Chapter 1.

STUDY ON PREVALENCE OF HUMAN PAPILLOMAVIRUS (HPV) DNA SEQUENCES IN ASYMPTOMATIC NORMAL WOMEN

Introduction

Since long extensive clinicoepidemiological as well as experimental studies have over and again reiterated sexual transmission of an infectious agent in the etiology of cancer of the uterine cervix in women (Purola and Savia 1977, Meisels et al., 1982, Durst et al., 1983, Reid et al., 1984, Roy, 1989, Schneider et al., 1988). Although several bacterial and viral agents, namely Chlamydia trachomatis, gonorrhoea, Herpes simplex virus type 2 (HSV2) and human papillomavirus (HPV) have been considered for this role for a long time, infection with HPV has emerged recently as a major causal agent for the development of cervical cancer (Schachter et al., 1982, Seth et al., 1988, Allerding et al., 1985, Schmauz et al., 1989, Alexander, 1973, Mc Cance, 1988, zur Hausen et al., 1977, zur Hausen et al., 1987, Das et al., 1989). The basis of such consideration are regular detection of specific HPV types in more than 90% of cervical cancers (Durst et al., 1983, Das et al., 1992a), occurrence of HPV DNA in cervical tumor cell lines (Boshart et al., 1984, Yee et al., 1985), integrated state of HPV in host cell genome (Durst et al., 1985, Matsukura et al., 1989, Cullen et al., 1991, Das et al., 1992b) and findings of transforming genes E\textsubscript{6} and E\textsubscript{7} (Schiller et al., 1984, Sarver et al., 1984) and their transcripts in tumor cells (Sarver et al., 1984). Papillomaviruses have also been shown to have oncogenic potential in animals, (Pflister et al., 1984) and there have been several reports of malignant progression of benign HPV-induced lesions in man (zur Hausen et al., 1977, zur Hausen et al., 1987, Das et al., 1989, Durst et al., 1985).

In absence of specific serological assays, deoxyribonucleic acid (DNA) cloning and modern molecular hybridization techniques that are commonly employed for detection of HPV, have so far demonstrated involvement of at least twenty different HPV genotypes in anogenital
cancers (de Villiers et al., 1989). Of these, HPVs 6, 11, 16, 18, 31, 33, 35 and 45 are the types most commonly detected in precancerous and cancerous lesions of the uterine cervix (Gissman et al., 1980, Gissman et al., 1983, de Villiers et al., 1989, Durst et al., 1983, Boshart et al., 1984, Lorincz et al., 1986, Beaudenon et al., 1986, McCance et al., 1985). Whereas HPV types 6 and 11 are associated predominantly with genital warts and low-grade dysplasias (Gissman et al., 1983), types 16 and 18 are found in a majority of invasive cervical carcinomas and in some premalignant lesions (zur Hausen et al., 1985, McCance et al., 1985, Durst et al., 1983, Scholl et al., 1985). Therefore HPV types 6 and 11 are generally considered to be "low risk", and the types 16 and 18 are termed as "high risk" for the development of cervical cancer. Like other DNA virus-induced tumors, it is not only the presence but integration of viral DNA into the host cell genome is also essential for the development of cervical cancer (Matsukura et al., 1989, zur Hausen et al., 1985, Das et al., 1992b). Therefore integration of high risk types HPV 16 and 18 DNA has been often observed in majority of high-grade dysplastic and malignant cervical tissues. Neither low risk HPVs (6 and 11) nor the low grade dysplastic lesions show integration of viral DNA (Durst et al., 1983, Das et al., 1992b).

In India where cancer of the uterine cervix is the major cancer in women, the frequency of HPV-16 is very high (~90%) in invasive carcinomas (Das et al., 1992b). The prevalence of HPV 18 and other types is very rare or nil. Majority of studies on HPV thus far have concentrated mainly on cervical tumors and there have not been much attempts to examine in normal population the extent and incidence of HPV infection, particularly the type 16 which is the most prevalent one throughout the world including India (Das et al., 1992a, b; Durst et al., 1983; Lorincz et al., 1986, Yoshikawa et al., 1985; Fuchs et al., 1988).

