Perinatal transmission and persistence of human papillomaviruses

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This thesis investigates whether human papillomaviruses transmitted from mothers to their infants.

Cervical/vaginal swabs were taken from 69 pregnant women. Buccal and genital samples were taken from their infants (all delivered vaginally) at 24 h (n=70; one set of twins), six weeks (n=49) and six months (n=19). All samples were examined for HPV -6, -11, -16, -18, -31 and -33 us polymerase chain reaction (PCR).

Thirty seven (54%) women had detectable detectable in sixteen (23%) infants at 24 h and seven (14%) at six weeks: a perinatal transmission rate of 23% (6/37) and persistence of 14% (7/37). All infants tested at six months were HPV negative.

At 24 h, HPV-16 was demonstrated in 9 mother-infant pairs, HPV-18 in 1 mother-infant pair, HPV -11 in 1 mother-infant pair, dual infection with HPV types 16 and 18 in 3 mother-infant pairs. Two infants with HPV-18 were delivered to mothers with HPV -16/18.

At 6 weeks, 5 infants remained HPV-16 positive, one infant r HPV -18 positive, whilst one infant who was HPV 16/18 positive was now HPV -16 positive.

To examine whether HPVs were acquired *in utero* or intrapartum, genital swabs were collected from 33 women who had amniotic fluid collect either at caesarean section (n=29) or at amniocentesis (n=4). Analysis of these samples demonstrated genital HPV in 10 (10%) of the women and none of the amniotic fluid samples.

To determine if a high maternal genital HPV load wa transmission, 15 women with genital HPV-16 densitometer. On average the mothers who transmit HPV 16 t infants had a higher viral load than non-transmitters ($p<0.05$).

To demonstrate persistence and source of infectivity at 2 years, the same group sequenced a 521 bp segment of the upstream regulatory (URR) of HPV-16 DNA isolated from 13 maternal samp samples taken from their infants.

In conclusion, HPVs can be transmitted perinatally. HPV DNA persisted up to 2 years of age. In addition women with a high genital HPV load were more likely to transmit the virus.

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Major publications arising from this work

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Glossary

^{0}C = degrees Celsius

ml= milliliter

DNA= deoxyribonucleic acid

μl= microliter

mM= micromole

KCl= potassium chloride

NaOH= sodium hydroxide

$MgCl_{2}$= magnesium chloride

HCl= hydrogen chloride

pH= potential of hydrogen

μg= microgram

h= hour

min= minute

EDTA= ethylenediaminetetraacetic acid

μM= micromole

ATP= adenosine triphosphatase

CTP= cytosine triphosphatase

GTP= guanine triphosphatase

TTP= thymine triphosphatase

pmole= picomole

pg= picogram

sec= second

ng= nanogram

%= per cent

M= molar

^{32}P= labeled oligonucleotide

bp= base pairs

CI= confidence interval

ORF= open reading frame

(w/v)= weight/volume

v.s= versus

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Chapter 1: Introduction and aims of the thesis

1.1 Malignant disease of the cervix

1.1.1 Epidemiology

The most recent estimate for the number of new cases of cervical cancer occurring in the world each year is 437, 000 (Parkin *et al.*, 1993). This accounts for 12% of all female cancers. Southern Asia contributes just over one quarter of this total. Cervix cancer is the most frequent cancer of women in almost all of the developing areas. In developed countries, the incidence rates are generally low, with age standardized rates less than 15 per 100,000, with the exception of Eastern Europe (17.7 per 100,000). Although methods in collating data may only be crude (e.g. lack of statistics and incorrect coding), these figures demonstrate the magnitude of the problem. This is further highlighted by the poor prognosis of cervical cancer i.e. a five year survival of only 60% (Jordan, 1982).

Invasive cancer of the cervix is the sixth commonest cancer in British women with an annual incidence of 16/100 000. Annually approximately 1800 women die in England and Wales (Williams, 1992). The cumulative rate (age-standardised rate indicating the probability [as a percentage] of a woman developing cervical cancer) up to age 74 in England and Wales is 1.2.

Although death rates from cervical cancer have fallen sharply in many developed countries, worldwide age-specific mortality rates show an increase in the younger age group. This is also true for the young adult population in the UK.

The highest rates of cervical neoplasia occur in Latin America, where the risk is six times greater than that of whites in the United States, whose rate is one of the lowest in the world. Even lower rates have been reported for Jewish women in Israel (Boyd & Doll, 1964). There are also distinct trends with race. In the United States rates tend to be higher in blacks than whites.

It may be argued that socioeconomic state is an important determinant for these varied rates. Indeed, when adjustments were made for socioeconomic differences, the excess risk of cervical cancer amongst blacks was reduced from more than 70% to less than 30% (Devasa & Diamond,1980).

1.1.2 Pathology

About 90% of invasive cervical tumours are squamous carcinomas, arising from the squamocolumnar junction. Grossly these take the form of either an exophytic cauliflower growth or ulcer with accompanying necrosis. The remainder are adenocarcinomas, arising from the endocervix, or mixed squamous adenocarcinomas.

Cervical tumours evolve as a sequential and multi-step process. The term cervical intraepithelial neoplasia (CIN) suggested by Richart (1967) has been widely used to describe all stages of premalignant disease which range from CIN I to CIN III (carcinoma *in situ*). Its diagnosis is based upon architectural and cytological appearances of the cervical epithelium. These include the loss of differentiation, nuclear pleomorphism and an increase in abnormal mitoses.

The stratified squamous epithelium of the vagina and ectocervix meet the columnar epithelium of the uterine cavity at the squamocolumnar junction (SCJ). This is the site where a vast majority of premalignant and malignant cervical disease arise. Premalignant lesions may disappear spontaneously or progress over a number of years. Progression from CIN I to CIN III, based on colposcopic examination without a punch biopsy was observed in 26% of women after 2 years (Campion *et al.*, 1986). The malignant potential of CIN III was also highlighted when 36% of a cohort of women developed invasive disease (McIndoe *et al.*, 1984)

Carcinoma of the cervix spreads predominantly by either direct invasion

into neighbouring structures or lymphatic permeation. The lymph nodes involved are the parametrial, internal iliac, obturator and external iliac groups.

Depending on the stage of the disease, treatment involves either surgery, radiotherapy or a combination of both (Averett *et al.*, 1969; Jampolis *et al.*, 1975). Success rates decrease with increasing tumour bulk and stage.

1.1.3 Aetiology

Evidence linking cervical cancer and sexual activity has been available for over a century. Rigoni-Stern (1842), a surgeon in Padua, observed a lower frequency amongst nuns and single women compared to married women. Gagnon (1950) recorded a similar protection from cervical cancer in women leading a celibate life. Rotkin (1973) has since suggested certain risk factors often associated with cervical cancer - multiple sexual partners and a young age at the onset of intercourse.

Brinton *et al.* (1987) reported a three fold increase in the incidence of cervical cancer in women with 10 or more sexual partners compared to women with one partner. Similarly, the risk of CIN associated with 6 or more partners has been reported to be six times higher than that associated with 1 partner (Harris *et al.*, 1980). In both of these studies, adjustments were made for age, race, and socioeconomic state.

Women with first sexual experience before the age of 20 are 50% more likely to develop cervical cancer compared to women who initiate sexual intercourse after the age of 20 years (Brinton *et al.* 1987).

The association of cervical cancer with sexual risk factors has stimulated the search for a venereal agent. Several transmissible agents have thus been postulated.

In the 1960s, there was evidence suggesting a link between Herpes simplex

Virus (HSV) 2 infections and cervical cancer, but in the last two decades, interest has focused on certain types of human papillomaviruses (HPVs). Other risk factors include the oral contraceptives and smoking.

1.1.3.1 Oral contraceptive steroids

The relationship between oral contraceptives (OC) and cervical neoplasia has been the subject of numerous epidemiological studies, which at times have been conflicting. The controversial results may be due to the fact that OC use is highly correlated with the key factors associated with cervical cancer i.e. age at first intercourse and number of sexual partners (Brinton *et al.* 1991).

Whilst most earlier studies have found no association between OC and cervical abnormalities (Boyce *et al.*, 1972), recent studies have (Harris *et al.*, 1980; Beral *et al.*, 1988).

There is some evidence that oestrogens promote wart virus infections. Genital warts are more commonly observed during pregnancy and frequently resolve postpartum (Schneider *et al.*, 1987). However, the relation between oral contraception and cervical neoplasia remain unclear.

Problems from previous studies include making adjustments for a variety of confounding factors, especially sexual behaviour. It is imperative that well designed follow up studies are carried out to allow for these.

