20 leukemia samples, 5 normal oral samples and 5 ovarian cancers were negative for HPV 16 and/or 18 sequences by Southern blotting.

4.1.7 Controls for PCR and Southern blot hybridization

DNA extracted from HPV-containing cell lines derived from cervical carcinoma, SiHa and HeLa and were included in every experiment as positive controls for HPV 16 and HPV 18, respectively. They were amplified using identical PCR procedures and conditions. Amplified products of both cell lines produced visible 450 bp of the HPV L1 fragment as well as the 268 bp β -globin gene. In addition to the positive controls, negative controls were included in every batch of PCR reactions, to monitor potential contamination of exogenous DNA materials. For the negative controls, sterile deionized water replaced the DNA template. Any batch of results with a contaminated negative control was dismissed and the whole procedure repeated. Results were confirmed and recorded only after both the positive and negative controls gave acceptable results.

The PCR products were subjected to Southern blot hybridization with HPV 16 and HPV 18 probes. DNA from HPV 16-containing SiHa cell line and from HPV 18-containing HeLa cell line hybridized intensely with HPV 16 probe and HPV 18 probe, respectively (Figures 4.2 and 4.4). Specificity of the probe were tested by crosshybridizing DNA from SiHa with HPV 18 probe and DNA from HeLa with HPV 16 probe. Neither cell line showed positive hybridization signal. As expected, the negative controls which did not contain any DNA template did not show any positive hybridization signal with any of the probes.

Table 4.1. Distribution of HPV 16 and HPV 18 in various cervical and non-cervical tissues as determined by PCR and Southern blot hybridization

Samples	PCR - positive				PCR - negative			Total
	16	18	16/18	HPV X	16	18	16/18	
Cervical tissues								
Cervical cancer - SCC	8	8	14	2	1	1	-	40
Normal pap smear	6	-	2	2	-	-	-	30
Non-cervical tissues								
Leukemia	-	-	-	-	-	-	-	20
Ovarian cancer	-	-	-	-	-	-	-	5
Normal oral	-	-	-	-	-	-	-	5

Note : HPV X represents HPV type unrepresented by probe for HPV 16 and HPV 18 DNA probes

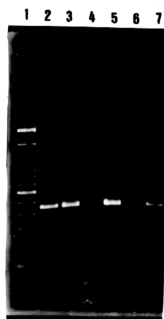


Fig. 4.1. Photograph of a 2% agarose gel showing PCR amplified HPV DNA with primers MY 11 and MY 09.

- Lane 1 : 100 bp molecular weight marker
- Lane 2 : HPV 16 positive control DNA (CaSki)
- Lane 3 : HPV 16 positive control DNA (SiHa)
- Lane 4 : Negative control (water as template for PCR)
- Lane 5 - 7 : Test DNA

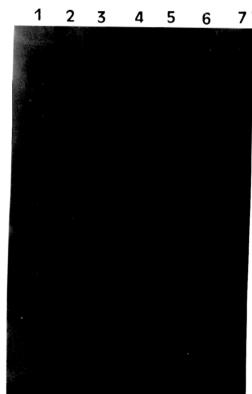


Fig. 4.2. Photograph of corresponding Southern blot hybridization of PCR amplified product to digoxigenin-labelled HPV 16 DNA probe.

- Lane 2 : HPV 16 positive control DNA (CaSki)
- Lane 3 : HPV 16 positive control DNA (SiHa)
- Lane 4 : Negative control (water as template for PCR)
- Lane 5 - 7 : Test DNA

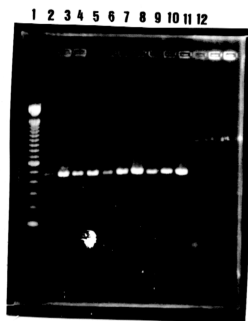


Fig. 4.3. Photograph of a 2% agarose gel showing PCR amplified HPV DNA with primers MY 11 and MY 09.

- | | | |
|-------------|---|---|
| Lane 1 | : | 100 bp molecular weight marker. |
| Lane 2 - 10 | : | Test DNA. |
| Lane 11 | : | HPV 18 positive control DNA (HeLa). |
| Lane 12 | : | Negative control (water as template for PCR). |

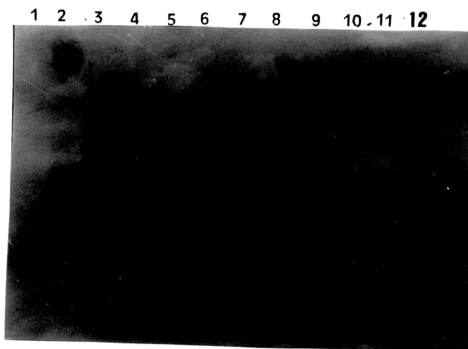


Fig. 4.4. Photograph of corresponding Southern blot hybridization of PCR amplified product to digoxigenin-labelled HPV 18 DNA probe.

- Lane 2 -10 : Test DNA
- Lane 11 : HPV 18 positive control DNA (HeLa)
- Lane 12 : Negative control (water as template for PCR)

4.2 *In situ* hybridization detection of HPV 16 and HPV 18 in cervical lesions

A total of 74 paraffin-embedded biopsies from various lesions of the cervix were analyzed for the presence and distribution of HPV 16 and 18 DNA by *in situ* hybridization. Thirty had the histological features of SCC, five were ADCs, 20 were CIN 3/CIS, 15 were CIN 2 and four were CIN 1. The tissues were analyzed separately using the 450 bp digoxigenin-labelled HPV 16 and HPV 18 DNA probes. Positive hybridization signals were localized in the nuclei of infected cells and confined within lesions. Hybridization was absent in the adjacent stromal tissues. Signals were widely variable in both intensity and topographical distribution within the lesions. Due to their close similarity, the distributions for both HPV 16 and 18 DNA were described together in the following section. The rates of detectable HPV 16 and 18 DNA varied according to the histologic diagnosis and are tabulated in Table 4.2.

4.2.1 Detection of HPV 16 and 18 DNA in CIN 1

Only one out of the four CIN 1 cases examined contained HPV 16 and 18 DNA (Table 4.2). HPV DNA was mostly detected within the hyperplastic zone of the proliferating basal and parabasal cells, comprising the lower one-third of the thickness of the entire epithelium. HPV DNA were also detected in koilocytes located in the superficial layers (Figure 4.5).

4.2.2 Detection of HPV 16 and 18 DNA in CIN 2

Of the 15 cases of CIN 2 examined, 10 were positive for HPV 16 and 18 DNA (Table 4.2). All 10 contained HPV 16 DNA. On the other hand, HPV 18 DNA was harboured by eight CIN 2 cases. Cells exhibiting the presence of the HPV genome were intensely stained, numerous and uniformly distributed. They mostly occupy the

overlying, more differentiated intermediate layers of the epithelia. Staining was the strongest in the koilocytes (Figure 4.6).

4.2.3 Detection of HPV 16 and 18 DNA in CIN 3/CIS

Of the 20 cases of CIN 3/CIS examined, 17 (85%) were found to harbour HPV DNA. HPV 18 DNA occurred in all 17 cases but HPV 16 was found in only 11 cases (Table 4.2). Intensely stained nuclei were abundant producing a crowded appearance throughout the entire thickness of the epithelium. In some cases, staining was either very weak or absent within cells located deep in the basal layers (Figure 4.7).

4.2.4 Detection of HPV 16 and 18 DNA in invasive cervical carcinomas

Twenty four out of the 30 SCC and four out of the five ADC examined contained HPV DNA. HPV 16 DNA was found in 20 (67%) SCC and one (20%) ADC. HPV 18 DNA was found in 20 (67%) SCC and four (80%) ADC (Table 4.3).

In general, cells harbouring HPV DNA were abundant and distributed uniformly throughout the lesions. The distribution of HPV DNA in the positive specimens were widely variable. In majority of the cases, staining intensities varied from cell to cell within the same lesion. Some nuclei were strongly stained while others had weaker intensities. Whereas the HPV DNA-positive cells were present only within the lesions, in many cases not all affected cells hybridized for HPV DNA. Thus, the HPV DNA-positive malignant cells did not differ morphologically and could not be distinguished from their adjacent HPV-negative counterparts (Figure 4.8).

Under comparable experimental conditions, two cell lines CaSki and HeLa which represented as positive controls hybridized intensely with HPV 16 and HPV 18 DNA probes, respectively (Figures 4.9 and 4.10). Specificity of the probes were

tested by cross-hybridization. HeLa did not react with HPV 16 DNA probe and CaSki did not react with HPV 18 DNA probe. Similarly, no hybridization was detected on the negative control slides which comprised of tissues untreated with the probe.

4.3 Prevalence of HPV 16 and 18 DNA in CIN and invasive cervical lesions as detected by PCR, Southern blot hybridization and *in situ* hybridization.

The overall detection frequencies of HPV 16 and 18 by combining the data obtained by PCR-Southern blot hybridization and *in situ* hybridization are as shown in Table 4.3. HPV DNA of these oncogenic viruses were detected in 88 out of the 114 preinvasive and invasive cervical lesions examined in this study. They were found in 80% (56/70) of SCC, 80% (4/5) of ADC, 85% (17/20) of CIN 3/CIS, 66.7% (10/15) of CIN 2 and 25% (1/4) of CIN 1. An increased rate of HPV DNA positivity was observed from CIN 1, CIN 2 to CIN 3/CIS. However the prevalence of HPV DNA dropped slightly between CIN 3/CIS and invasive carcinomas.

Analyzed separately, HPV 16 was detected in 61% (43/70) of SCC, 20% (1/5) of ADC and 58% (44/75) of all cervical carcinomas considered together. HPV 16 DNA was also detected in 55% (11/20) of CIN 3/CIS, 66.7% (10/15) of CIN 2 and 25% (1/4) of CIN 1. HPV 18 DNA on the other hand, was found in 61% (43/70) of SCC, 80% (4/5) of ADC and 62.6% (47/75) of all cervical carcinomas considered together. HPV 18 DNA was also detected in 85% (17/20) of CIN 3/CIS, 53.3% (8/15) of CIN 2 and 25% (1/4) of CIN 1.

The prevalence of HPV 16 DNA not differ significantly from that of HPV 18 DNA. Table 4.3 shows that the prevalence of HPV 16 was identical to that of HPV 18 in SCC. In contrast, a more noticeable difference between the prevalence of the two viruses were observed in ADC and CIN 3/CIS (Tables 4.2 and 4.3). In these two

cases, HPV 18 DNA occurred more frequently than HPV 16 DNA. The prevalence of HPV 18 DNA increased from CIN 1 through CIN 2 and CIN 3/CIS but dropped slightly between CIN 3/CIS and invasive carcinomas. HPV 16 DNA increased in prevalence from CIN 1 through CIN 2 to CIN 3/CIS and invasive carcinomas.

Based on the virologic data, the CIN/CIS cases were assigned to two groups - the low grade and the high grade lesions (Table 4.4). HPV DNA was detected in 77% of the high grade lesions (CIN 2 and CIN 3/CIS) and in much lower proportion of 25% in the low grade lesions (CIN 1).