Early reports suggest that HPV16 DNA is rarely found in normal cervical epithelium (Schneider et al., 1985), but recent studies reveal that these viruses can be detected in at least 8 - 35% of normal women (Toon et al., 1986, Das et al., 1989, de Villiers et al., 1987). Recently Sanjose et al., (1992) have reported the average type specific distribution of viral DNA in normal women with latent infections (HPV 6/11 = 9.2%; HPV 16/18 =
Various prevalence estimates have been reported for subclinical or latent HPV infection in different age groups. In a cohort of 8755 women with normal Pap smears, the highest HPV rate (13%) was found in the age group between 20 and 25 years with a constant decline with increasing age to as low as 5% in women above 55 years (de Villers et al. 1987; de Villers et al., 1992; Ley et al., 1991). Another report from Meanwell et al., (1987) suggests that the frequency of HPV 16 DNA is lowest in patients with CIN, but it rises in invasive cervical cancer patients. It has been suggested that HPV infection may alter the behaviour of cervical lesions in women under 40 years of age (Toon et al., 1986; Mitchell et al., 1986) and that preneoplastic lesions with HPV-16 infection have a high rate of progression to malignancy (Cuzick et al., 1992).

An in-depth research on HPV has been hindered due to the lack of adequate detection methods. Standard viral detection techniques involving serological markers are precluded due to the lack of an in vitro culture system for HPV and non-availability of type specific antibodies of HPV. Although recently bacterially expressed fusion proteins, synthetic peptides and propagation of HPV in a mouse xenograft system are available, the detection of HPV mainly relied on various modern molecular DNA hybridization methods (Howley et al., 1979, Gissmann et al., 1976, Lorincz et al., 1986). Southern blot hybridization is considered as the gold standard for HPV assay because of its well defined analytical sensitivity in terms of amount of target viral DNA detected and because of optimal HPV type identification provided by combined hybridization and restriction fragment analysis. Southern blot hybridization has yielded enormous data supporting a relationship between HPV and cervical neoplasia (Howley et al., 1979, Gissman et al., 1976, Cullen et al., 1991, Durst et al., 1987, Lorincz et al., 1990). Recently, polymerase chain reaction (PCR), a DNA amplification technology (Saiki et al., 1985; Mullis & Faloona, 1987) has provided a highly sensitive method for detection of various gene sequences of interest including viral infections in a large number of specimens at a time. PCR method has been used for the detection of HPV DNA by several authors and enormous amount of data has
been generated within a short span of time. (Manos et al., 1989, Snijders et al., 1990, Vanden Brule et al., 1990, Yoshikawa et al., 1990). Its main advantage is its high level of sensitivity, but a disadvantage is its vulnerability to false positives resulting from contamination with HPV DNA from other sources. Specific precautions and modifications are available to minimize the chances of contamination in PCR reaction (Longo et al., 1990, Bauer et al., 1991).

Detection of HPV infections in 5% to as high as 50% of normal population (Meanwell et al., 1987, de Villiers et al., 1987, Toon et al., 1986) and findings of 2 to 20% of invasive cervical cancers without any HPV infection (Crook et al., 1991, Scheffner 1991, Higgins et al., 1991, Das et al., 1992a) raise more controversy rather than resolving the issue of HPV as a principal causal agent for the development of cervical cancer. In order to understand the natural history and biological behaviour of HPV infection during cervical carcinogenesis, it is an essential prerequisite to know the prevalence of HPV infection, particularly of high risk types in asymptomatic normal population of women. This would also allow early identification of high risk women who are likely to progress to invasive cancer.

In the present study detection of HPV has been carried out in the normal population using HPV 16 oligoprimers and probes of HPV16 representing the high risk group and of HPV 11 as the low risk group. The type specific E6/E7 primers have been chosen to detect HPV infection in asymptomatic normal individuals. Besides, an attempt has been made to correlate the effect of other epidemiological risk factors, namely early age of marriage, smoking, number of sexual partners and use of oral contraceptives in the development of cervical cancer in presence of HPV infection.
MATERIALS AND METHODS

Study Population and biological Specimens

A total of 89 women with complaints of menstrual irregularities, pruritus vulvae, infertility, repeated abortions and dysmenorrhea were enrolled in the present study. These women aged between 20-35 years, were reporting for gynaecological examinations in the out-patient Department of Gynaecology, All India Institute of Medical Sciences, (AIIMS) New Delhi (India). Vaginal smears/cervical smears were obtained with the help of wooden spatula. Pap smear slides were prepared for cytological and histopathological examination in the Department of Pathology, AIIMS. The rest of the material along with spatula was put into a tube containing PBS (pH 7.2). Cells were washed off from spatula into the PBS solution and were stored in -70°C until use. In addition, 83 women with invasive cervical cancer were investigated for HPV prevalence in relation to their age and influence of other associated risk factors.