1.1.3.2 Other contraceptives

The use of barrier contraceptives should theoretically have a protective function against sexually transmitted agents. Wright *et al.* (1978) reported a lower incidence of cervical neoplasia amongst users of the diaphragm compared to those using the oral contraceptive pill. Protection is not only conferred by the barrier method, but also from the concurrent use of spermicides which have anti-viral properties (Hildesheim *et al.*, 1990 a)

1.1.3.3 Smoking

An association between cigarette smoking and cervical cancer was postulated fifteen years ago (Winkelstein 1977). Of the 33 epidemiological studies that have provided information about cervical cancer and cigarette smoking, 26 have shown a positive correlation (review by Winkelstein 1990). In the other six, patient selection was different, and the overall aim of the study was not specific for smoking and cervical cancer.

An increased risk has also been noted with the number of cigarettes smoked and the duration of smoking. Brinton *et al.* (1986) reported a two fold increase amongst current smokers compared with non-smokers.

It may be argued that people who smoke may share similar characteristics i.e. start intercourse at an earlier age, and have more sexual partners, but after adjustment for these factors a significant risk still persisted (Harris *et al.*, 1980).

A case-control study of cigarette smoking and dysplasia and carcinoma *in situ* of the cervix was conducted by Trevathan *et al.*, (1983). Results were adjusted for age, number of sexual partners, age at first intercourse, socioeconomic status and oral contraceptive use. The authors concluded that cigarette smoking was significantly associated with carcinoma *in situ* , severe dysplasia, and mild-moderate dysplasia (relative risks, 3.6, 3.3 and 2.4 respectively. There was also evidence that the risk was greatest in women who began smoking in their early teenage years.

A chemical carcinogen in tobacco smoke may be responsible for carcinogenesis, as high levels of smoke derived cotinine is present in cervical mucus of smokers. This may be responsible for changes in the cervical immune system (Schiffman *et al.*, 1987).

Sasson *et al.*, (1985), examined the cervical mucus of smokers and non-smokers for the presence of nicotine and its major metabolite, cotinine, and compared the data with those obtained from analyses of serum and urine.

The results of these analyses demonstrate that nicotine and cotinine can be detected in the uterine cervix of the cigarette smokers.

Cigarette smoking is associated with a significant and dose-dependant decrease in the concentration of Langerhans' cells. These are antigen presenting cells in the cervical epithelium (Tay *et al.*, 1987). The reduction in the number of Langerhans' cells available to detect and present viral antigens to T lymphocytes may facilitate the establishment and persistence of local viral infection. This in turn could increase the likelihood of the development of a virally induced neoplastic transformation (Singer & McCance, 1986).

Smoking appears to be linked to squamous cell carcinomas and not the rarer adenocarcinomas (Brinton *et al.*, 1986b).

1.1.3.4 Infectious agents other than the Human papillomaviruses

In the 1960s, there was great interest in the oncogenic potential of Herpes simplex virus (HSV) 2. Sero-epidemiological studies reported higher rates of antibodies against HSV 2 in cervical cancer patients than controls.

A prospective study in Czechoslovakia failed to confirm these findings (Vonka *et al.*, 1984). In the latter study, serum was obtained from patients with cervical abnormalities, and from controls matched on the basis of their age, age at first intercourse, number of sexual partners and smoking habits. The two groups were followed up for at least 4 years and only those controls that remained free of any colposcopic abnormality were included in the study. The author concluded that there were no differences in HSV antibody levels in controls and patients. However, the number of cases were small, and the possibility of over-matching could not be excluded.

Over the years, uncertainty has persisted as neutralizing antibodies to HSV 2 have been found more frequently among cases than controls (Dale *et al.*,

1988; Slattery *et al.* 1989). It may be that the serological tests used were not able to measure HSV 2 type-specific antibodies but mainly antibodies common to HSV 1 and HSV 2.

An aetiological role between HSV 2 and cervical carcinoma cannot be excluded, particularly as a potential interactive factor with HPV (Zur Hausen, 1983).

There has been an interest in other infectious diseases such as Chlamydia, Epstein-Barr virus, bacterial vaginosis, gonorrhoea and syphilis. However no consistent association has been demonstrated. Recently, infection with human immunodeficiency virus (HIV) has been observed to be correlated with detection of HPV related cytological changes (Feingold *et al.*, 1990).

1.2 Papillomaviruses and tumours

1.2.1 Introduction

A viral aetiology for human warts was first suggested by Ciuffo (1907), who demonstrated transmission of wart lesions using cell-free preparations.

Subsequently, Shope (1933) described the first papillomavirus as being the aetiological agent of infectious papillomatosis in rabbits. This virus recovered from the cutaneous horns in cotton tail rabbits produced papillomas in domestic rabbits which often progressed to malignancy. The application of coal tar as a cofactor accelerated the rate of progression to malignancy (Rous & Kidd, 1936). It took an additional 40 years before the first experimental studies linked HPVs with cancer of the cervix (zur Hausen *et al.*, 1974).

Progress in HPV research was slow because HPVs could not be propagated in tissue culture (Rous & Kidd, 1936). HPV replication and production requires cells to be terminally differentiated and as tissue cultures are

undifferentiated replication of viral DNA and production of infectious viral particles would not be feasible.

In the 1970s, advances became more rapid. The advent of recombinant DNA technology enabled the cloning and sequencing of HPV DNA (Law *et al.*, 1979). This led to identification of multiple HPV types exhibiting a wide range of tissue tropism and clinical manifestations.

In 1979 specific HPV types were identified in a rare form of human carcinoma arising in patients with epidermodysplasia verucciformis (EV) at sun light exposed areas (Orth *et al.*, 1979). EV is an autosomal recessive condition with a state of immunodeficiency. Papillomas develop on the skin following infection with HPV types 5 and 8 (Orth *et al.* 1978; Pfitser *et al.* 1981). These papillomas have the potential to convert to malignant squamous carcinomas following exposure to sunlight (Ruiter, 1973).

In 1980, the first genital isolate (HPV -6) was characterised (Gissmann & zur Hausen, 1980). This was soon followed by additional types i.e. types -11 (Gissmann *et al.*, 1982), type -16 (Durst *et al.*, 1983) and -18 (Boshart *et al.*, 1984).

Today, over seventy types of HPVs have been characterised (de Villiers, 1989). Of these, 28 have been isolated from benign and malignant genital lesions.

1.2.2 General description of Papillomaviruses

Papillomaviruses (PVs) [Latin: papilla, nipple; Greek: oma, tumour] belong to a genus of small DNA viruses, the papovaviridae. Other viruses that belong to this genus, include polyomavirus and simian virus type-40 (SV40). One differences between PVs and other viruses in the same genome is that PVs code for proteins via one DNA strand (Broker & Botchan,1986). Moreover, papillomaviruses have a narrow host range and are tissue specific (Lancaster & Olsen, 1982)

PVs are epitheliotropic, double stranded DNA viruses. They are enclosed within an icosahedral capsid made up of 72 capsomeres. The molecular weight of the genome is 5 x 10^6 daltons, with a length of approximately 7.9 Kilobases. There are now over 70 different types of HPVs, based on the differences in the nucleic acid homology. If there is less than 50% homology in the nucleotide sequences between a known HPV and an unknown one, then the latter is assigned a new number. Types sharing greater than 50% homology are divided into subsets under the same number (Coggin & zur Hausen, 1979).

1.2.3 HPV genomic structure and function

Papillomavirus genomes contain two main coding regions, designated as early (E) or late (L), and a non-coding region (NCR) located between the end of the L and beginning of the E region (figure 1.1). The (E) region is believed to be involved in viral replication and transformation, whilst the (L) region in maturation and assembly.

The early region contains up to eight open reading frames (ORFs). The E1, E2, E4, E6 and E7 ORFs are common to all papillomaviruses, but E3 is restricted to BPV-1 (Danos *et al.*, 1983). The late region contains two ORFs, L1 and L2 which respectively code for the major and minor capsid proteins. The L1 ORF contains the sequences conserved in all papillomaviruses (Firtzlaff *et al.*, 1988)

1.2.3.1 Early proteins

E1 has a molecular weight of 23 KD and is involved in replication and maintenance of episomal DNA.

The E2 ORF codes for at least two proteins. The full length protein is a trans-activator (Hirochika *et al.*, 1987), and the other (carboxy-terminal half)

product which inhibits transactivation and influences HPV transcription (Lambert *et al.*, 1987). It is therefore designated as a gene with regulatory functions. The majority of HPV integrated cervical carcinomas, the genome is disrupted within the E1/E2 ORFS. When the ability of E2 to repress transcription is lost, increased expression of E6 and E7 transcripts may contribute to the cell's progression toward malignancy (Schwartz, 1985).

The E4 ORF has a molecular weight of 10 KD, and is the most abundant HPV protein, but to date its function is unknown. Doorbar *et al.*, (1986) have postulated that E4 gene product may be involved in virus maturation.