4.3.1 Occurrence of single and mixed infections with HPV 16 and 18 in cervical lesions.

Prevalence rates of single and mixed infections with HPV 16 and 18 are shown in Tables 4.2 and 4.3. Single infections with either HPV 16 or 18 were found to occur in 32.4% (37/114) of all cervical lesions examined. They occurred in 37.1% (26/70) of the SCC, 60% (3/5) of the ADC, 30% (6/20) of CIN 3/CIS, 13.3% (2/15) of CIN 2 but none in CIN 1. The rate of single infection increased from CIN 1 to CIN 3/CIS but dropped in invasive carcinomas.

Mixed infections with HPV 16 and 18 were present in all histologic diagnoses. Of all the cervical lesions examined, 44.7% (51/114) were found to harbour both HPV 16 and 18 DNA. Mixed infections were recorded in 42.8% (30/70) of the SCC, 20% (1/5) of the ADC, 55% (11/20) of CIN 3/CIS, 53.3% (8/15) of CIN 2 and 25% (1/4) of CIN 1. The rate of mixed infections increased from CIN 1 through CIN 2 and CIN 3/CIS but dropped slightly in invasive carcinomas.

The frequencies of single and mixed infections with HPV 16 and 18 between preinvasive and invasive cervical disease were further analyzed statistically using the

chi-square test. It is found that in relation to the CIN/CIS lesions, cancer occurrence was independent of the nature of infection, either singly or as mixed infection ($\chi^2 = 1.954$, $p > 0.05$).

In most mixed infections the hybridization signal of one probe was accompanied by a weaker signal of the other probe. In others, both probes produced signals of similar intensities.

4.3.2 Age of patients at diagnosis and histologic features of cervical lesions

The mean age of the study population is shown in Table 4.5. The mean age of patients with cervical cancer was 49.5 years as opposed to 40.3 years of patients with preinvasive diseases. Considered in detail, the mean ages of patients with SCC and ADC were 49 and 58.8 years, respectively. For patients with preinvasive diseases, it increased from 29.5 years for CIN 1 to 38.14 years for CIN 2 and 44 years for CIN 3/CIS.

4.3.3 Prevalence of HPV DNA and age of patients

Table 4.6 correlates the HPV genotype with mean ages of HPV-positive patients. The mean age of the 66 patients positive for HPV 16 DNA was 47.8 years, almost similar to the 48.4 years of the 73 patients positive for HPV 18 DNA.

For further analysis, the study population was divided into four age groups. The details of HPV 16 and 18 prevalences in the age groups are as shown in Figure 4.11. HPV 16 and 18 were shown to prevail in all age groups listed. The patterns of detection rates for both HPV 16 and 18 were similar. They increased from age group 25-34 to age group 35-44 but remained almost consistent in the older age groups.

Table 4.2. Distribution of HPV 16 and HPV 18 in CIN lesions as determined by *in situ* hybridization

Histological diagnosis	No of cases	No of tissues positive for HPV DNA (%)				
		Total no of tissues positive for HPV DNA	16	18	16/18 (%)	Total
CIN 3/CIS	20	17 (85.0)	-	6 (30.0)	11 (55.0)	11 (55.0)
						17 (85.0)
CIN 2	15	10 (66.7)	2 (13.3)	-	8 (53.3)	10 (66.7)
						8 (53.3)
CIN 1	4	1 (25.0)	-	-	1 (25.0)	1 (25.0)
						1 (25.0)

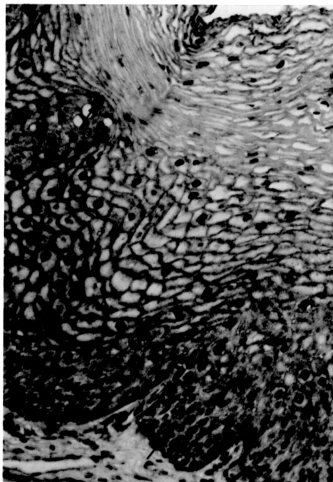


Fig. 4.5. *In situ* hybridization analysis of paraffin-embedded CIN 1 tissue using digoxigenin-labelled HPV 16 and HPV 18 DNA probes. The sections were counterstained with hemotocilin. Positive hybridization signals can be clearly identified in the nucleus of cells (arrow) in the basal (B) and parabasal (P) layers extending upwards to superficial cells with koilocytotic features (K) (x 250)

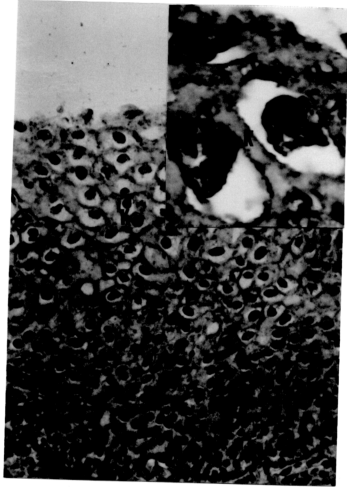


Fig. 4.6. *In situ* hybridization of HPV 16 and HPV 18 DNA in CIN 2. Positive hybridization signals of HPV DNA are seen in the nucleus of cells throughout the epithelium. Hybridization signals vary in intensities (arrow) (x 250). Insert shows that the koilocytes (K) are often reactive to the HPV DNA probe (x 1250).



Fig. 4.7. Localization of HPV 16 and HPV 18 DNA in nucleus of cells of CIN 3/CIS tissues. The hybridization signals of various intensities are identified in the nucleus of cells overlying the basal (B) and parabasal layers extending towards the surface of the epithelium. (x 500).

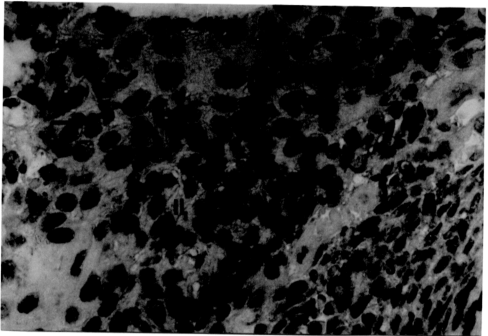


Fig. 4.8. Presence of HPV DNA in squamous cell carcinoma of the cervix. HPV DNA are found scattered uniformly throughout the invasive lesion (IL). Cells in the stroma (S) do not hybridize with the HPV probe. Hybridization signals vary in intensities in the transformed malignant cells (arrow) (x 500)

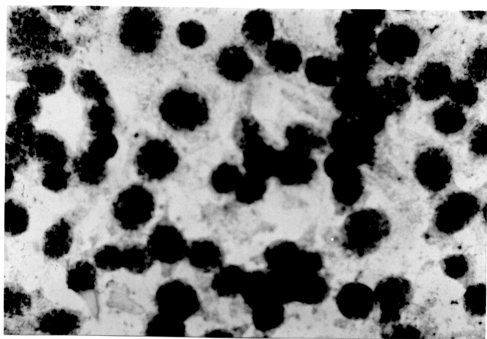


Fig. 4.9. DIG detection of HPV 16 genome in HPV 16-carrying CaSki cells fixed onto glass slides (x 1250).

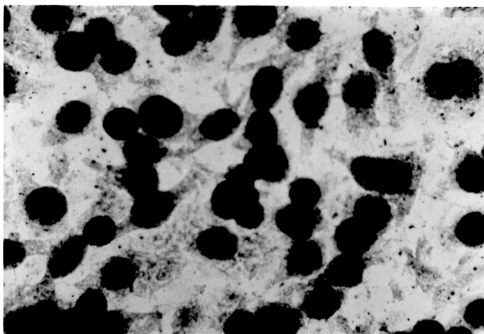


Fig 4.10. : DIG detection of HPV 18 genome in HPV 18-carrying HeLa cells grown onto glass slides (x 1250).

Table 4.3. Overall frequency of HPV 16 and HPV 18 DNA in cervical lesions as determined by PCR-Southern blot hybridization (PCR-SB) and *in situ* hybridization (ISH)

Histology	No of cases	No of cases containing HPV DNA (%)					HPV X /
		16	18	16/18	Total 16	Total 18	HPV negative (%)
SCC							
PCR-SB	40	9 (22.5)	9 (22.5)	14 (35)	23 (57.5)	23 (57.5)	8 (20)
ISH	30	4 (13.3)	4 (13.3)	16 (53.3)	20 (66.6)	20 (66.6)	6 (20)
Total SCC	70	13 (18.5)	13 (18.5)	30 (42.8)	43 (61.4)	43 (61.4)	14 (20)
		26 (37.1)					
ADC							
ISH	5	-	3 (60)	1 (20)	1 (20)	4 (80)	1 (20)
Total invasive carcinoma	75	13 (17.3)	16 (21.3)	31 (41.3)	44 (58.6)	47 (62.6)	15 (20)
		29 (38.7)					
Total CIN / CIS	39	2 (5)	6 (15.3)	20 (51.2)	22 (56.4)	26 (66.7)	11 (28.2)
		8 (20.5)					
Total cervical lesions	114	15 (13.1)	22 (19.2)	51 (44.7)	66 (57.9)	73 (64)	26 (22.8)
		37 (32.5)					

Table 4.4. Distribution of HPV 16 and HPV 18 in preinvasive lesions

Histological Diagnosis	HPV 16	HPV 18	Total positive for HPV DNA
High grade (CIN 2, CIN 3/CIS)	21/35 (60%)	35/35 (71%)	27/35 (77%)
Low grade (CIN 1)	1/4 (25%)	1/4 (25%)	1/4 (25%)

Table 4.5. Correlation of age of patients at diagnosis and histologic types of cervical lesions

Histological type of lesion	No of cases	Mean age of patients (years)
SCC	70	49
ADC	5	58.8
Total invasive carcinoma	75	49.6
CIN 3/CIS	20	44
CIN 2	15	38.14
CIN 1	4	29.5
Total dysplasia	39	40.3

Table 4.6. Correlation of age of patients at diagnosis and HPV genotype

HPV genotype	No of patients	Mean age of patients (years)
HPV 16	66	47.8
HPV 18	73	48.4

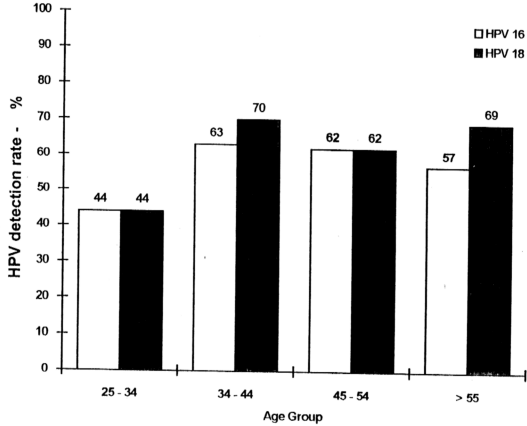


Fig. 4.11 Distribution of HPV types according to age group of patients with cervical lesions

4.4 Detection of HPV antigens by immunohistochemistry

The 74 paraffin-embedded cervical biopsies comprising of 30 SCC, five ADC, 20 CIN 3/CIS, 15 CIN 2 and four CIN 1 previously screened for HPV 16 and 18 DNA by *in situ* hybridization were further analyzed for the expression of HPV 18 E6 and HPV 16 L1 protein in the tissues by immunohistochemistry.