DNA Extraction from Cervicovaginal Smears

Genomic DNA was isolated by centrifuging cervicovaginal smear samples at 6000rpm. After washing the cell pellet with PBS (pH 7.2) twice, fresh and chilled CLB (0.32 mM sucrose, 10mM Tris-HCl pH 7.6 5mM MgCl2, 1% Triton X-100) was added to the cell pellet. The cell pellet was treated twice with CLB and then once with PLB (10mM Tris-Hcl pH 8.0, 10mM NaCl, 10mM EDTA). Finally 250µl of PLB and proteinase K (1mg/ml)were added to the cell pellet and an incubation was carried out for 3 hours at 65°C with shaking at an interval of 15 minutes. The DNA preparation was then analysed by gel electrophoresis.

Polymerase Chain Reaction (PCR)

PCR amplification (Saiki et al., 1985) was carried out using 50 to 100 ng of purified DNA in 50 or 100µl reaction volume, containing 1uM of each primer, 50 mM KCl, 100 mM Tris-HCl pH 8.3, 2.5 mM MgCl2, 75µg/ml BSA, 200 uM of each dNTP and 1 to 2 units of Taq DNA polymerase.
Table 2 Specification of HPV 16, HPV 11 & human β - globin gene specific oligonucleotide sequences used as primers in PCR

<table>
<thead>
<tr>
<th></th>
<th>HPV 16</th>
<th>Position</th>
<th>Amplimer length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H 1</td>
<td>5'ATT-AGT-GAG-TAT-AGA-CAT-TA 3'</td>
<td>320-339</td>
<td></td>
</tr>
<tr>
<td>H 2</td>
<td>5' GGC-TTT-TGA-CAG-TTA-ATA-CA 3'</td>
<td>429-410</td>
<td>110bp</td>
</tr>
<tr>
<td>H 3</td>
<td>ATG-GAA-CAA-CAT-TAG-AAC-AGC, AAT-ACA-AAC-AGC CGT-TGT-C</td>
<td>354-393</td>
<td></td>
</tr>
<tr>
<td>HPV 11</td>
<td></td>
<td>Position</td>
<td></td>
</tr>
<tr>
<td>NH 1</td>
<td>5' ATT-AAC-CAA-TAT-AGA-CAC-TT 3'</td>
<td>321-340</td>
<td></td>
</tr>
<tr>
<td>NH 2</td>
<td>5' GGC-TTG-TGA-CAC-AGG-TAA-CA 3'</td>
<td>430-411</td>
<td>110bp</td>
</tr>
<tr>
<td>NH 3</td>
<td>ATG-CAC-CTA-CAG-TAG-AAG-AAG-AAA-CCA-ATG-AAG-ATA-TTT-T</td>
<td>355-394</td>
<td></td>
</tr>
<tr>
<td>β-globin-1</td>
<td>5'-GAA-GAG-CCA-AGG-ACA-GGT-AC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-globin-2</td>
<td>5' CAA-CTT-CAT-CCA-CGT-TAC-ACC-3'</td>
<td></td>
<td>268bp</td>
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</tbody>
</table>

H1, H2, NH1 and NH2 are the primers to amplify E6 ORF of HPV16 and HPV11, H3 & NH3 are the oligonucleotides used as probes to hybridize specifically with HPV16 and HPV11. The base positions are numbered from their published sequences.
(Cetus corporation, USA). This reaction mix was overlayered with 50μl of mineral oil in order to avoid evaporation.

Two pairs of specific oligomers (20mers) from E6/E7 ORFs of HPV 16 and HPV 11 were used to amplify 110 bp of HPV 16 and 11 in patient DNA samples. HPV plasmid DNA was used as a positive control. A pair of primers from the human β-globin gene were used as internal controls to check the purity of tumor DNA for the PCR test. Oligoprobes were then radiolabelled using γ-32p dATP (Dupont-NEN USA). The specific activity of the probes was 0.5-1x10^8 cpm/ug of DNA. The primer sequences and probes are shown in Table 2.