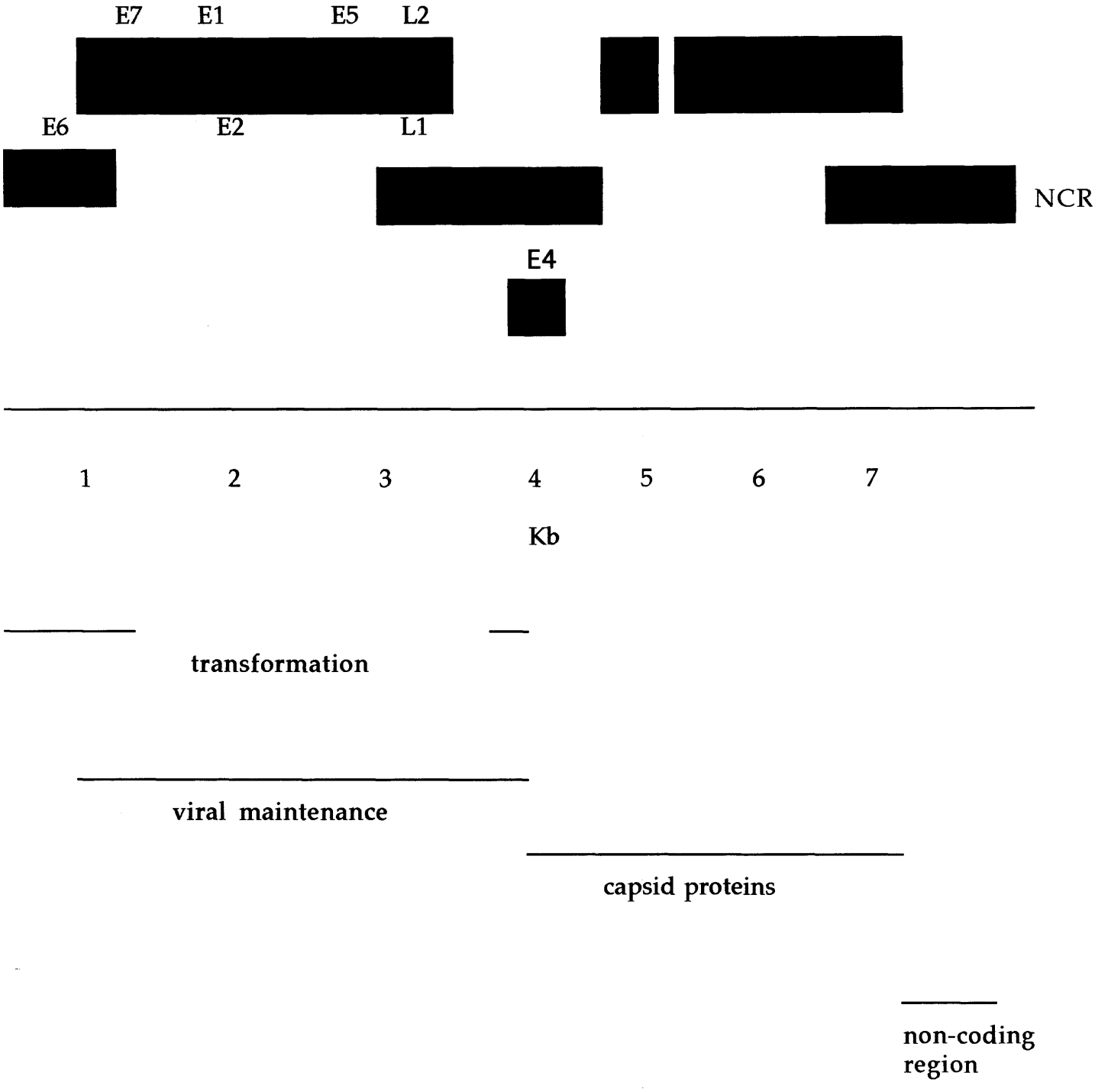

Figure 1.1.

Genomic organisation (linearised coding strand) of HPV-16 and summary of the function of various open reading frames.

1.2.3.2 ORFs with transforming ability

The ORFs encoding proteins with potential transforming function include the E6, E7, and E5.

HPV E7 encoded proteins have been isolated in the cytoplasm of HPV 16 related cancers and cell lines derived from cervical cancers (Smotkin & Wettstein, 1986). It has been shown that E7 gene product has the capacity to bind the retinoblastoma gene product (Dyson *et al.*, 1989). This ability is superior to the analogous gene product of the non-cancer related HPV ie types 6 and 11. The E6 gene product of HPV-16 can bind the p53 gene product (Werness *et al.*, 1990). Recent studies have identified HPV-16 E5 as another ORF with transformation capacity (Leptak *et al.*, 1991).

1.2.3.3 Late proteins

The papillomavirus capsid consists of proteins transcribed from the late region. L1, which is 500 amino acids long codes for the major capsid protein (80% of the total capsid proteins) and highly conserved between different papillomavirus types. The L2, which is less well conserved codes for the minor capsid protein.

1.2.4 Pathogenesis

HPVs induce hyperplasia of cells in the lower epithelial layers, but formation of complete viral particles appears to be restricted to the upper layer of the epidermis. Naturally occurring papillomavirus infections may be initiated by abrasion of an epithelial surface. A wound may provide the virus access to the lower layer of permissive cells. The basal layer of the epithelium appears to be a reservoir until replication starts (Schneider *et al.*, 1987). The interval from exposure to development of lesion ranges from 3 weeks to eight months (Oriel, 1971).

HPVs can be divided according to their target epithelium (Table 1.1). The most common clinical manifestation due to HPV is the common hand wart, typically caused by HPV types 1 and 2. The benign anogenital lesions, condylomata acuminata (genital warts) are often due to HPV types 6 and 11 (Gissmann *et al.*, 1983).

Table 1.1

Target epithelia for individual human papillomavirus types.

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(1) Cutaneous epithelia.

1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 36, 37, 38, 41, 46, 47, 48, 49, 50

(2) Genital mucosa

Condylomata acuminata: 6, 11, 42, 44, 51, 55, 58, 67

Intraepithelial neoplasia: 6, 11, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40.

Carcinoma: 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 66.

(3) Oral mucosa

11, 13, 30, 32, 57.

Adapted from de Villiers 1989.

1.2.5 Papillomaviruses and carcinoma of the cervix

There is now increasing evidence that certain human papillomavirus type-16 (HPV-16) and related types (HPV-18, HPV-31 and HPV-33) play an essential role in the pathogenesis of most cases of severe dysplasia and cervical carcinoma (Durst *et al.*, 1983; Galloway & McDougall, 1990; zur Hausen, 1991, Bosch *et al.*, 1995, Kjaer *et al.*, 1996,Lehtinen *et al* ., 1996). HPV types 6 and 11 are found in genital warts (condylomata acuminata), and to a lesser extent in premalignant lesion, usually CIN grade 1. The evidence linking HPVs with cervical carcinomas has been derived from clinical, epidemiological and molecular investigations.

Several reviews linking HPV-16 infection with cervical cancer have been published (zur Hausen, 1989; Galloway & McDougall, 1990).

1.2.5.1 Clinical

The rate of HPV-16 DNA detection in CIN lesions increases directly in proportion to their severity. McCance *et al.* (1985), reported that the rate of HPV-16 detection increased from 55% in CIN grade 1 to 71% in CIN III and 90% in malignant disease. Another conclusion from his study was, the prevalence of HPV type 6 decreased with increased severity of cervical abnormality.

O'Leary *et al.*, (1997), also concluded that HPV 16 and 18 were predominantly identified in low grade glandular intraepithelial lesions, high grade glandular intraepithelial lesions and adenocarcinomas with HPV prevalence increasing with grade of dysplasia.

HPV-16 DNA occurs in 90% of cervical biopsies, but in only about 10-30% of women without cervical abnormalities (van den Brule *et al.*, 1989; Pao *et al.* 1990, zur Hausen, 1994; Alani & Munger, 1998).

1.2.5.2 Epidemiological

Based on a case-control study in Spain and Columbia, Munoz *et al* (1992), concluded an association between HPV and cervical cancer. The choice of the countries was based on differences in risk of cervical cancer, as the prevalence in Columbia was 8 times greater than that of Spain. Cases were women with histologically confirmed invasive squamous cell carcinomas and controls were those that had not received previous treatment for cervical cancer or had normal cervical cytology following recruitment. The latter group were randomly selected from the general population. Cervical scrapes obtained from these women were analysed for the presence of HPV by using the polymerase chain reaction (PCR), southern hybridisation (SH) and Vira Pap (VP). The prevalence of HPV-DNA using these three techniques was higher in cases than in controls in both countries. For all cases and controls, the prevalence of HPV-DNA detected by PCR was 73.8% and 7.4% respectively. By far the most common type of HPV detected was type-16, but types 18, 31, 33 and 35 were also prevalent. In this study both VP and SH appeared to be less sensitive than the PCR method.