4.4.1 Correlation between cervical histology and the detection frequencies of HPV 18 E6 protein

Infected cells were recorded as positive for the E6 protein when reddish-brown precipitate was clearly observed in the nuclear and / or cytoplasmic regions. Detection rates for HPV 18 E6 protein varied according to histologic diagnoses (Table 4.7).

Of the 74 cervical lesions examined, a total of 56 cases (75.7%) exhibited the presence of the E6 protein. E6-positivities were detected in 25 of 30 (83.3%) cases of SCC, all five (100%) cases of ADC, 18 of 20 (90%) cases of CIN 3/CIS and eight of 15 (53.3%) cases of CIN 2. There was an increase in the relative expression of the E6 protein with severity of the CIN lesions (Table 4.7, Figure 4.12). The protein was not detectable in low grade CIN lesions (CIN 1) but increased to 75.7% of the high grade lesions and continued to be highly expressed in invasive carcinomas (85.7%). The detection rate of E6 protein in invasive carcinomas was higher than that of the total CIN/CIS (85.7% versus 66%). However, chi-square analysis revealed that the difference was not significant ($\chi^2 = 2.67$, $p > 0.05$).

4.4.1.1 Correlation between the expression of E6 protein and the presence of HPV 18 DNA

The correlation between the expression of HPV E6 protein and the presence of the HPV 18 genome is shown in Table 4.7 and Figure 4.13. A total of 45 of 50 (90%) cases of cervical lesions which harboured HPV 18 DNA were also positive for HPV 18 E6 antigen. Nineteen of 20 (95%) cases of HPV 18-associated SCC, all four (100%) cases of HPV 18-associated ADC, all 17 (100%) cases of HPV 18-associated CIN 3/CIS and five of eight (62%) cases of HPV 18-associated CIN 2 exhibited the expression of the E6 protein. An increase in E6 positivities among HPV 18-positive lesions was seen with increasing severity of the lesions.

Table 4.7 also revealed that a total of 11 of 24 (45.8%) cervical lesions which were HPV 18 DNA-negative by *in situ* hybridization showed the presence of the E6 protein. Six were SCC, one was an ADC, one was a CIN 3/CIS, and three were a CIN 2. Meanwhile 13 lesions which were negative for the HPV 18 genome remained negative for the E6 protein.

R is the ratio correlating the E6 positivities (%) with the presence of HPV 18 DNA (%). It increased from 0 (0:25) for CIN 1 to 1 (53:53) for CIN 2, 1.05 (90:85) for CIN 3/CIS and 1.26 (85.7:68) for invasive carcinomas (Figure 4.13).

4.4.1.2 Topographical distribution of HPV 18 E6 protein in cervical lesions

Intranuclear and/or cytoplasmic signals indicating the expression of the E6 protein were widely variable in intensity and distribution within the lesions. The degree of staining intensities reflect the extent of protein positivity or amount of protein expressed in infected cells. Cells with intense staining were considered strongly positive while those with faint staining were weakly positive. Cells positive for the

protein were confined within the lesions and were comprised of those with large, round nuclei to koilocytes. Occasionally very faint and diffuse unspecific staining was seen but was easily distinguished from the characteristic staining of the protein in tumour cells.

Positive and negative controls were included in every batch of experiment. HPV 18-containing HeLa cell line which presented as the positive control was subjected to identical experimental conditions. Intense stainings in both intranuclear and cytoplasmic regions were seen, indicating the presence of the E6 protein in abundance (Figure 4.14). Negative controls were cervical sections untreated with the monoclonal antibody specific for the HPV 18 E6 protein. Upon examination, none of these sections stained positive.

4.4.1.3 Distribution of HPV 18 E6 protein in CIN 2

Cells containing the E6 protein were abundant and uniformly distributed within the lesions. The E6-positive cells were seen in variable staining intensities scattered throughout the entire thickness of the epithelia and were mixed with unstained ones (Figure 4.15).

4.4.1.4 Distribution of HPV 18 E6 protein in CIN 3/CIS

In E6-positive specimens, cells expressing the protein were present in abundance and mixed with unstained ones throughout the entire thickness of the affected epithelia. As compared to those of CIN 2, these cells were more numerous and intensely stained (Figure 4.16).

4.4.1.5 Distribution of HPV 18 E6 protein in invasive carcinoma

In positive specimens, cells exhibiting the presence of the E6 protein were numerous and uniformly distributed throughout the lesions. In many tissues the staining were intense. Although they were cytomorphologically similar yet not all malignant cells stained for the protein (Figure 4.17).

4.4.1.6 Intracellular localization of HPV 16 E6 protein in CIN and invasive carcinoma

The intracellular localization of the E6 protein as determined by the type of staining was analyzed in Table 4.8. Staining in both nuclear and cytoplasmic regions were seen in 13 of 30 (43%) cases of invasive carcinoma, nine of 18 (50%) cases of CIN 3/CIS and five of eight (55%) cases of CIN 2. Cytoplasmic stainings in the absence of nuclear stainings were identified in three of 30 (10%) cases of invasive carcinoma, two of 18 (11%) cases of CIN 3/CIS and three of eight (37.5%) cases of CIN 2. The third type of staining pattern which is exclusively intranuclear was demonstrated only among the CIN 3/CIS and invasive carcinoma, with frequencies of seven of 18 (38.8%) and 14 of 30 (46%), respectively.

Table 4.7. Detection of HPV 18 E6 protein in relation to the status of HPV 18 DNA in the cervical intraepithelial neoplasia and cervical cancer

Type of lesions	No of cases	HPV 18 DNA positive		HPV 18 DNA positive		Total no of E6-positive cases (%)
		+ve	-ve	+ve	-ve	
SCC	30	19	1	6	4	25 (83.3)
ADC	5	4	-	1	-	5 (100)
Total cervical cancer	35	23	1	7	4	30 (85.7)
CIN 3/CIS	20	17	-	1	2	18 (90)
CIN 2	15	5	3	3	4	8 (53.3)
CIN 1	4	-	1	-	3	0
Total dysplasia	39	22	4	4	9	26 (66.7)
Total	74	45	5	11	13	56 (75.7)

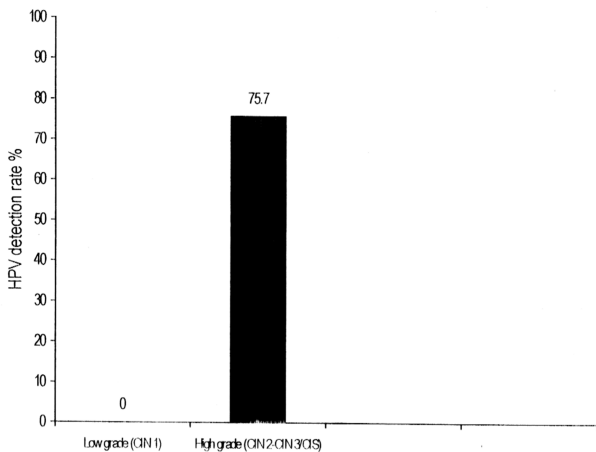


Fig. 4.12 Correlation between HPV 18 E6 protein positivity and grades of CIN lesions

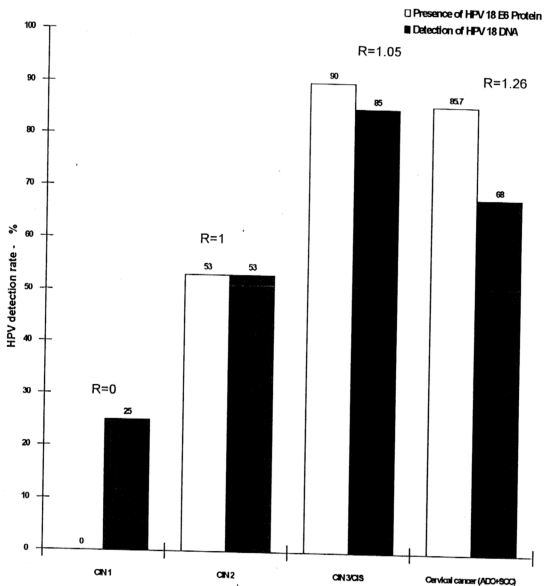


Fig. 4.13 Correlation between the expression of HPV 18 E6 oncoprotein and the detection rate of HPV 18 DNA in CIN lesions and cervical cancer.

$$R = \frac{\text{Presence of HPV 18 E6 protein}}{\text{Detection of HPV 18 DNA}}$$

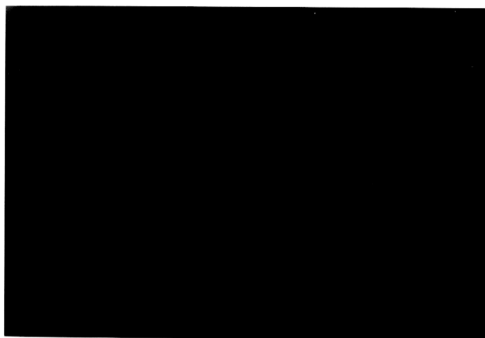


Fig. 4.14. Detection of HPV 18 E6 protein in HPV 18-containing HeLa cells using the Labelled Streptavidin Biotin (LSAB) peroxidase, the AEC substrate system and anti-HPV 18 E6 monoclonal antibody (dilution 1:80). Intense stainings are exhibited in the nucleus and cytoplasm (x 1250).

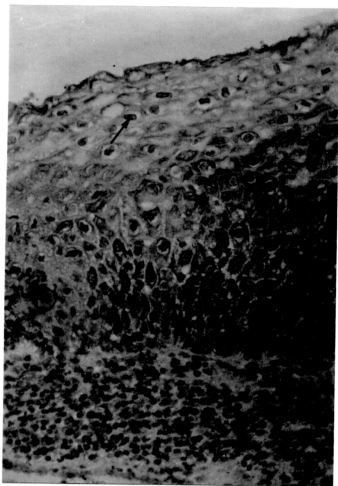


Fig. 4.15. Immunohistochemical detection of HPV 18 E6 protein in paraffin-embedded CIN 2 tissue
HPV 18 E6-positive cells are distributed throughout the thickness of the epithelium (arrow) (x 250).

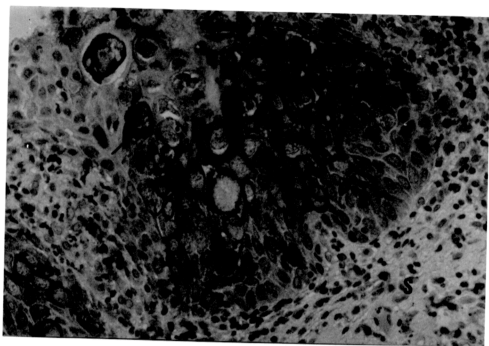


Fig. 4.16. Detection of HPV 18 E6 protein in cells of CIN 3/CIS . The E6 protein is confined in cells of the lesion (arrow) and is absent in cells of the stroma (S) (x 500).

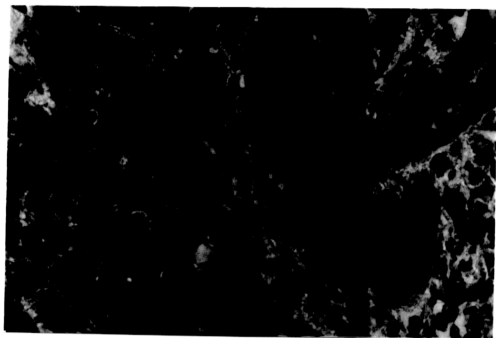


Fig. 4.17. Localization of HPV 18 E6 protein in the intranuclear and cytoplasmic regions of cells of squamous cell carcinoma (arrow). No staining is observed in cells of the stroma (S) (x 500).