**Synthesis and purification of oligonucleotide probes and primers**

The oligonucleotides were synthesized by the solid-phase triester method. The oligomers were end-labeled using (γ-32p) ATP and T4-polynucleotide kinase (Bos et al., 1986).

After inactivation of the bacteriophage T4 polynucleotide kinase by heat, 40μl H₂O, 240μl of 5M solution of ammonium acetate & 750μl of ice-cold ethanol was added to the tube. After mixing the contents, the tube was held for 30 minutes at 0°C.

The radiolabelled oligonucleotides were recovered by centrifugation at 12,000 g for 20 minutes at 0°C in a microfuge. Using an automatic pipetting device equipped with a disposable tip, the supernatant (which contains most of the unincorporated γ-32p ATP) was carefully removed.

500μl of 80% ethanol was added, the tube vortexed briefly, and recentrifuged. The supernatant was carefully removed. After the residual ethanol had evaporated, the radiolabeled oligonucleotides were redissolved in 100 μl of TE (pH 7.6).

**Southern blot hybridization of PCR product**

The prehybridization and hybridization of the nitrocellulose/nylon
membranes were carried out as described by Maniatis et al. (1982). Prehybridization was done in a buffer containing 5 X Denhardt's solution, 6 X SSC and 1% SDS for 2 hours at 65°C. The prehybridized filter was then incubated in hybridization buffer (50% formamide, 6 X SSC, 5 X Denhardt's solution and 1% SDS containing 10^6 cpm/ml of probe) for 16-24 hours at 42°C with shaking. After hybridization, the filter was washed in 2X SSC and 1% SDS at room temperature for 15 minutes and then at 65°C for 1 hour with two changes of buffer. Finally the filter was washed under high stringency washing conditions (0.1 X SSC, 1% SDS) at 65°C for 1 hour. The wet filter was wrapped in a plastic bag and exposed to an X-ray film in a X-ray cassette which contained either one or two intensifying screens. The cassette was kept at -70°C for 1-7 days after which the film was developed.

RESULTS

PCR amplification of HPV DNA sequences:

All the DNA samples isolated from cervicovaginal smears were analysed along with positive (HPV) plasmid DNA and negative controls (male lymphocyte DNA) by PCR, using HPV types 16 and 11 specific oligo-primers. Amplification of a 110bp fragment was detected for both HPV 16 and 11 DNA sequences (Fig 3A and 4A respectively). The authenticity of the amplified DNA product was checked by hybridizing with HPV 16 & 11 oligo probes respectively (Fig 3B & 4B). The sequences for the type specific oligo primers and oligo probes are mentioned in Table 2. Amplification by control β-globin gene using specific primers of β-globin was done(Fig 5). Fig 5 shows the amplification of a 268 bp β-globin gene specific fragment of the samples which were found to be positive for HPV 16 & 11. None of the samples demonstrated positivity for both HPV 16 & 11. Of the 89 patients, HPV-16 were detected in about 30% (27 out of 89) of asymptomatic normal women. The frequency of HPV-16 infection in invasive carcinoma was found to be as high as 97.6 % (81 out of 83). HPV-11 infection was found in 7% (8 out of 89)normal asymptomatic women while frequency of HPV -11 in invasive carcinoma was extremely low 6% (5 out of 83). The persistence of the virus was found till a period of six weeks (Fig 6A,6B).
Fig 3 (A) - Ethidium bromide stained agarose gel (2%) showing HPV-16 DNA amplified by PCR in asymptomatic women. Lane 1 shows Hae III digested φ X 174 DNA size marker. Lane 2 shows amplification of HPV-16 plasmid DNA. Lane 3,5 are cervicovaginal DNA samples show no amplification. Lanes 4 & 6 are showing specific amplification of 110 bp (arrow) of HPV-16.

(B) Southern blot hybridization of PCR product (in Fig 3A) showing presence of HPV-16 DNA sequences in normal cervicovaginal smears. Southern blot was hybridized using HPV-16 oligoprobe mentioned in Table 2. Lane 1: Hac III digested φx174 DNA size marker. Lane 2 shows 110 bp amplified DNA product from HPV-16 DNA used as positive control. Lane 3 & 5 show no amplification in sample No 32 & 34. Lane 4 & 6 shows 110 bp amplified DNA from non suspect cases 38 & 40.
Fig 4 (A)- Ethidium bromide stained (2%) agarose gel showing HPV-11 DNA amplification by PCR in normal asymptomatic cases. Lane 1 shows Hae III digested φ X 174 DNA size marker. Lane 3, 4 and 6 show no amplification in case No 45,46 & 48. Lane 5 shows 110 bp amplification in normal cervicovaginal smear sample No 52.