HPV 16 has been detected in samples of lymph nodes and pelvic metastases from women with cervical carcinoma associated with HPV-16 (Lancaster *et al.*, 1986).

HPV type 18 is the second most prevalent viral type, often associated with adenocarcinomas and highly aggressive small cell undifferentiated carcinoma of the cervix (Wilczynsk *et al.*, 1988).

In contrast to HPV-16, type 18 is not typically associated with a spectrum of precancerous lesions (Kurman *et al.*, 1988), and geographically appears to be widely distributed. In cervical cancer tissues from Austria, Columbia and Spain, HPV 18 was detected in only 4-5% of the samples (Munoz *et al.*, 1992), whereas in 14-24% of the tumours in France, Africa and the United States

(Riou *et al.*, 1990).

Lorincz *et al.*, (1992) suggested the HPV types associated with anogenital lesions be divided into those with high (types 16, 18, 45 and 56), intermediate (31, 33, 35, 51 and 52) and low (6, 11, 42, 43, 44) cancer risk. In a series of patients with cervical cancers, the 'high risk' HPVs were present in 74%, whereas no HPV in the 'low risk' category were present.

1.2.5.3 Molecular

HPV-16 DNA in cervical cancers is often integrated into host DNA (Gissmann & Schwarz, 1986). Integration is defined as the covalent attachment of HPV DNA to chromosomal DNA. In contrast, the episomal form of viral DNA is free and circular (Durst *et a*l., 1985 & Cullen *et al.*, 1991). The site of integration is non-specific (Durst *et al.* 1985; Smotkin & Wettstein, 1986), although specific integration into chromosome 8q21-q22 near the c-myc gene has been described (Durst *et al.*, 1987).

Integration of viral DNA is frequently associated with cervical cancers and cases of severe dysplasias. It has not been demonstrated in benign HPV infections e.g. genital warts commonly caused by HPV 6 and 11. Integration usually disrupts the E1 and E2 open reading frames (ORFs), causing over expression of the E6/E7 genomes. Whilst integration may be an important factor in carcinogenesis, Cullen *et al.*. (1991) reported that it was not obligatory. In a series of cases of cervical neoplasia, the latter author concluded that 19% of patients had HPV DNA in an episomal form. Fuchs *et al.* (1989) also concluded that up to 36% of cervical cancers had HPV DNA in an episomal form.

HPV type 18 is nearly always present in an integrated form, whereas HPV 16 may exist as an episomal or an integrated form. This probably explains the more aggressive nature of HPV-18 associated adenocarcinomas

(Walker *et al.* 1989).

1.2.5.4 Early genes E6 & E7

Two early genes, E6 and E7, are usually retained in cervical carcinomas (Schneider-Gadicke & Schwartz, 1986; Smotkin & Wettstein 1986). The E6 and E7 proteins have been shown to play an essential role in the *in vitro* transformation of primary human keratinocytes and rodent cell lines (Jewers *et al.*, 1992) through their ability to interact with cellular growth regulatory proteins. The E6 and E7 genes of the high risk HPVs (16 and 18) immortalise human foreskin and cervical keratinocytes. These cells acquire an unlimited life span and quickly become aneuploid. Low risk HPVs cannot immortalise human keratinocytes *in vitro* (Woodworth *et al.*, 1989). The immortalised cells are however non tumorigenic when transplanted into nude mice. In contrast, cell lines obtained from malignant tumour of the cervix are tumorigenic.

HPV-16 E6 and E7 proteins inactivate the endogenous tumour suppressor proteins p53 and pRB, respectively (Tidy & Wrede, 1992, Scheffner *et al.*, 1993, Slebos *et al.*, 1994).

It is now known that HPV E7 forms a complex with a cellular protein (Dyson *et al.*, 1989) which is coded by the retinoblastoma (Rb) gene. The Rb gene consists of 27 exons which span 200 Kilobases (Kb) of chromosomal DNA (Lee *et al.* 1987). The associated mRNA transcript is 4.7 Kb and encodes a 105 Kd nuclear protein which shows cell cycle dependant phosphorylation. The active form of Rb is thought to be the dephosphorylated form of the protein which accumulates in the cell in the G0/G1 phase of the cell cycle. The ability of E7 to form a complex with pRB suggests that these tumour viruses transform cells through their ability to prevent a cellular growth-suppressing mechanism, and hence mimicking the state seen in spontaneous

human tumours that have lost pRB function. The loss of function contributes to oncogenic transformation.

Another cellular protein known to bind to the HPV E6 is p53 gene product (Werness *et al.* 1990). The human p53 gene locus is located on the short arm of chromosome 17 and spans 20Kb of genomic DNA. Transcription of this gene yields a 2.5 Kb mRNA which encodes a 53 KDa protein of 393 amino acids (Lamb & Crawford, 1986). The p53 proteins is thought to play an important role in the regulation of the cell cycle. It is likely that they function to control negatively entry or progress through the cell cycle, possibly at the G1/S boundary. The E6 forms a complex with the cellular p53 protein and directs an increase in the rate of p53 degradation (Scheffner *et al.*, 1991).

The cell regulatory function of both p53 and Rb may also be affected as a result of mutations (Bartek J *et al.*, 1990). Mutations of the p53 protein have been detected in cancer of the bladder, colon, breast and ovary. In fact, p53 is the most common mutated gene in human cancer.

As pRB and p53 play an essential role in cellular growth, it becomes apparent that the binding to HPV oncoproteins may inhibit its normal functions. The E7 proteins coded by the oncogenic HPVs, in particular type 16, has a greater affinity for Rb gene product when compared to the more benign HPVs. This is also true for the E6 coded proteins and p53 (Gage *et al.*, 1990).

1.3 Identification of Papillomaviruses

1.3.1 Clinical

Genital warts is the most widely recognised genital HPV infection, usually visible to the naked eye. They are typically present on the shaft of the penis, on the prepuce and the glans. In the female, it affects the posterior vaginal

fornix and the labia. These lesions are also frequently seen in the perianal skin and on the adjacent sites of the inner thigh. Grossly, the lesions appear as a soft pedunculated papule and typically range from 3 to 10 mm in size. They are often multiple and occur in clusters. In the vagina condylomata acuminata are localised by means of acetic acid and colposcopy, appearing as white raised lesions.

Grossly visible condylomata are usually associated with HPV types 6 or 11. Condyloma acuminata may occasionally be confused with such as condylomata lata of syphilis.

1.3.2 Electron microscope

HPV particles from skin warts were first demonstrated by negative staining (Strauss *et al.*, 1949), and by transmission electron microscopy of tissue sections (Almedia *et al.*, 1962, plate 1.1). The virions are arranged in crystalline arrays and scattered throughout the nucleus. After the rupture of the nuclear membrane, virions may also be present in the cytoplasm.

The concentration of viral particles varies considerably in different wart types. Palmar and plantar warts show the highest number of viral particles, whereas common warts contain a lower virus concentration (Almedia *et al.*, 1962; Laurent *et al.*, 1975). Warts that are 6-12 months old contain the highest number of viral particles. Compared to cutaneous lesions, a lower number of HPV particles are found in penile and vulvar warts (Dunn & Ogilvie, 1968).

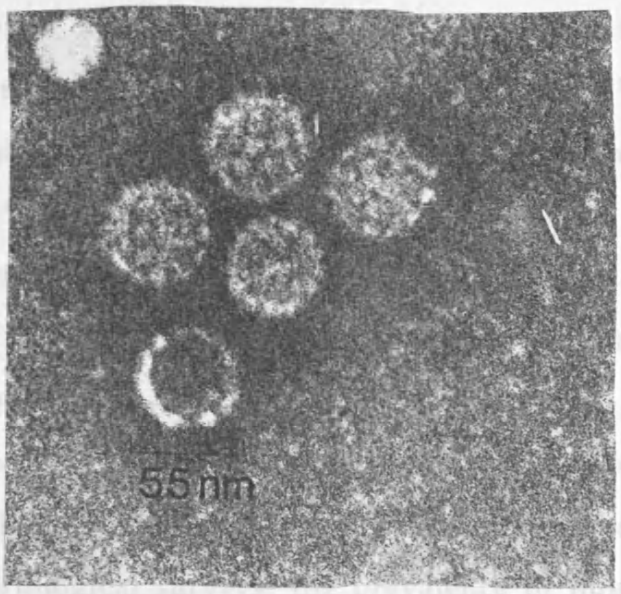

Plate 1.1

Structure of papillomavirus particles. Electron microscope showing approximately x 110, 000 magnification. A large clump of virions observed when stock BPV-2 was mixed (1:1) with undiluted antisera to disrupt BPV-1.

1.3.3 Cytology and Colposcopy

Cytological diagnosis depends on the finding of minute changes in the structure of nuclear chromatin, the outline of the nuclear membrane, presence or absence of nucleoli and the texture of the cytoplasm. Three cell types are diagnostic in the presence of condyloma:

The Koilocyte - a mature squamous cell characterized by large perinuclear cavity. The cytoplasm surrounding the cavity is dense. The nucleus may be single, often double. There is no nucleolus. No inclusion bodies are observed in HPV infections.