Table 4.8. Intracellular localization of HPV 18 E6 protein in cervical lesions as determined by immunohistochemistry

Type of lesions	No of E6- positive cases	Type of staining		
		Intranuclear only	Cytoplasmic only	Intranuclear + cytoplasmic
Invasive carcinoma	30	14 (46 %)	3 (10 %)	13 (43 %)
CIN 3 / CIS	18	7 (38.8 %)	2 (11%)	9 (50 %)
CIN 2	8	-	3 (37.5 %)	5 (55 %)
CIN 1	0	-	-	-

4.4.2 Correlation between cervical histology and the detection frequencies of HPV 16 L1 protein

Infected cells were accounted as positive when staining was clearly identified in the nuclei. As seen in Table 4.9, the rate of detectable HPV 16 L1 protein varied according to histologic diagnoses.

Of the 74 cervical lesions examined, a total of 39 cases (52.7%) exhibited the presence of the L1 protein. They were 21 of 30 (70%) cases of SCC, four of five (80%) cases of ADC, 10 of 20 (50%) cases of CIN 3/CIS and four of 15 (26.6%) cases of CIN 2. There was an increases in the relative expression of the L1 protein with severity of the cervical lesions. The protein was not detectable in the low grade CIN lesions (CIN 1) but increased to 40% in the high grade lesions (CIN 2 and CIN 3/CIS). The detection rate of the protein was higher in invasive carcinoma than that of the

preinvasive lesions, being 71.4% and 35.8% , respectively. Chi-square analysis revealed that the difference was highly significant ($\chi^2 = 7.97$, $p < 0.05$).

4.4.2.1 Correlation between the expression of the HPV 16 L1 protein and the presence of HPV 16 DNA

Table 4.9 shows the expression of the L1 protein in relation to the presence of the HPV 16 genome in cervical lesions. A total of 29 of 43 (67.4%) cases of cervical lesions which harboured HPV 16 DNA were also positive for HPV 16 L1 protein. Seventeen were SCC, one was an ADC, seven were CIN 3/CIS and four were CIN 2. An increase in L1 protein positivities was seen between HPV 16-associated preinvasive and invasive carcinoma, being 50% (11/22) and 85.7% (18/21), respectively. Chi-square analysis revealed that the increase was significant ($\chi^2 = 4.72$, $p < 0.05$).

Table 4.9 also revealed that 10 of 15 (40%) cervical lesions which were HPV 16-negative by *in situ* hybridization showed the presence of the L1 protein. Four were SCC, three were ADC and three were CIN 3/CIS. Meanwhile 15 lesions which were negative for HPV 16 genome remained negative for the L1 protein.

R is the ratio correlating the L1 positivities (%) with the presence of the HPV 16 DNA (%). The ratio increased from 0 (0:25) for CIN 1 to 0.399 (26.6:66.7) for CIN 2, 0.91 (50:55) for CIN 3/CIS and 1.22 (71.4:58.6) for invasive carcinoma (Figure 4.18).

4.4.2.2 Topographical distribution of HPV 16 L1 protein in cervical lesions

Positive stainings indicating the presence of HPV 16 L1 protein varied widely in intensities as well as topographical distribution within lesions. The degree of staining intensities reflect the extent of protein positivity or the amount of the L1 protein

expressed by infected cells. Cells with intense staining were strongly positive while those with faint staining were considered weakly positive. Although diffuse cytoplasmic staining was also noted in several cervical lesions, infected cells were accounted as positive for the L1 protein only when staining was clearly identified in the nuclei. Cells positive for the protein were confined within lesions and were comprised of those with large, round nuclei to koilocytes. Staining was absent in adjacent stromal tissues in all cases.

Positive and negative controls were included in every batch of experiment. HPV 16-containing CaSki and SiHa cell lines were subjected to identical experimental conditions. None of these cell lines stained positive and therefore inappropriate as positive controls for the L1 protein (Figure 4.19). It was noted that one cervical lesion which was strongly positive for this protein and it was selected as a routine positive control in the tests. Negative controls were adjacent cervical sections which were treated with water instead of the L1-specific monoclonal antibody. These sections always gave negative staining.

4.4.2.3 Distribution of HPV 16 L1 protein in CIN 1

The one case of CIN 1 which harboured HPV 16 DNA did not exhibit the characteristic intranuclear precipitate and was accounted as negative for the L1 protein. However, diffuse cytoplasmic staining was demonstrated in the the intermediate and superficial layers of the epithelium of this particular tissue.

4.4.2.4 Distribution of the HPV 16 L1 protein in CIN 2

The distribution of the protein was seen as patchy. Cells containing the L1 protein were not abundant and included koilocytes in the superficial layers of the epithelia (Figure 4.20). A few were also detected in the basal and parabasal layers.

Diffuse cytoplasmic stainings were noted in 11 of 15 (73%) cases and were widespread in the intermediate and superficial layers. In fact, all the five cases which were negative for both HPV 16 genome and L1 protein displayed similar stainings.

4.4.2.5 Distribution of the HPV 16 L1 protein in CIN 3/CIS

Cells containing the L1 protein in positive cases were more numerous than those of CIN 2. In five of seven positive cases, cells containing the L1 protein were seen scattered and mixed with unstained cells throughout the entire epithelia (Figure 4.21). One other contained L1-positive cells in the intermediate and superficial layers and the remaining one case only contained few L1-positive cells.

Diffuse cytoplasmic stainings were observed in 11 of 20 (55%) cases. When present, such stainings occupied the layers overlying the basal and parabasal zones.

4.4.2.6 Distribution of the HPV 16 L1 protein in invasive carcinoma

In positive cases, cells exhibiting the presence of the L1 protein were found to be more numerous and more intensely stained than those of the preinvasive lesions. These cells which were confined within the lesions were often mixed with unstained cells (Figures 4.22a and 4.22b). Observations revealed that cells which stained positive for the protein could not be differentiated cytomorphologically from unstained ones. Distribution of the L1-positive cells in invasive lesions varied. In some specimens, they were uniformly distributed. In others, the distributions were patchy.

In contrast to CIN 1, CIN 2 and CIN 3/CIS, diffuse cytoplasmic staining was not observed in any of the cervical lesions examined.

Table 4.9. Detection of HPV 16 L1 protein according to the status of HPV 16 DNA in cervical intraepithelial neoplasia and cervical cancer

Type of lesion	No of cases	HPV 16 DNA positive		HPV 16 DNA negative		Total no of L1-positive cases (%)
		+L1	-L1	+L1	-L1	
SCC	30	17	3	4	6	21 (70)
ADC	5	1	-	3	1	4 (80)
Total cervical cancer	35	18	3	7	7	25 (71.4)
CIN 3/CIS	20	7	4	3	-	10 (50)
CIN 2	15	4	6	-	5	4 (26.6)
CIN 1	4	-	1	-	3	0
Total dysplasia	39	11	11	3	8	14 (35.8)
Total	74	29	14	10	15	39 (52.7)

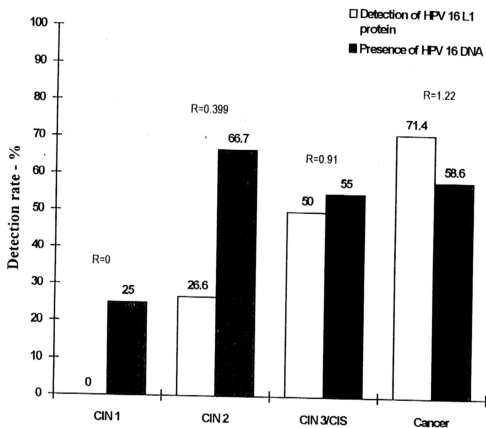


Fig. 4.18 Correlation between the expression of HPV 16 L1 protein and the detection rate of HPV 16 DNA in CIN lesions and cervical cancer

$$R = \frac{\text{Detection of HPV L1 protein}}{\text{Presence of HPV 16 DNA}}$$

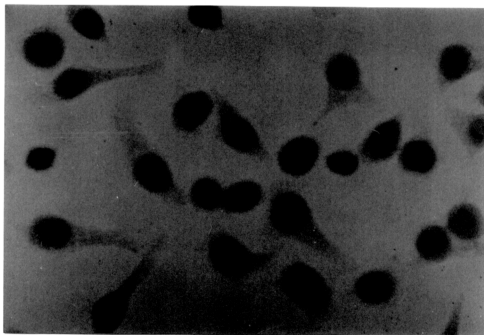


Fig. 4.19. HPV 16-containing CaSki cells showing no staining to anti-HPV 16 L1 monoclonal antibody (diluted 1:40) indicating the absence of L1 protein expression in these cells (x1250).



Fig. 4.20. Presence of HPV 16 L1 protein in cells of CIN 2. Intense stainings are noted in koilocytes (K) (x500).

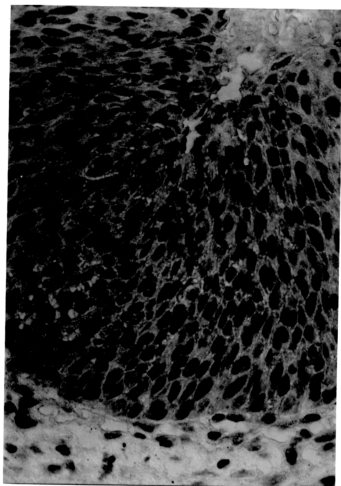


Fig. 4.21. Distribution of HPV 16 L1 protein in cells of CIN 3/CIS. L1-positive cells are distributed throughout the entire thickness of the epithelium, mixed with unstained cells (arrow) (x500).

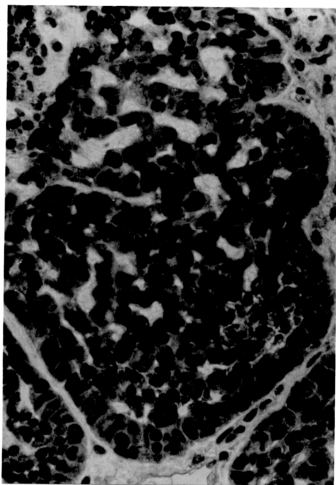


Fig. 4.22a. Intracellular localization of HPV 16 L1 protein in cells of adenocarcinoma. Cells positive for the protein are found in abundance, often mixed with unstained cells (arrow) (x500).

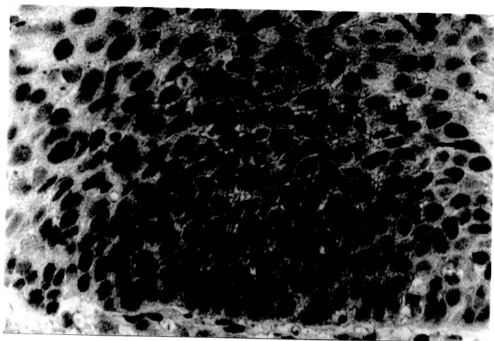


Fig. 4.22b. Localization of HPV 16 L1 protein in the nucleus of cells of squamous cell carcinoma. Cells positive for the protein are numerous and confined within lesions. Positive stainings vary in intensities (arrow) (x 500).