(B)- Southern blot hybridization of PCR products (in Fig 4 A) showing presence of DNA sequences in normal cervicovaginal smears. Southern blot was hybridized using HPV-11 oligoprobe mentioned in Table 2 Lane 1 is Hae III digested φ X 174 DNA size marker. Lane 2 shows amplification of HPV-11 plasmid DNA. Lane 3, 4 & 6 show no amplification in non suspect cases 45,46 & 48. Lane 5 shows 110 bp amplified DNA from non suspect case No 52.
Fig 5 - Ethidium bromide stained (2%) agarose gel showing amplification of human β-globin gene in normal women using specific set of oligonucleotide primers, as a experimental control. Lane 1 shows Hae III digested φX 174 DNA size marker. Lane 2 shows human placenta DNA showing 268 bp β-globin gene amplification. Lanes 3 & 4 show 268 bp β-globin amplification in normal cervicovaginal smear DNAs positive for HPV-16. Lane 5 & 6 show similar amplification (arrow) from normal cervicovaginal smear DNAs positive for HPV-11.
Fig 6 (A) - Southern blot hybridization of PCR products positive for HPV-16 in normal cases on day 1. Lane 1 shows 110 bp amplified DNA product from HPV-16 plasmid DNA used as positive control. Lanes 2-5 show 110 bp amplified DNAs from non suspect cases (sample nos 11, 22, 31 & 42) hybridizing with HPV-16 oligoprobes. Lane 6 shows male lymphocyte DNA sample used as negative control showing no amplification.

(B) - Southern blot hybridization showing viral persistence in same cases shown in Figure 6 A after six weeks of follow up. Lane 1 shows 110 bp amplified DNA product from HPV-16 DNA used as positive control. Lanes 2-5 show 110 bp amplified DNA from non suspect cases. (Sample nos 11, 22, 31 & 42) hybridizing with HPV-16 oligoprobes. Lane 6 showing normal male lymphocyte DNA sample used as negative control show no amplification.
Table 3: Presence of HPV type 16 and 11 in normal cervicovaginal smear samples

<table>
<thead>
<tr>
<th>Type of HPV</th>
<th>Hybridization condition</th>
<th>Normal cervix</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>By Southern</td>
<td>By PCR</td>
</tr>
<tr>
<td>11</td>
<td>Stringent</td>
<td>0</td>
<td>8 (7%)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Stringent</td>
<td>0</td>
<td>27 (30%)</td>
<td></td>
</tr>
<tr>
<td>16+11</td>
<td>Stringent</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
**Epidemiological factors and HPV 16 infection**

In view of recent speculation regarding the role of HPV in cervical neoplasia in young women, we examined in detail the relation between age and the presence of HPV 16 DNA in patients & controls. Two groups were included in the present study. Group 1 includes 83 invasive cancer patients and severe dysplasia cases. In this group 81 women out of 83 were positive for HPV infection checked by PCR and Southern or dot blot hybridization. Group 2 included a control population of 89 women with normal healthy cervix. Out of 89 control women 35 were found to have HPV infection by PCR.

Other exogenous factors like early marriage, age at first coitus, smoking history and number of sexual partners seem to be playing significant roles in the development of cervical cancer. A significant number of population 27 (22%) out of 83 cancer patients were married before 20 years compared to 10 (3.5%) out of 35 women having latent HPV infection in control group. Age at first coitus seems to be an alarming contributing factor since 38 (31%) women out of 83 had an onset of sexual activity before 18 years of age. Where as in control group 7 (3%) women out of 35 controls positive for latent HPV infection started sexual activities at an age of 18-19 years. A significant number of patients 28 (23%) were smokers. Most of the patients belonged to low socioeconomic class living in unhygienic conditions. Most of the patients were nonusers of oral contraceptives and had more than two children.