The dyskeratocyte - mature squamous cells with nuclei identical to those of koilocytes. The cytoplasm is however more dense and stains a brilliant orange with the OG6 of the Papanicolaou technique.

Condylomatous parabasal cells - with an amphophilic cytoplasm containing nuclei with smudged chromatin may sometimes be observed.

In recent cytological surveys, the prevalence of human papillomavirus ranged from 3 per cent to 7 per cent (Syrjanen *et al.*, 1990).

The colposcopic criteria for abnormal epithelium are leukoplakia (a white area detected before the application of acetic acid), or iodine negative areas not reacting to the Lugol's Iodine.

1.3.4 Molecular biology

Methods for identification PVs have been based on different hybridisation techniques. These include southern blot, dot blot, filter *in situ*. The most recent and sensitive technique, based on DNA amplification is the polymerase chain reaction (PCR). Each of the techniques has advantages and disadvantages. There are also differences in sensitivity and specificity.

1.3.4.1 Southern Blot Hybridisation (SBH)

The Southern blot hybridisation (Southern, 1975) has been the method to which all DNA detection techniques are compared. The cellular DNA extracted from a biopsy sample is initially digested with restriction endonucleases (this cleaves double-stranded DNA at specific sites). These fragments are then separated on agarose gel with each of the HPV types having a characteristic pattern. Following electrophoresis, the sample is depurinated in hydrochloric acid and denatured into single strands using an alkali (NaOH). This is then transferred overnight by capillary action to a nitrocellulose filter. In evaluating and typing HPV DNA, the filter is hybridised with a cocktail containing several radioactively labelled HPV types. Permanent binding to the filter is achieved by baking at 80^0C under vacuum. The principal goal being maximum sensitivity. Under these conditions, two to four days of exposure will give a strong hybridisation signal on a photographic film (Figure 1.2).

Southern blotting is a relatively sensitive method that can detect HPV in over 90% of condylomata, intraepithelial neoplasms and carcinomas (Reid *et al.*, 1987). Its sensitivity depends on the amount of HPV DNA present, and the degree of base pairing with the HPV sequences in the probe. Typically, one copy of HPV-DNA is detected per 100 cells (Maitland *et al.*, 1987). It is a more sensitive technique than other types of filter hybridisation techniques. However a disadvantage is that with preparation and photography, it may take up to 10 days to analyse results . This is impractical in large scale studies.

Other problems with Southern blot hybridisation were demonstrated when identical samples were analysed by five different laboratories, there was only a 45% correlation (Brandsma, 1989).

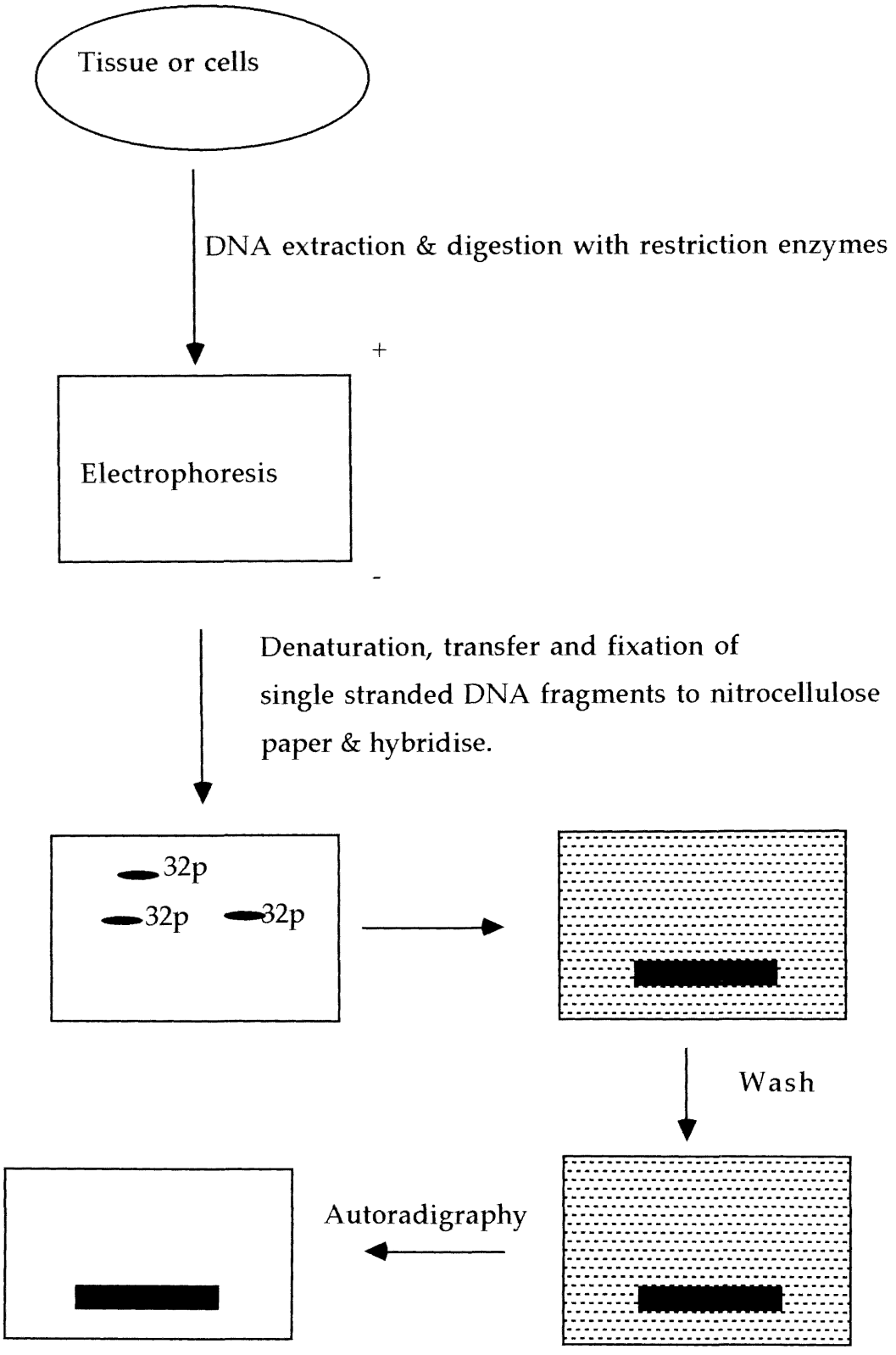

Figure 1.2

Principles of Southern Blot Hybridisation

1.3.4.2 Slot blot hybridisation

Slot blot hybridization is similar to SBH. The only difference is that in the former case the DNA is not electrophoresed and transferred to the filter. Slot blot hybridization has a lower threshold in detecting DNA. Whereas 0.1 to 1 pg of HPV DNA can be detected by SB analysis, 0.01 pg can be detected by slot blot (Nuovo & Richart, 1989) and may therefore be useful in detecting samples such as cervical scrapes in which DNA may be minimal. It is also quicker than SBH in analysing results. The major draw back is non specific hybridization, resulting in false positive and false negatives.

1.3.4.3 Filter *in situ* hybridisation (FISH)

The first HPV assay method which was designed for population studies was FISH. In this case, cells are applied directly to a filter, and the cells are disrupted and denatured (McCance *et al.*, 1986). After washing, auto radiography is carried out and results read after 1-5 days. An advantage is the ease by which a large number of samples can be analysed. However, this method is not as sensitive as Southern Blotting.

1.3.4.4 Polymerase chain reaction

The polymerase chain reaction (PCR) is a relatively new DNA amplification technique, first described in 1985 (Saiki *et al.*, 1985), which generates millions of copies of a specific DNA fragment in a few hours (Ehrlich *et al.*, 1991).

The PCR method is useful in molecular virology, in particular HPV studies. The reaction is based on annealing and extension of two oligonucleotides primers that flank the region of the DNA of interest. The genomic sequence of the DNA to be amplified must be known in sufficient detail to design primers which will anneal to the ends (Figure 1.3).

A PCR is carried out on an aliquot of sample plus target-specific primers, thermostatable DNA polymerase, deoxyribonucleotides and a buffer (Saiki *et al.*,1988). There are three steps in the PCR reaction: separating the strands of the specimen DNA at near melting point temperature; annealing of the complementary sequences on the separated strands by specific primers; and synthesis of the new DNA by extension of the primers. The temperature changes are brought about by an automated thermal cycler. Each cycle is completed in a few minutes and results in a two fold increase in the amount of target DNA. Therefore, a million fold increase in DNA is achieved within a few hours. The amplified product is then identified on an agarose gel. HPV-PCR detects ten to hundred copies of the genome in a sample compared to a hundred thousand by other techniques.