4.5 Serology to HPV

Five synthetic peptides (L1:13, L1:30, L2:49, E2:245 and E7:5) representing the deduced amino acid sequences of HPV 16 L1,L2, E2 and E7 ORF and one synthetic peptide (E2:245) of HPV 18 ORF were evaluated for their reactivity with human sera. Three serum pools were prepared (i) one pool of 80 sera were obtained from patients with histologically confirmed cervical carcinoma, (ii) one pool of 32 sera were obtained from healthy pregnant ladies and (iii) one pool of 30 sera were obtained from healthy female university students. The history of these subjects concerning past or present HPV infections were unknown. Groups (ii) and (iii) consisted of women not known to have either CIN or cervical cancer at the time the study commenced.

The 142 sera were tested in ELISA for IgA, IgG and IgM antibodies against peptides. IgA antibodies were detected with anti-human IgA horseradish peroxidase-labelled monoclonal antibody, IgG with rabbit anti-human IgG monoclonal antibody with alkaline phosphatase conjugate and IgM with goat anti-human IgM monoclonal antibody with glucose oxidase conjugate. After 15 minutes following the addition of substrate the absorbances were recorded at 415 nm for IgA and IgM and at 405 nm for IgG. OD values of 0.1 or more above the background (duplicate blank without any peptide coated to plate) were scored as positive.

4.5.1 Immune response to HPV 16 L1:13 peptide

The proportions of sera from cervical cancer patients, pregnant women and university students with anti-HPV 16 L1:13 antibodies are shown in Table 4.10.

The intensity of antibody reaction to the peptide in the different subject groups is illustrated in Figures 4.23a, b and c.

Anti-HPV 16 L1: 13 IgA reactivity

Anti-HPV 16 L1:13 IgA antibodies were exclusive to the cervical cancer group. Eleven of 80 (13.8%) patients tested were reactive to the peptide. The absorbance values of seropositive sera ranged from 0.331 to 1.383 (Figure 4.23a). Of the 11 seropositives, 10 (91%) showed OD values larger than 0.5.

Anti-HPV 16 L1:13 IgG reactivity

Anti-HPV 16 L1:13 IgG antibodies were found in the sera from 10 of 80 (12.5%) cervical cancer patients, five of 32 (15.6%) pregnant women and two of 30 (6%) university students (Table 4.10). It follows from these data that no difference exist in the prevalent rates of IgG antibodies between the cervical cancer group and healthy women. Positive reactions were somewhat more frequent among cancer and pregnant women as compared to the students. However the differences were again not statistically evident.

The levels of IgG antibodies in the three groups were compared (Figure 4.23b). The OD values of seropositive sera ranged from 0.154 to 0.996 for the sera from cervical cancer patients, 0.261 to 1.003 for the sera from pregnant women and 0.859 to 1.246 for the sera from university students. Analysis revealed that there was no significant difference in the IgG antibody levels between the three groups ($0.10 < p < 0.25$, Kruskal-Wallis test).

Anti-HPV 16 L1:13 IgM reactivity

Anti-L1:13 IgM antibodies were detectable in a majority of cervical cancer patients (67/80, 83.8%) , pregnant women (27/32, 84.4%) and university students (22/30, 73.3%) (Table 4.10).

The levels of IgM in the sera of the three groups were compared (Figure 4.23c). The OD values ranged from 0.132 to 1.29 for the sera from cervical cancer patients , 0.185 to 1.39 for the sera from the pregnant women and 0.187 to 0.776 for the sera from university students. Strong IgM reactivities were observed in all groups. Forty five of 67 (67%) seropositives from cervical cancer group, 20 of 27 (74%) seropositives from the pregnant group and 20 of 22 (91%) seropositives from the students group showed OD values larger than 0.5. Nonparametric multiple comparison analysis revealed that IgM reactivity was significantly elevated in the group of pregnant women as compared with the group of university students ($0.005 < p < 0.01$). However, there was no significant difference in the IgM reactivity to L1:13 between cancer patients and university students ($0.05 < p < 0.1$) and between cancer patients and pregnant women ($0.20 < p < 0.5$).

Table 4.10. Detection of antibodies against HPV 16 L1:13 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university student

Serum	No of sera tested	Antibody response (%)					χ^2 (df=1)
		Ig A	χ^2 (df=1)	Ig G	χ^2 (df=1)	Ig M	
Cervical cancer	80	11 (13.75)	3.45	10 (12.5)	0.02	67 (83.8)	0.00
Healthy pregnant women	32	0 (0)		5 (15.6)		27 (84.8)	
Healthy university students	30	0 (0)		2 (6)		22 (73.3)	
Total healthy women	62	0 (0)	7.42	7 (11.3)	0.00	49 (79)	0.25

χ^2 values are based on the percentage of the positive sera compared to cervical cancer.

► denotes significance at $p < 0.05$

χ^2_{11} values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

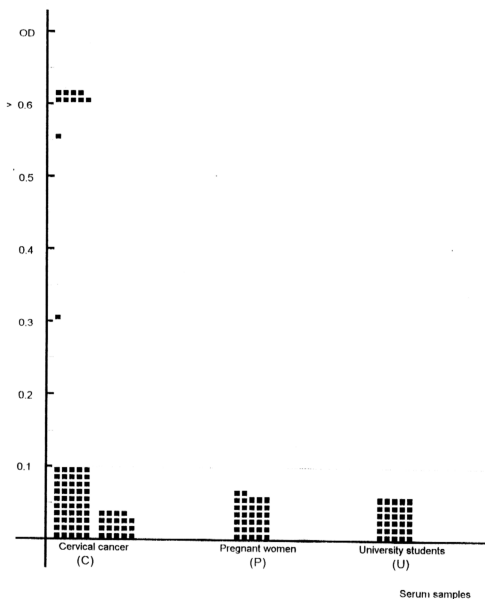


Fig 4.23a IgA reactivity to HPV 16 L1:13. IgA antibodies were detected with an IgA (alpha chain)-specific HRP conjugated monoclonal antibody. Each datum represents the OD value for each serum (diluted 1:30) reacted with peptide L1:13 after subtraction with the OD of the same serum when reacted with the uncoated well. The horizontal dash line represent the cut-off for seropositivity.

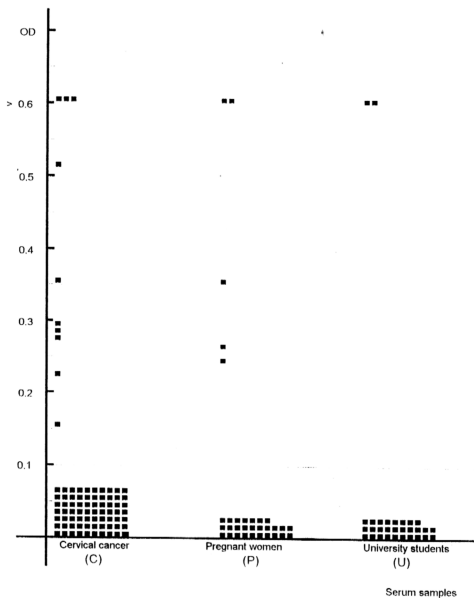


Fig. 4.23b IgG reactivity to HPV 16 L1:13. IgG antibodies were detected with an IgG (gamma chain)-specific alkaline phosphatase conjugated monoclonal antibody.

C versus P versus U = 0.10 < p < 0.25 (Kruskal-Wallis test)

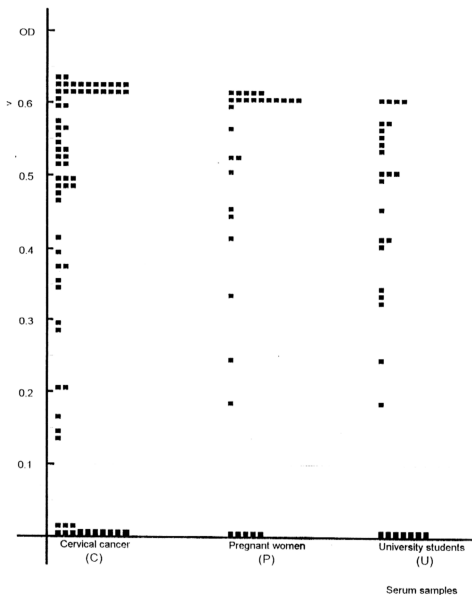


Fig. 4.23c IgM reactivity to HPV 16 L1:13. IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibody.

C versus P versus U = $0.005 < p < 0.001$ (Kruskal-Wallis test)

C versus U = $0.05 < p < 0.1$

C versus P = $0.20 < p < 0.50$

P versus U = $0.005 < p < 0.01$

nonparametric multiple comparison test

4.5.2 Immune response to HPV 16 L1:30 peptide

The proportion of sera from cervical cancer patients, pregnant women and university students with antibodies to HPV 16 L1:30 peptide are shown in Table 4.11. The intensity of antibody response to the peptide in the different subject groups is illustrated in Figures 4.24a, b and c.

Anti-HPV 16 L1:30 IgA reactivity

Anti-L1:30 IgA antibodies were found to be infrequent. Antibodies were detected in only five of 80 (6.3%) cervical cancer patients, one (3.1%) pregnant women and one (3.3%) university students (Table 4.11). The anti-L1:30 IgA reactivities among cervical cancer patients were strong. Four out of 5 (80%) seropositive sera had OD values larger than 0.5 (Figure 4.24a).

Anti-HPV 16 L1:30 IgG reactivity

Twenty one of 80 (26.3%) cervical cancer patients, seven of 32 (21.9%) pregnant women and two of 30 (6.6%) university students showed the prevalence of anti-L1:30 IgG reactivities. As depicted in Table 4.11, the prevalence rate of IgG seropositivities among cervical cancer patients was similar to that among pregnant women but was significantly higher than the rate among university students ($p < 0.05$).

The anti-L1:30 IgG antibody levels in cervical cancer patients, pregnant women and university students were compared (Figure 4.24b). OD values ranged from 0.201 to 0.829 for sera from cervical cancer patients, 0.136 to 0.492 for sera from pregnant women and 0.181 to 0.404 for sera from university students. Five (24%) seropositive sera from cervical cancer group but none from the other two

groups showed OD values larger than 0.5. However, further analysis revealed that there was no significant difference in the IgG antibody levels between the three groups ($0.1 < p < 0.25$, Kruskal-Wallis test).

Anti-HPV 16 L1:30 IgM reactivity

Only the cervical cancer group (14/80, 17.5%) and the students group (3/30, 10%) demonstrated elevated levels of anti-L1:30 IgM antibody. None of the pregnant women showed IgM reactivities to the peptide (Table 4.11).

The anti-L1:30 IgM antibody levels in cervical cancer patients and university students were compared (Figure 4.24c). The OD values ranged from 0.101 to 0.408 for sera from cervical cancer patients and 0.148 to 0.597 for sera from the students. Further analysis confirm that there was no difference in the IgM antibody levels between the 2 groups ($p > 0.2$, Mann Whitney test).