Table 4 describes the relationship of age of women with cervical intraepithelial neoplasia and HPV infection. The figures shown in Table 4 indicate that out of 83 cancer cases, 11 were CIN Ia - Ib in the age group of 28 to 38 years. 24 cases were found in the category of CIN IIa - IIb and were over 40 years of age. 40 cases (50%) fell in the category of CIN IIIa - IIIb and they belonged to age group 50 years or above. Stepwise discriminant analysis identified increasing age as the single most significant independent risk factor contributing towards HPV16 infection in cervical intraepithelial neoplasia and invasive cervical cancer.
<table>
<thead>
<tr>
<th>Clinical Stages</th>
<th>HPV 16 DNA in Invasive/dysplasia cases</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN Ia - Ib</td>
<td>11 of 83</td>
<td>under 40 yrs.</td>
</tr>
<tr>
<td>IIA - IIB</td>
<td>24 of 83</td>
<td>above 40 yrs.</td>
</tr>
<tr>
<td>IIIA - IIIB</td>
<td>40 of 83</td>
<td>above 50 yrs.</td>
</tr>
</tbody>
</table>
Cervical cancer constitutes a substantial health problem, as it is the second most common female cancer world-wide and the dominant cancer in Indian women. The epidemiological studies on cervical cancer strongly point to involvement of an sexually transmitted infectious agent as being involved in the aetiology. Human papillomavirus (HPV) has been implicated as the most likely causal infectious agent on the basis of results from several molecular biological investigations which have clearly indicated the carcinogenic potential of specific HPV types (zur Hausen, 1989a). The natural history of HPV infections in the cervical epithelium has suggested the stages as "clinical", when there are signs visible to the unaided eye, "subclinical", when HPV-related changes are only apparent with the aid of the colposcope or microscope and "latent", when there is no morphological abnormality of the squamous epithelium. However, HPV could be detected through molecular hybridization assays. Latent HPV infections are expected to retain intact viral genome. PCR systems using primer sequences derived from the most conserved L1 region or transforming E6/E7 region should be able to detect most HPV infections.

The prevalence of HPV DNA is higher among women whose smears show morphological signs of HPV infection, but in none of the studies, the presence of HPV-related morphology was fully confirmed by the hybridization assay. Similar comparisons in studies that used non-PCR based hybridization methods showed lower correspondence (Bauer et al., 1991; Ward et al., 1990). These results indicate that as the sensitivity (detectability threshold) of the PCR method increases, the specificity for detection of HPV infection also increases.

The type specific HPV DNA prevalence among women with and without morphological signs of HPV infections have been reported extensively (Vanden Brule et al., 1989; 1990, 1991). HPV types reported in most of these studies have been grouped on the basis of extent of severity of cervical lesions induced by them. HPV types 6 and 11 are considered as low-risk types whereas the high risk HPV types are 16, 18 and occasionally 31, 33, 35.
If HPV is indeed the major cause of or it is strongly associated with the development of cervical cancer, the early identification of women who are infected by HPV might well be a reasonable way to determine which women need intensive follow-up and management. Estimates of the prevalence of human papillomavirus (HPV) infection of the genital tract have been based primarily on the detection of HPV DNA. A variety of serological assays to detect general antibodies to the genital-type HPVs have been developed but the use of general (non-type-specific) serological assays could give false positive results that are not fully reflective of specific HPV antibodies. Therefore DNA hybridization methods such as dot blot, filter in situ hybridization, Southern blot and polymerase chain reaction based assays are the most preferred techniques for HPV detection. Southern blot hybridization is considered to be the standard HPV assay because of its well defined analytical sensitivity.

Gopalkrishna et al. (1992) compared the sensitivity of various molecular methods including Polymerase Chain Reaction (PCR) that are being employed for detection of HPV. Their results indicate that PCR is obviously a highly sensitive method but Southern blot hybridization is certainly more specific and sensitive than any other DNA hybridization methods except radioactive in situ hybridization which is comparable to southern blotting if a single copy gene localization method is used (Das et al., 1992b).

A widely used system is the L1 consensus primer method developed by Manos and colleagues (Manos et al., 1989). It has been suggested that consensus primers are not as sensitive for specific HPV types as the corresponding type-specific primers might be. This is perhaps due to mutual interference of the different primers in the reaction mixture.