In a study by Melchers *et al.*, (1989) cervical samples were obtained from 80 women. These were analysed for the presence of HPV DNA by using PCR, SBH and FISH. It was noted that the sensitivity of the PCR was superior when compared to SBH and the FISH. Whereas the two latter methods were similar in their sensitivity i.e. a detection rate of HPV in 46%, the PCR detected HPV in 70% of the cases.

There are however disadvantages associated with PCR. The process is susceptible to contamination, giving rise to false positive results (Kwok & Higuuchi, 1989).

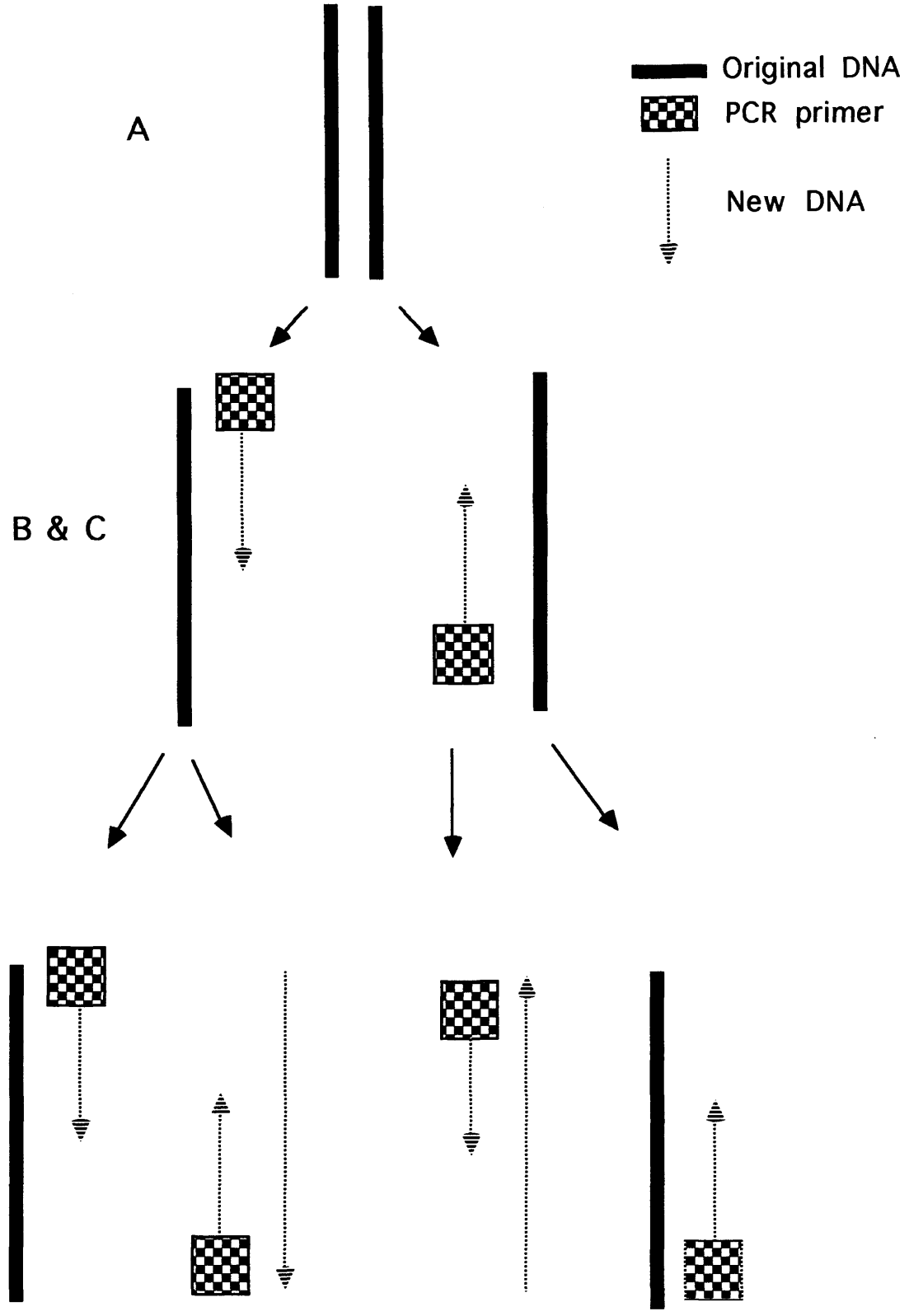

Figure 1.3

Graphic representation of the polymerase chain reaction. It demonstrates DNA denaturing, primer annealing and extension.

1.3.5 Serological assays for detection of HPV antibodies

HPV serology has been hampered by the lack of appropriate target antigens for use in serological assays. HPVs cannot readily be propagated *in vitro* (Taichman *et al.*, 1984) and premalignant and malignant cervical lesions contain few intact HPV-16 virions or capsid proteins.

Xenograft-implanted nude mouse models for the replication of HPV-11 (Kreider *et al.*, 1992) and for the production of HPV-16 lesions (Sterling *et al.*,1990) have been developed. However, the yield of virus particles from this system is low.

In recent years, investigators have utilized recombinant DNA technology to express fusion proteins from different HPV ORFs or have used chemically synthesized peptides to obtain antigens for a study of the immune responses to HPV (Carter *et al.*, 1994, Kirnbauer *et al.*, 1994).

Indeed antibodies to recombinant proteins and to synthetic peptides corresponding to HPV-16 early and late proteins have been detected in sera from patients with CIN, but these antibody concentrations been generally low (Cason & Best, 1991).

The lack of a permissive system for the propagation of viral stocks containing abundant HPV particles has recently been overcome by the organotypic (raft) culture system. This has permitted the study of a number of the differentiation specific aspects of HPV, including amplification of viral DNA, expression of late genes and viral morphogenesis (Meyers *et al.*,1992, Frattini *et al.*,1996, Meyers *et al.*, 1997).

1.3.5.1 Immune response to HPVs

The composition and the competence of the host's immune response play major roles in influencing the outcome of HPV infections. Immunosuppression, whether hereditary, acquired or iatrogenic, permits the

development of HPV lesions. Patients with common variable immunodeficiency (characterised by the failure to produce antibodies) appear not to be unduly susceptible to the development of HPV lesions (Benton *et al.*, 1992). Thus cell-mediated immune (CMI) response seems to be particularly important for the regression of HPV induced lesions. Nevertheless, neutralising antibodies to HPVs in cervical secretions may prevent reinfections. Effective CMI responses probably explain why the majority of untreated CIN lesions do not progress to malignancy (Nasiell *et al.*, 1976).

1.4 Perinatal transmission of other organisms other than human papillomaviruses

1.4.1 Human immunodeficiency virus (HIV) and pregnancy

HIV, the main cause of acquired immunodeficiency syndrome (AIDS) was identified in 1984. It is a major world health concern with the highest prevalence in East and Central Africa, where in certain areas, up to 30 % of pregnant women are infected. Heterosexual intercourse accounts for 60% of transmission; and that 40 % of those infected are female.

HIV is a retrovirus with a simple structure consisting of a protein core (containing the RNA complex and reverse transcriptase) surrounded by an inner matrix protein coat (p18), itself surrounded by a glycoprotein membrane (gp120, gp41). The RNA genome contains three genes, one of which, 'env', encodes for envelope proteins including gp 120, responsible for "locking in" to the CD4 cell receptors. This protein is highly variable and therefore evades immune surveillance and its depletion is a measure of spread of infection within the individual.

Primarily in the United Kingdom, HIV is a problem in large cities such

as Edinburgh, Dundee and London. The prevalence of HIV, based on anonymised screening of heel prick samples from newborn infants , was 1 in 360 in inner London, 1 in 1200 in Outer London and 1 in 7200 in the remainder of England and Wales (Department of Health, 1995).

Perinatal transmission of this virus has been one of the main concerns. The estimated rate of transmission from mother to the fetus varies from 15 % to 20 % in Europe, 15 % to 30 % in United States of America and 25 % to 35 % in Africa (Newell & Peckham, 1993). Indeed over the past 15 years, HIV infection has become a major cause of illness and death in young children. In the USA, HIV infection is now the seventh leading cause of death in children one to four years of age. The World Health Organisation (WHO) estimates that over one million children are infected world wide.

It is believed that fetal infection commonly occurs in the latter part of pregnancy. There is good evidence that babies can also be infected by breast feeding (Dunn *et al.*, 1992). However attention is now mainly focused on events around delivery and current belief is that as much as two thirds of vertical transmission may occur around this time (Dunn *et al.*, 1995). It is suggested that the degree of exposure to infected maternal blood and cervical mucus during delivery is a main determinant of transmission. Viral load is also an important determinant of transmission and therefore is greatest early and late in the natural history of infection (Peckham & Gibb, 1995).