Table 4.11. Detection of antibodies against HPV 16 L1:30 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university students

Serum	No of sera tested	Antibody response (%)				χ^2 (df=1)
		Ig A	Ig G	Ig M	χ^2 (df=1)	
Cervical cancer	80	5 (6.3)	21 (26.3)	14 (17.5)		
Healthy pregnant women	32	1 (3.1)	7 (21.9)	0 (0)	$\chi^2_1 = 1.79$	$\chi^2_1 = 1.54$
Healthy university students	30	1 (3.3)	2 (6.6)	3 (10)	$\chi^2_1 = 0.00$ 0.02	0.45
Total healthy women	62	2 (3.2)	9 (14.5)	3 (4.8)	0.03 2.22	4.9 4.18

χ^2 values are based on the percentage of the positive sera compared to cervical cancer.

► denotes significance at $p < 0.05$

χ^2_1 values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

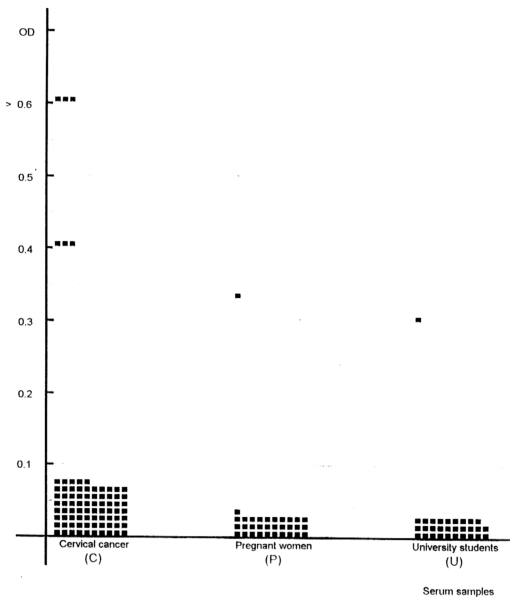


Fig. 4.24a IgA reactivity to HPV 16 L1:30. IgA antibodies were detected with an IgA (alpha chain)-specific HRP conjugated monoclonal antibody. Each datum represents the OD value for each serum (diluted 1:30) reacted with peptide L1:30 after subtraction with OD of the same serum when reacted with the uncoated well. The horizontal dash line represents the cut-off for seropositivity.

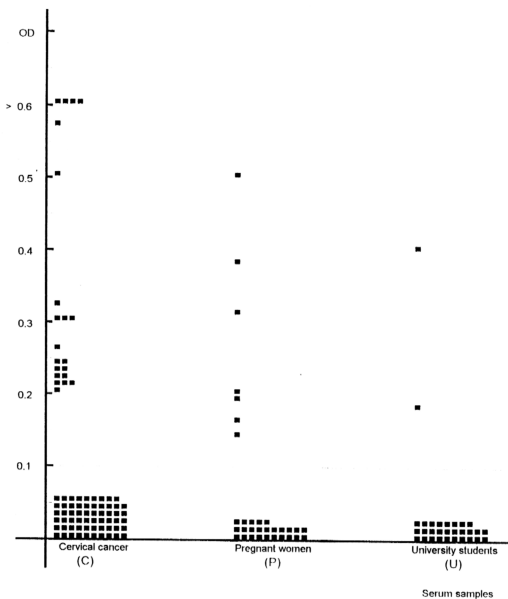


Fig. 4.24b IgG reactivity to HPV 16 L1:30. IgG antibodies were detected with an IgG (gamma chain)-specific alkaline phosphatase conjugated monoclonal antibody

C versus P versus U = $0.25 < p < 0.5$ (Kruskal-Wallis test)

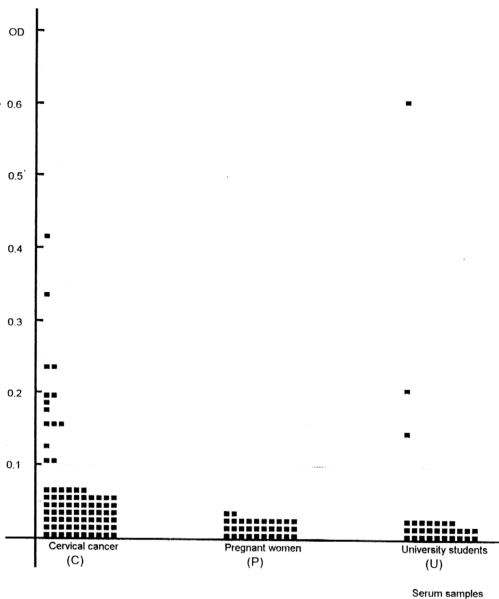


Fig. 4.24c IgM reactivity to HPV 16 L1:30. IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibody.

C versus U = $p > 0.2$ (Mann Whitney test)

4.5.3 Immune response to HPV 16 L2:49 peptide

The proportions of sera from cervical cancer patients, pregnant women and university students with antibodies to HPV 16 L2 : 49 peptide are shown in Table 4.12. The intensity of antibody response to the peptide in the different subject groups is illustrated in Figures 4.25a, b and c.

Anti-HPV 16 L2:49 IgA reactivity

Anti-L2:49 IgA antibodies were found in similar proportions in the sera of cervical cancer patients (22/80, 27.5%) pregnant women (9/32, 28.1%) and university students (6/30, 20%) (Table 4.12).

The anti-L2:49 IgA antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.24a). OD values ranged from 0.101 to 1.657 for sera from cervical cancer patients, 0.104 to 1.526 for sera from pregnant women and 0.127 to 1.051 for sera from university students. Four out of 22 (18%), two out of 9 (22%) and one out of 6 (16.7%) seropositive sera from cervical cancer, pregnant women and students groups, respectively, showed OD values larger than 0.5. Further analysis revealed there was no significant difference in the IgA antibody levels between the three groups ($0.10 < p < 0.25$, Kruskal-Wallis test).

Anti-HPV 16 L2:49 IgG reactivity

Anti-L2:49 IgG antibodies were much more common among cervical cancer patients, pregnant women and university students, being 52/80 (65%), 20/32 (62.5%) and 14/30 (46.7%), respectively. Although positive reactions were more frequent in

both cervical cancer patients and pregnant women as compared with university students, the differences were not statistically evident (Table 4.12).

The anti-L2:49 IgG antibodies in the cervical cancer patients, pregnant women and university students were compared (Figure 4.25b). OD values ranged from 0.102 to 1.893 for sera from cervical cancer patients, 0.115 to 1.622 for sera from pregnant women and 0.115 to 2.023 for sera from university students. Seventeen of 52 (32.7%) seropositives from cervical cancer group, eight of 20 (40%) seropositives from the pregnant group and six of 14 (43%) seropositives from the students group showed OD values larger than 0.5. Further analysis revealed that there was no significant difference in the IgG antibody levels between the three groups ($0.75 < p < 0.9$, Kruskal-Wallis test).

Anti-HPV 16 L2:49 IgM reactivity

IgM reactivity with L2:49 was almost non-existent. Low IgM response was seen in only one cervical cancer patient (Figure 4.25c).

Table 4.12. Detection of antibodies against HPV 16 L2:49 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university students

Serum	No of sera tested	Antibody response (%)			
		χ^2 (df=1)	Ig G	χ^2 (df=1)	χ^2 (df=1)
Cervical cancer	80	22 (27.5)	52 (65)	1 (1.25)	
Healthy pregnant women	32	9 (28.1)	20 (62.5)	0 (0)	
Healthy university students	30	6 (20)	14 (46.7)	0 (0)	
Total healthy women	62	15 (24.2)	34 (54.8)	1 (1)	

χ^2 values are based on the percentage of the positive sera compared to cervical cancer.

► denotes significance at $p < 0.05$

χ^2_1 values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

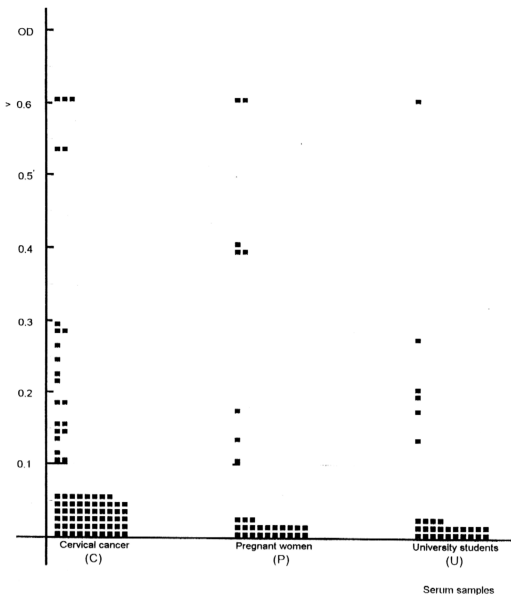


Fig. 4.25a IgA reactivity to HPV 16 L2:49. IgA antibodies were detected with an IgA (alpha chain)-specific HRP conjugated monoclonal antibody. Each datum represents the OD value for each serum (diluted 1:30) reacted with peptide L2:49 after subtraction with OD of the same serum when reacted with the uncoated well. The horizontal dash line represents the cut-off for seropositivity.

C versus P versus U = $0.90 < p < 0.95$ (Kruskal-Wallis test)

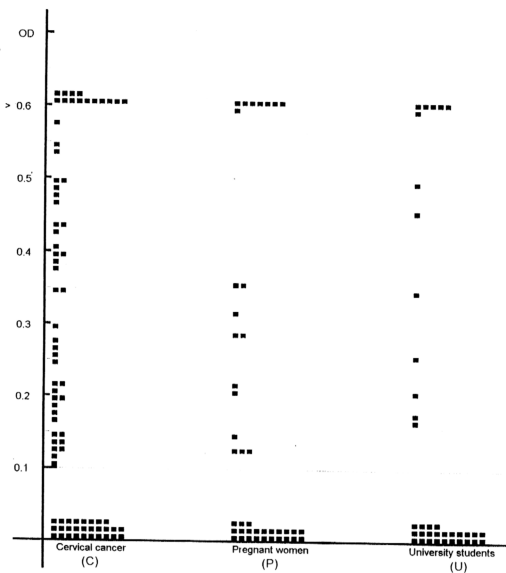


Fig. 4.25b IgG reactivity to HPV 16 L2:49. IgG antibodies were detected with an IgG (gamma chain)-specific alkaline phosphatase conjugated monoclonal antibody.

C versus P versus U = $0.75 < p < 0.90$ (Kruskal-Wallis test)

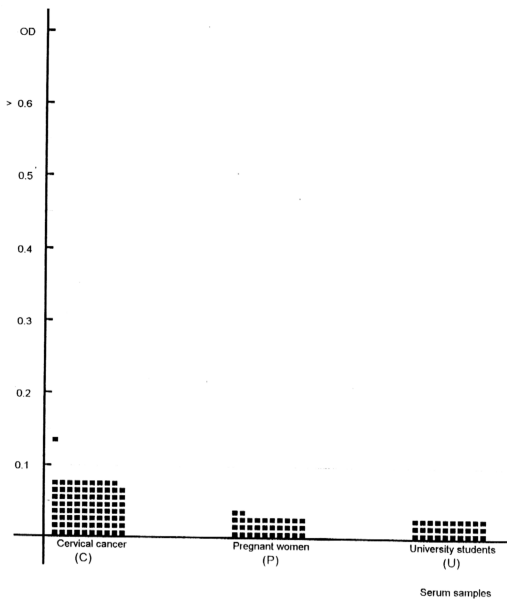


Fig. 4.25c IgM reactivity to HPV 16 L2:49. IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibody.