PCR based methods have revealed that over 80% of specimens from invasive cervical cancer and advanced preneoplastic lesions contain specific types of human papillomavirus (Riou et al., 1990; van den Brule et al., 1991; Higgins et al., 1991). Studies in younger age groups are inconsistent, showing that the majority of HPV infections detected at ages below 30 is mostly of low-risk HPV types. Bauer et al. (1991) reported a 3.0%
prevalence rate of HPV 6/11 as compared to 10.9% of high-risk HPV types. Hallam et al. (1991) reported a prevalence of 12.6% of HPV types 6/11 and 27.7% of HPV types 16/18/31. Both studies examined women in their twenties.

In the present study DNA samples from 89 non-suspect, asymptomatic individuals were analysed using type specific primers. Type specific primers were designed screening E6/E7 regions of HPV types 11 (low risk) and HPV-16 (high risk). The PCR system detected presence of HPV16 in 27(30%) of 89 where as HPV 11 was found only in 8 (7%) confirming the earlier findings that HPV 11 is rarely found in cytologically normal smears and most latent infections are mainly due to high risk HPV-16. (Van den Brule et al., 1990). The authenticity of the type specific PCR amplified DNA product was checked following hybridization with the type specific HPV probes. Sensitivity of detection may depend upon the method employed for analysing the PCR product. It has been shown by Bauer et al., (1991), that gel visualization alone may miss some of the HPV-positive samples which could be identified only after hybridization with a specific HPV probe.

The data from the present study suggests that the frequency of finding latent HPV 16 DNA is lowest in control cases with age between 20 years to 35 years. The frequency of HPV16 DNA was found significantly lower in patients with cervical intraepithelial neoplasia grade I, higher in CIN II and III and still higher in patients with invasive carcinoma since the mean ages of patients with CIN I, CIN III and invasive cancer are around 33, 43 and 52 years respectively (Table 4). Our study enabled us to conclude that increasing age as the single most significant independent risk factor for discriminating between patients and controls. This observation is in good agreement with the data of Meanwell et al., (1987).

Although increasing age has been observed as the most significant factor for the development of cervical cancer, other exogenous factors like early marriage and age at first coitus have also been found to be the major contributing factors in the development of cervical neoplasia. This may be because of the fact that initial infection of human papillomavirus is based on exposing the young immature cervix which are most susceptible to various
infectious agents by starting sexual activities at an early age. Similar observations have been reported earlier by Murthy et al., (1990). Number of sexual partners has been found to have a minimal effect as a risk factor in the present study whereas use of oral contraceptives has been found to be insignificant. Smoking history of the patients showed a significant correlation with CIN. In earlier studies also high levels of smoke derived nicotine and cotinine were found in the cervical mucus of smokers suggest a possible biological mechanism for the smoking associated carcinogenic effects, particularly with respect to enhancing the effects of infectious agents, including HPV (Schiffman et al., 1987).

In a follow up study, after an interval of six weeks, analysis of DNA samples showed presence of HPV type 16 in majority of patients. Based on these results we suggest that the persistent high risk viral infection could be important and the carriers may be identified as high risk population for developing cervical cancer. This observation is in sharp contrast to what Pao et al., (1990) has reported. The reason could be that latent intracellular infection persists in some cases if not all. Results obtained from the present study confirm that HPV infections persist as latent infections, possibly with intermittent cycles of replication or viral production.

The possibility of HPV being a significant risk factor gains further support from a recent report of a long term follow up study carried out to understand the biological behaviour and natural history of precancerous and early cancerous lesions of the uterine cervix in India (Das et al., 1989). When compared with a number of potential risk factors, HPV was found to be a highly significant independent risk factor for development of cervical cancer (Das et al., 1989; Murthy et al., 1990). The frequent detection of specific HPV types in premalignant and malignant lesions of the uterine cervix indicates that infection of these HPVs may play an important role in the etiology and in the clinical progression of precancerous lesions (Das et al., 1989). It is therefore, suggested that early identification of HPV types may provide important information on the tumourogenic potential of a lesion and may facilitate the identification of individuals at high risk. In the present study prevalence of high risk HPV16 in normal population has been found much higher compared to low risk HPV type 11. Although it is
important to establish the prevalence of HPV infection in normal population and in invasive cervical cancer cases, evidence exists, especially at the molecular biological (zur Hausen, 1991a) and epidemiological (Ley et al., 1991) levels which suggests that HPV infection alone is perhaps not sufficient for the development of malignant lesion, but that additional host cell gene modifications are needed for the cascade of events required for malignant conversion. These modifications may be mediated by endogenous as well as by exogenous factors.