An early diagnosis of HIV infection in infants born to HIV infected mothers is made after the detection of virus in culture, the HIV genome by PCR, the presence of viral antigen, or the persistence of HIV antibody beyond the age of 18 months. To confirm the diagnosis, positive results on two separate blood samples are required with one or a combination of tests (viral culture and/or the PCR). The diagnosis is supported by the persistence of HIV antibody after 18 months.

1.4.2 Rubella

In 1941, Gregg described the occurrence of congenital cataracts after maternal Rubella. Since Gregg's report, Rubella's effects on the embryo, fetus and neonate has become more apparent.

Rubella is a small (58 nm) single stranded RNA togavirus. It infects only humans and is transmitted to seronegative subjects by aerosolized respiratory secretions. The virus replicates in the respiratory system and then enters a viraemic phase. The placenta and the fetus are affected at that time.

Rubella seroprevalence is age related. More than 90 % of Caucasian women of child bearing age have rubella antibodies. In total, 0.5-2 % of susceptible pregnant women are infected each year. Congenital disease occurs in 0.2-0.5 per 1000 births. Women whose immunity is vaccine-induced experience re-infection more frequently (50 %) than those whose immunity is naturally acquired (5 %).

Rubella is a mild childhood infection of little consequence except when it occurs during pregnancy. One third of primary infections are subclinical. Prodromal symptoms of malaise, headache, low-grade fever, coryza and conjunctivitis may precede the characteristic pinpoint macular rash by 1-5 days. The contagious period extends from 8 days before to 8 days after the onset of the rash. Rubella antibodies are present by the time the rash appears.

Maternal viraemia associated with either symptomatic or asymptomatic primary disease is a prerequisite for fetal infection. The likelihood of fetal infection and damage is dependant on the gestation of maternal infection. When maternal infection is acquired during organogenesis (the first 12 weeks), cardiac, eye and ear defects may develop. Hearing defects are common up to 17 weeks gestation. Indeed, deafness may be the only anomaly when rubella is acquired after the first trimester. The risk of rubella defect after the 17th week of pregnancy is small but real.

Growth deficiency is common and is not corrected by delivery. Viraemia is rare during re-infection. The risk of fetal infection is probably below 10%, especially in women with vaccine induced immunity (Miller, 1990).

1.4.3 Parvovirus

Human parvovirus B19 was discovered in 1975 by Cossart. Its adverse effect on pregnancy outcome was first recognised in 1984 (Carrington *et al.*, 1987) and the first prenatal diagnosis was made in 1987 by Naides and Weiner (1989).

It is a small (20-25 nm), non-enveloped, single stranded DNA virus. It infects only humans usually *via* aerosolized droplets.

B 19 seroprevalence is age-related; about 50% of women of child bearing age are immune. Fifth disease, or erythema infectiosum is characterized by high fever and a rash and is moderately infectious, with an incubation period of 4-14 days. The virus causes mild illness in children. In adults, infection is more serious with adenopathy and mild arthritis as common complications. Other B 19 syndromes include; transient aplastic crisis (TAC), which occurs in patients with either sickle cell disease or chronic haemolytic anaemia.

Fetal infection is explained by the duration of viraemia in the infected pregnant woman. Fetal death may occur in the first, second or third trimester of infection. The overall fetal loss due to intrauterine B 19 infection is 10% with the highest fetal risk probably in the first 18 weeks of pregnancy.

The effect of B 19 infection on the fetus ranges from no effect to growth deficiency to chronic hepatitis with the development of isolated ascites to non-immune hydrops fetalis. After delivery, these children continue to shed virus for a variable duration.

1.4.4 Cytomegalovirus (CMV)

CMV infects most people at some time during their life. The infection is of little consequence except in the fetus and the immunocompromised subject. Most primary and recurrent infections are subclinical. Regardless of whether or not symptoms are present, the fetus may be infected and damaged.

CMV is a large DNA virus of the Herpesvirus group. It replicates in the nucleus of infected cells producing the characteristic nuclear inclusion.

Congenital CMV infection has a high incidence compared to other intrauterine viral infections (0.2-2.2% of live births). In total, 10% of congenitally infected neonates will have sequelae; 50% of these are severe (mental retardation, sensorineural hearing loss, cerebral palsy).

The incidence of congenital infection is fairly stable (0.3%) in the UK. During primary infection the rate of transmission to the fetus is 40% and is not influenced by gestational age. The severity of congenital infection occurs in the first half of pregnancy. Recurrent maternal infection accounts for 20-30% of congenital CMV infection in the UK.

Among neonates infected in utero, about 10% are symptomatic at birth, of which 15% have classic cytomegalic inclusion disease. Altogether, 15-20% of symptomatic infants die and 90% of the survivors develop sequelae. among infected but asymptomatic newborns, 5-15% develop sequelae, especially sensorineural hearing loss. In England and Wales, about 400 neonates will be damaged either at birth or by age 1 year.

1.4.5 Varicella

Varicella-zoster virus (VZV) is a DNA virus of the herpesvirus family which produces two distinct clinical syndromes. In temperate climates, VZV is responsible for the childhood exanthematous disease commonly known as chickenpox. Clinical chickenpox develops 10-20 days after respiratory

exposure and consists of fever, malaise and a pruritic rash which develops initially as crops of maculopapules which rapidly vesiculate and finally crust over. Chickenpox in children is a highly infectious but a mild infection. Primary VZV in adults, however, is occasionally more severe, leading to complications such as pneumonia, encephalitis and myocarditis.

In the pregnant woman VZV infection has a number of implications for the fetus. Generally, acute chickenpox in a pregnant woman, if systemic symptoms are significant, may incite premature uterine activity and subsequent premature delivery. More specifically, however, a syndrome of intrauterine VZV infection has been described which includes a number of structural abnormalities which correspond to effects of cutaneous vesicles occurring in a small fetus. In the first 20 weeks, 0.5 and 2 per cent of fetuses are affected, with more severe damage found in babies infected at earlier gestational age. In addition babies infected in utero may develop Zoster within the first 2 years of life. The fetal abnormalities include cutaneous scarring, limb hypoplasia, missing/hypoplastic digits, convulsions, microcephaly, chorioretinitis and cataracts.

1.4.6 Herpes Simplex Virus (HSV)

HSV is a DNA virus of the herpes family. HSV-1 has been considered the cause of orolabial herpes; HSV-2 has been considered the cause of genital herpes infection.

Genital herpes may occur after sexual contact with an infectious person. The incubation period is less than 1 week, and persons transmitting the virus may be asymptomatic. In the absence of circulating antibody, primary HSV genital infection may be quite severe, with systemic symptoms of fever, malaise, myalgias and aseptic meningitis.

HSV infection in pregnant women has two important components:

primary infection in the mother, and transmission of infection (either primary or recurrent) to the neonate and subsequent disseminated infection. Primary HSV infection in the pregnant woman may be severe enough to cause systemic illness, and fever may stimulate premature uterine contractions. Thus premature labour and delivery may be serious complications of HSV infection in pregnancy, depending on the gestation of the pregnancy.

The fetus acquiring HSV in the perinatal period may suffer severe neonatal morbidity, including dissemination leading to chorioretinitis, meningitis , encephalitis, mental retardation, seizures and deaths. There are no consistent reports of a congenital syndrome due to intrauterine infection with HSV. However the spectrum includes miscarriage, prematurity and intrapartum infections with resultant disseminated HSV infection (Whitley *et al.*, 1988).

1.4.7 Hepatitis B

Hepatitis B, is a viral liver disease caused by hepatitis B virus (HBV), a member of the Hepadnaviridae family of DNA viruses. HBV is tropic for human hepatocytes, thus the major clinical disease caused by the virus is hepatitis. Clinical illness is generally manifest after a 2-6 month incubation period. Approximately two-thirds of acute HBV infections are subclinical and asymptomatic, or associated with a mild influenza like illness. Symptoms, if present include nausea, vomiting, anorexia and right upper quadrant pain.

In adults, 90% of acute HBV infections resolve completely within 6 months. In the other 10% of individuals , hepatitis B surface antigen (HBs Ag) remain in the serum, the patient considered as a chronic carrier and most will develop chronic active hepatitis, chronic persistent hepatitis or, rarely, fulminant HBV infection which progresses rapidly to hepatic failure and

death.

Acute HBV Infection occurring in late pregnancy and around delivery favours vertical transmission from mother to child before or peri-partum. In one study, the risk of vertical transmission for mothers with acute hepatitis B manifest during the first (0 per cent) and second trimester (6 per cent) respectively was significantly less than that for third trimester (67 per cent) and within 5 weeks post-partum (100 per cent).

Up to 40 per cent of all chronic carriers of HBV arise following vertical transmission. Babies born of HBsAg positive mothers with serological evidence of virus replication- HBeAg and HBV DNA- have between a 20 and 95 per cent chance of becoming infected, depending on maternal ethnic origin.