4.5.4 Immune response to HPV 16 E2:245 peptide

The proportions of sera from cervical cancer patients, pregnant women and university students with antibodies HPV 16 E2:245 are shown in Table 4.13. The intensity of antibody response to the peptide in the different subject groups is illustrated in Figures 4.26a, b and c.

Anti-HPV 16 E2:245 IgA reactivity

Anti-HPV 16 E2:245 IgA were significantly more frequent among cervical cancer patients (37/80 (46.3%)) than among pregnant women (8/32 (25%)) and university students (3/30 (10%)) ($p < 0.05$) (Table 4.13).

The IgA antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.26a). OD values ranged from 0.118 to 0.607 for sera from cervical cancer patients, 0.102 to 0.516 for sera from pregnant women and 0.11 to 0.143 for sera from university students. Nonparametric multiple comparison analysis revealed that anti-E2:245 IgA response were elevated among cervical cancer patients as compared to university students ($0.02 < p < 0.05$).

Anti-HPV 16 E2:245 IgG reactivity

Only two groups had IgG antibodies to HPV 16 E2:245. The antibodies were detected in 21 of 80 (26.3%) cervical cancer patients and in 11 of 32 (34.4%) pregnant women (Table 4.13).

The IgG antibody levels in the cervical cancer patients and pregnant women were compared (Figure 4.26b). OD values ranged from 0.131 to 1.281 for sera from cervical cancer patients and 0.101 to 0.982 for sera from pregnant women. Five of 21 (23.8%) seropositives from cervical cancer group and four of 11 (36.4%) seropositives

from the pregnant group showed OD values larger than 0.5. Further analysis revealed that there was no significant difference in the anti-E2:245 IgG levels between the two groups ($p > 0.2$, Mann Whitney test).

Anti-HPV 16 E2:245 IgM reactivity

Anti-HPV 16 E2:245 IgM antibodies were much more common among cervical cancer patients, pregnant women and university students, being 43/80 (53.8%), 25/32 (78.1%) and 20/30 (66.6%) respectively. IgM antibodies to the peptide were significantly more frequent among the healthy women than in cervical cancer patients ($p < 0.05$) (Table 4.13).

The IgM antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.26c). OD values range from 0.133 to 1.496 for sera from cervical cancer patients, 0.103 to 0.546 for sera from pregnant women and 0.148 to 0.75 for sera from university students. Only a minority of seropositivities showed OD values larger than 0.5. Nonparametric multiple comparison analysis revealed that IgM responses to peptide E2:245 were significantly elevated among pregnant women and university students ($0.02 < p < 0.05$). In contrast, no significant difference is noted in IgM levels between cancer patients and university students ($p > 0.5$) and between cervical cancer patients and pregnant women ($0.05 < p < 0.10$).

Table 4.13. Detection of antibodies against HPV 16 E2:245 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university students

Serum	No of sera tested	Antibody response (%)				
		Ig A	χ^2 (df=1)	Ig G	χ^2 (df=1)	Ig M
Cervical cancer	80	37(46.3)		21 (26.3)		43 (53.8)
Healthy pregnant women	32	8 (25)	3.45 $\chi^2_1 = 1.47$	11 (34.4)	0.39 $\chi^2_1 = 10.29$	25 (78.1)
Healthy university students	30	3 (10)	10.87	0 (0)	8.1	20 (66.6)
Total healthy women	62	11 (17.7)	11.44	11 (17.7)	1.00	45 (72.6)

χ^2 values are based on the percentage of the positive sera compared to cervical cancer.

► denotes significance at $p < 0.05$

χ^2_1 values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

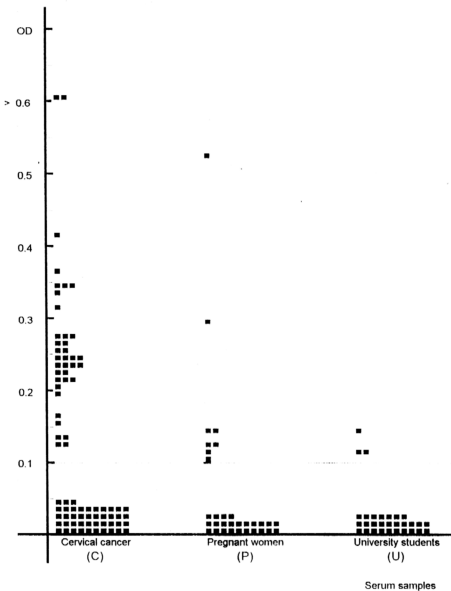


Fig 4.26a. Ig A reactivity to HPV 16 E2 : 245 Ig A antibodies were detected with an Ig A (alpha chain) - specific HRP conjugated monoclonal antibody. Each datum represents the OD value for each serum (diluted 1: 30) reacted with peptide E2 : 245 after subtraction with the OD of the same serum when reacted with the uncoated well. The horizontal dash line represents the cut-off for seropositivity.

C versus P versus U = 0.005 < p < 0.01, Kruskal-Wallis test.

C versus U = 0.02 < p < 0.05 | Nonparametric multiple comparison test.

C versus P = 0.05 < p < 0.1 |

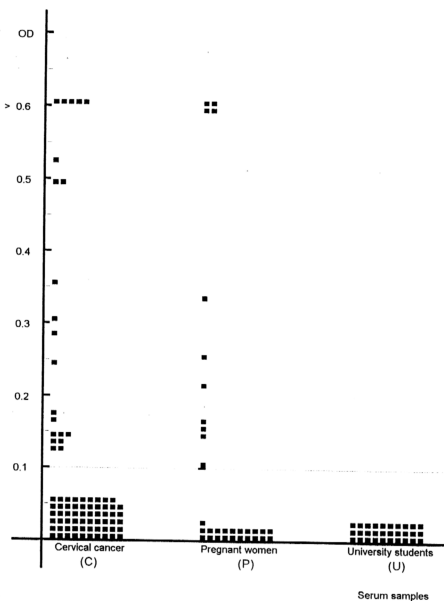


Fig. 4.26b IgG reactivity to HPV 16 E2 : 245. IgG antibodies were detected with an IgG (gamma chain)-specific alkaline phosphatase conjugated monoclonal antibody.

C versus P = $p > 0.2$, Mann Whitney test.

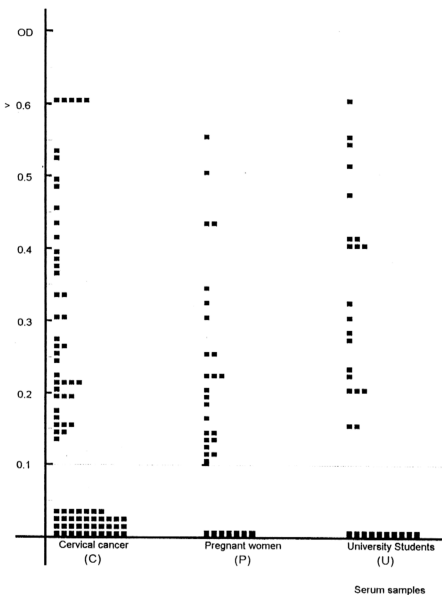


Fig. 4.26c IgM reactivity to HPV 16 E2:245 IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibody.

C versus P versus U = $0.01 < p < 0.025$, Kruskal-Wallis test.

C versus U = $p > 0.5$

C versus P = $0.05 < p < 0.10$

P versus U = $0.02 < p < 0.05$

nonparametric multiple comparison test

4.5.5 Immune response to HPV 16 E7:5 peptide

The proportions of sera from cervical cancer patients, healthy pregnant women and healthy university students with antibodies HPV 16 E7:5 peptide are shown in Table 4.14. The intensity of antibody response to the peptide in the different subject groups is illustrated in Figures 4.27a, b and c.

Anti-HPV 16 E7:5 IgA reactivity

Anti-E7:5 IgA seropositivities were found in near similar proportions among cervical cancer patients (21/80 (26.3%)), pregnant women (5/32 (15.6%)) and university students (7/30 (23.3%)) (Table 4.14).

The IgA antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.27a). OD values ranged from 0.212 to 0.948 for sera from cervical cancer patients, 0.151 to 0.481 for sera from pregnant women and 0.114 to 0.331 for sera from university students. Four cancer sera but none from the other two groups showed OD values larger than 0.5. Nonparametric multiple comparison analysis revealed that the levels of anti-E7:5 IgA antibodies among cervical cancer patients were significantly higher than those in university students ($0.002 < p < 0.005$). However, there was no significant difference in the IgA antibody levels, between cervical cancer patients and pregnant women ($0.2 < p < 0.5$) and between pregnant women and university students ($p > 0.5$).

Anti-HPV 16 E7:5 IgG reactivity

Like IgA, anti-E7:5 IgG seropositivities were found in nearly similar proportions among cervical cancer patients (17/80 (21.3%)), pregnant women (6/32 (18.8%)) and university students (7/30 (23.3%)) (Table 4.14).

The IgG antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.27b). OD values ranged from 0.12 to 0.802 for sera from cervical cancer patients, 0.126 to 0.655 for sera from pregnant women and 0.184 to 0.356 for sera from university students. Only four sera from the cancer group and one from the pregnant group showed OD values larger than 0.5. Further analysis revealed that there was no significant difference in the anti-E7:5 IgG antibody levels between the three groups ($0.99 < p < 0.995$, Kruskal-Wallis test).

Anti-HPV 16 E7:5 IgM reactivity

Anti-E7:5 IgM antibodies were more common among cervical cancer patients, pregnant women and university students, being 42/80 (52.5%), 20/32 (62.5%) and 20/30 (66.7%), respectively (Table 4.14).

The IgM antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.27c). OD values range from 0.101 to 0.683 for sera from cervical cancer patients, 0.115 to 0.594 for sera from pregnant women and 0.12 to 0.607, for sera from university students. Six sera from the cancer group, five sera from the pregnant group and three from the students group showed OD values larger than 0.5. Further analysis revealed that there was no significant difference in the level of anti-E7:5 IgM antibodies between the three groups ($0.5 < p < 0.75$, Kruskal-Wallis test).

Table 4.14. Detection of antibodies against HPV 16 E7.5 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university students

Serum	No of sera tested	Antibody response (%)			
		χ^2 (df=1)	Ig G	χ^2 (df=1)	χ^2 (df=1)
Cervical cancer	80	21(26.3)	17 (21.3)	42 (52.5)	
Healthy pregnant women	32	5 (15.6)	5 (18.8)	20 (62.5)	0.56 $\chi^2_1 = 0.006$
Healthy university students	30	7 (23.3)	7 (23.3)	20 (66.6)	1.25
Total healthy women	62	12 (19.4)	13 (21)	40 (64.5)	1.60

χ^2 values are based on the percentage of the positive sera compared to cervical cancer.

▲ denotes significance at $p < 0.05$

χ^2_1 values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

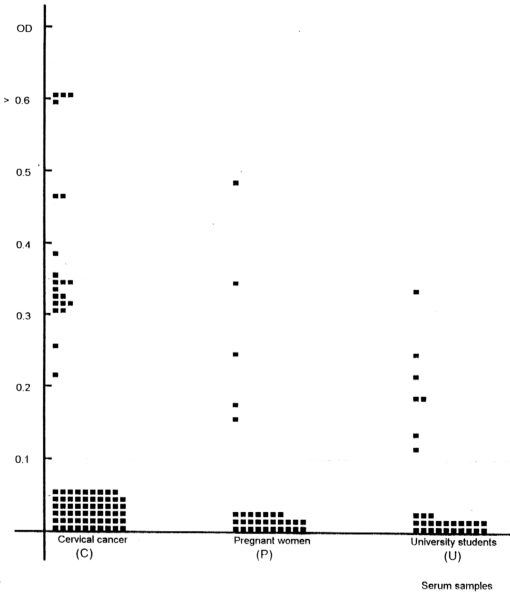


Fig. 4.27a IgA reactivity to HPV 16 E7.5. IgA antibodies were detected with an IgA (alpha chain)-specific HRP conjugated monoclonal antibody. Each datum represents the OD value for each serum (diluted 1:30) reacted with peptide E7.5 after subtraction with OD of the same serum when reacted with the uncoated well. The horizontal dash line represents the cut-off for seropositivity.

C versus P versus U = $p < 0.001$ (Kruskal-Wallis test)

C versus P = $0.2 < p < 0.5$

C versus U = $0.02 < p < 0.05$

P versus U = $p > 0.5$

nonparametric multiple comparison test

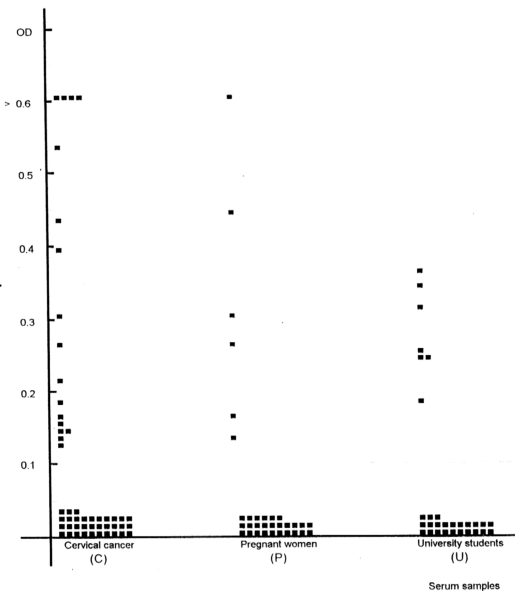


Fig. 4.27b IgG reactivity to HPV 16 E7:5. IgG antibodies were detected with an IgG (gamma-chain)-specific alkaline phosphatase conjugated monoclonal antibody.

C versus P versus U = $0.99 < p < 0.995$ (Kruskal-Wallis test)

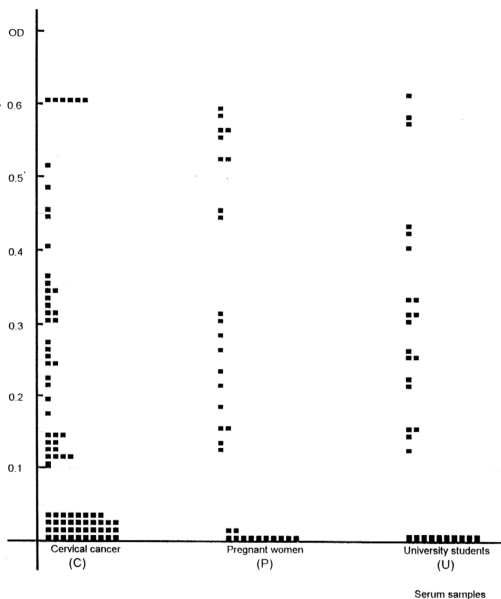


Fig. 4.27c IgM reactivity to HPV 16 E7:5. IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibodies.

C versus P versus U = $0.5 < p < 0.75$ (Kruskal-Wallis test)

4.5.6 Immune response to HPV 18 E2:245 peptide

The proportions of sera from cervical cancer patients, pregnant women and university students with antibodies to HPV 18-E2:245 peptides are shown in Table

4.14 . The intensity of antibody response to the peptide in the different subject groups is illustrated in Figures 4.28a, b and c.

Anti-HPV 18 E2:245 IgA reactivity

The prevalence of the IgA antibodies were significantly more frequent among cervical cancer patients than among the healthy women ($p < 0.005$). Anti-HPV 18 E2:245 IgA antibodies were detected in 18 of 80 (22.5%) cervical cancer patients and two of 32 (6.3%) of pregnant women. No reactivity was noted among university students (Table 4.15).

The anti-HPV 18 E2:245 IgA antibody levels in the cervical cancer patients, pregnant women and university students were compared (Figure 4.28a). OD values ranged from 0.11 to 0.778 for sera from cervical cancer patients and 0.149 to 0.366 for sera from pregnant women. Only three sera from the cervical cancer group showed OD values larger than 0.5. Further analysis revealed that there was no difference in the levels of anti-E2:245 IgA antibodies between the two groups ($p > 0.20$, Mann Whitney test).

Anti-HPV 18 E2:245 IgG reactivity

The presence anti-HPV 18 E2:245 IgG antibodies were significantly more common among cervical cancer patients than among healthy women ($p < 0.005$). Reactivities were identified in 21 of 80 (26.3%) cervical cancer patients and four of 32

(12.5%) pregnant women. Like IgA, no reactivity was noted among university students (Table 4.15).

The anti-HPV 18 E2:245 IgG antibody levels in the cervical cancer patients and pregnant women were compared (Figure 4.28b). OD values ranged from 0.102 to 1.001 for sera from cervical cancer patients and 0.129 to 0.301 for sera from pregnant women. Only three cancer sera showed OD values larger than 0.5. However, further analysis revealed that there was no difference in the levels of anti E2:245 IgG antibodies between the two groups ($p > 0.2$, Mann Whitney test).

Anti-HPV 18 E2:245 IgM reactivity

IgM reactivities were more common among cervical cancer patients (50/80 (62.5%)), pregnant women (15/32 (46.9%)) and university students (14/30 (46.6%)). There was no significant difference in the prevalence rates between the three groups (Table 4.15).

The anti-HPV 18 E2:245 IgM levels in the cervical cancer patients, pregnant women were compared (Figure 4.27c). OD values ranged from 0.11 to 0.735 for sera from cancer patients, 0.118 to 0.59 for sera from pregnant women and 0.121 to 0.566 for sera from university students. Eight sera from the cancer group, one sera from the pregnant group and two sera from the students group showed OD values larger than 0.5. Further analysis revealed that there was no significant difference in the anti-E2:245 IgM antibody levels among the three groups ($0.1 < p < 0.25$, Kruskal-Wallis test).

Table 4.15 : Detection of antibodies against HPV 18 E2:245 in (a) cervical cancer patients (b) healthy pregnant women (c) healthy female university students

Serum	No of sera tested	Antibody response (%)			
		Ig A	Ig G	Ig M	X ² (df=1)
Cervical cancer	80	18 (22.5)	21 (26.3)	50 (62.5)	
Healthy pregnant women	32	2 (6.3)	4 (12.5)	15 (46.9)	
Healthy university students	30	0 (0)	0 (0)	14 (46.6)	
Total healthy women	62	2 (3.2)	4 (6.5)	29 (46.8)	

X² values are based on the percentage of the positive sera compared to cervical cancer.

► denotes significance at p < 0.05

X²₁ values are based on the percentage of positive sera from healthy pregnant women compared to healthy female university students

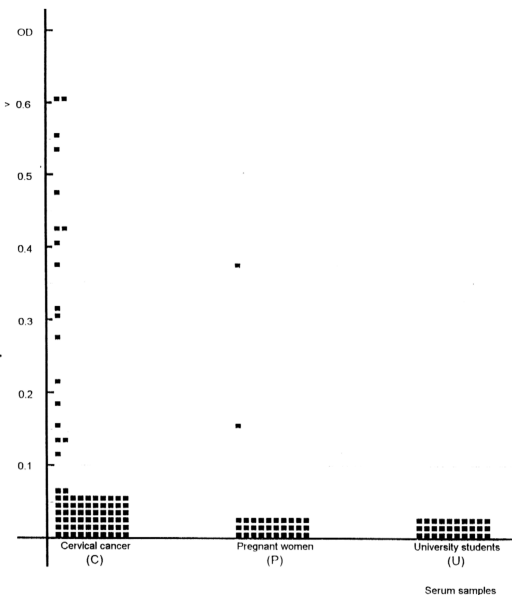
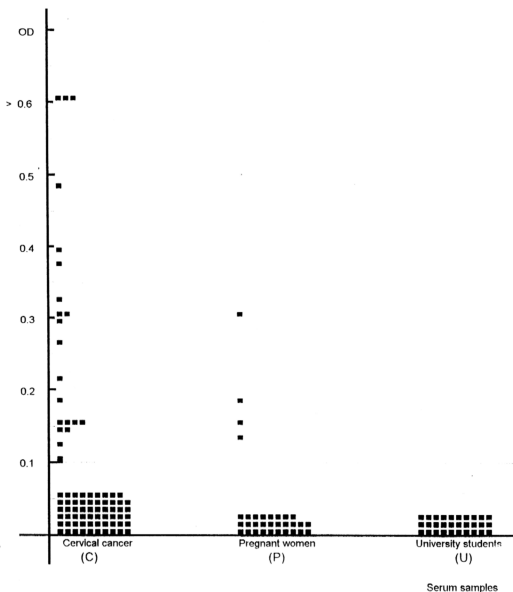


Fig. 4.28a IgA reactivity to HPV 18 E2:245. IgA antibodies were detected with an IgA (alpha chain)-specific HRP conjugated monoclonal antibody. Each datum represents the OD for each serum (diluted 1:30) reacted with peptide E2:245 after subtraction with OD of the same serum when reacted with the uncoated well. The horizontal dash line represents the cut-off for seropositivity.

C versus U = $p > 0.20$ (Mann Whitney test)



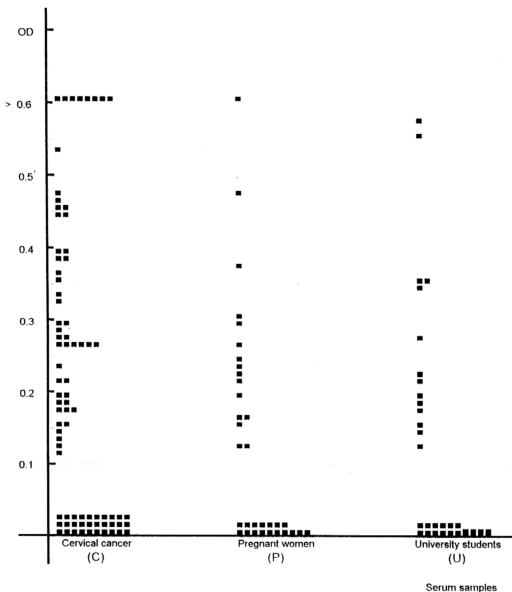


Fig. 4.28c IgM reactivity to HPV 18 E2.245. IgM antibodies were detected with an IgM (mu chain)-specific glucose oxidase conjugated monoclonal antibody.

C versus P versus U = 0.10 < p < 0.25 (Kruskal-Wallis test)