As part of a large scale controlled prospective study on the efficacy of the HBV vaccine amongst babies born of HBeAg positive mothers in Taiwan, 95 per cent who received no immunoprophylaxis became infected acutely and 93 per cent became infected chronically by the sixth month of follow up.

1.5 Modes of transmission of genital Human papilloma viruses

1.5.1 Sexual transmission

The sexual route of transmission of HPVs was noted in 1954, when Barrett *et al.* (1954) reported cases of 24 women with a history of genital warts. These women noted the appearance of genital warts four to six weeks after their husbands had returned from the Far East. All the consorts had admitted to sexual contact with women whilst overseas, and all recently had penile warts.

Condyloma acuminatum is the most commonly diagnosed viral sexually

transmitted disease (Becker *et al.*, 1987; DHSS, 1984), being more prevalent than Herpes simplex viral infections. Data collected from office based private practitioners in the United States demonstrated that in 1984 there were 156,720 first time visits for genital herpes and 224,900 cases of genital warts (Becker *et al.*, 1987).

In the U.K there has also been an increase in the incidence of genital warts. Annual reports of the chief medical officer of the Department of Health and Social Security, reported an increase of over 250 per cent between 1971 and 1982. A direct correlation exists between sexual activity and HPV infection. Daling *et al.* (1986) reported an increased prevalence of apparent HPV infection amongst women with a greater number of sexual partners.

1.5.2 Perinatal transmission

Juvenile laryngeal papillomatosis (JLP) remains a rare but sometimes an extensive, HPV related lesion. It can develop at any time between birth and young adulthood (Benjamin & Parson, 1988). The rate has been estimated at approximately 1 in 1500 births, although the actual number may be less (Mounts *et al.*, 1984; Shah *et al.*, 1986;).

Quick *et al.* (1980) reported that a high proportion of affected children were delivered to mothers with a history of genital warts. Similarly, Hallden *et al* (1986) reported a history of maternal condyloma in 54% of cases of JLP.

The morphology of laryngeal papillomas is similar to genital warts (Abramson *et al.*, 1987), with over 90% of JLP containing either HPV-6 or HPV-11 DNA (Gissmann *et al.*, 1983). In addition, there has been a case report of a 12 year old child with a laryngeal carcinoma that contained HPV -18 and -33 (Simon *et al.*, 1993)

Infants with laryngeal papillomatosis often present with a history of airway obstruction, with 15 % presenting prior to 2 month of age (Benjamin

&Parson, 1988). Of concern with JLP is the risk of developing invasive cancer. Predisposing factors include smoking and irradiation, although these factors are not absolutely necessary (Helmuth *et al.*, 1987; Bewtra *et al.*, 1982).

No satisfactory treatment for this clinical condition has yet been discovered, despite a multitude of approaches including systemic chemotherapy, excision, cryotherapy, carbon dioxide laser and interferon therapy (Singleton & Adkins, 1972; Bone *et al.*, 1976; Benjamin *et al.*, 1988).

HPV types 6 and 11 are not the only viruses implicated in vertical transmission as antibodies to HPV-16 occur in 25% of children under the age of ten (Cason *et al.*, 1992) and HPV-16 DNA has been reported in buccal swabs from 19% of children under 6 years with no evidence of sexual abuse (Jenison *et al.*, 1990).

In one study of 1707 sera from individuals aged 1 to 95 years, anti-HPV-16 E4 antibodies were common (20%) in sera from children and teenagers, but not from adults (1.14%), which led the authors to conclude that HPV -16 infections may occur frequently in early life (Muller *et al.*, 1995).

Pao *et al.* (1992) detected genital HPV-DNA in vulval swabs from 9 of the 61 (15%) women who never had sexual intercourse. Sedlacek *et al.* (1989) documented the presence of HPV-DNA in the nasopharyngeal aspirates of neonates delivered to women that had HPV-DNA in their exfoliated cervical cells.

Roman & Fife (1986) demonstrated HPV-DNA in foreskins of infants that had undergone circumcision. When the type of HPV was analysed in these specimens, HPV 16 was demonstrated in 2 and HPV 6 in one. Unfortunately, the authors did not type the HPV DNA in maternal cervical cells and therefore contamination from other sources could not be excluded.

Smith *et al.* (1991) reported transmission of cancer associated HPVs in oropharyngeal of only 2 of 72 infants, a perinatal transmission rate of 2.8%.

However, the author may have failed to detect HPV-DNA in infant samples as ViraPap/ViraType kit was used, which is not as sensitive as the Southern blot used by Sedlacek *et al.* (1989).

One report of three year old children and their HIV infected mothers in Zaire revealed that while 10/81 children (12.3%) were positive for high risk HPV DNA, this did not correlate with HPV infection in the mothers (St Louis *et al.*, 1993). This ambiguity may again be explained by the insensitivity of the Virapap/Viratype kit used.

A summary of these studies is given in table 1.2

Table 1.2

Summary of evidence for the transmission of HPVs from mother to infants

Authors	No.infants positive /no. tested	Specimen	HPV types detected	Method
Sedlacek *et al*	11/23 (47.8%)	NP aspirate	Untyped	SB
Smith *et al.*	2/11 (18%)	OP cells	16/18 (1) 31/33/35 (1)	ISH
Roman & Fife	3/70 (4.3%)	Foreskin	16(2), 6(1)	SB

OP: Oropharyngeal aspirate

NP: Nasopharyngeal aspirate

1.5.3 Other modes of transmission

Other routes of infant infection with high risk HPVs may exist. Some viruses such as HIV may be transmitted *via* breast milk, but it seems unlikely that HPVs are present in breast milk since infections with these viruses do not have a viraemic phase.

Certain retroviruses can be transmitted vertically by infecting the gametes, and there is one report of HPV DNA sequences in Percoll-purified human sperm cells (Chan *et al.*, 1994): this seems unlikely given the high degree of tissue specificity of genital HPVs for keratinocytes.

Some HPV infections may occur in the absence of physical contact between individuals, possibly *via* minor abrasions. HPVs are stable to desiccation and heat (Smotkin, 1989) and may therefore be acquired from infected clothing. HPV-16 DNA has been isolated from surgical instruments and even smoke from the laser ablation of CIN lesions. Indeed, another study has shown that infectious bovine virions can be isolated from fomites and laser smoke (Sawchuck & Felten, 1989).

1.6 Rationale and aims of the thesis

It is apparent that in addition to sexual contact, HPVs may also be transmitted by other routes.

Perinatal transmission with HPV types 6 and 11 are well documented, and although HPV-16 is more prevalent among the general population (Soares *et al.*, 1990), the possibility that HPV type 16 and other cancer associated HPVs are transmitted perinatally has been ignored. Perhaps, one explanation could be that these genotypes are not associated with any identifiable lesions in infancy. Indeed, several authors have suggested that prospective studies are required to resolve this question (Ingram *et al.*, 1992; Schneider & Koutsky 1992; Smith *et al.*, 1991).

Some authors have investigated perinatal transmission with HPVs, but have used methods not as sensitive as the new DNA amplification techniques.

No previous study has i) used the PCR to demonstrate perinatal transmission and ii) followed up infants to two years to demonstrate persistence of HPVs.

It is the aim of this study to determine:

(i) The frequency of perinatal transmission of the cancer associated HPVs

(ii) Factors that may influence transmission.

(iii) The persistence of these viruses at six weeks, six months and two years (by Cason *et al.*) after birth.

Chapter 2:
The frequency of perinatal transmission of human papillomaviruses

Chapter 2 The frequency of perinatal transmission of human papillomaviruses

2.0 Objectives

i) To determine the frequency of perinatal transmission of human papillomaviruses using the polymerase chain reaction (PCR).

2.1 Background

Genital HPV infections are more common during pregnancy as a result of an increase in HPV replication (Schneider *et al.*, 1987; Czegledy *et al.*, 1989; Rando *et al.*, 1989) and show a high regression rate after delivery (Jablonska *et al.*, 1982; Garry & Jones, 1985; Rando *et al.*, 1989). Rando *et al.* (1989), reported that 52.5% of women had genital HPV-DNA detected during pregnancy when compared to 17.5% postnatally.

Studies using different hybridisation techniques have demonstrated HPV types 16, 18, 31, 33 or 35 in exfoliative cervical cells from between 2 and 36% of pregnant women (Table 2.1). The differences in the prevalence could be a result of differences in the population size, the sensitivities of the different assay techniques used, the period during pregnancy when samples were taken and the population studied.

Using Southern blots, Schneider *et al.* (1987) analysed cervical smears of 92 pregnant and 96 non-pregnant women matched by age for the presence of HPV-DNA. Twenty six (28%) of the pregnant women and 12 (12.5%) of the non-pregnant women were positive for HPV. HPV 16 accounted for 42% of all positive pregnant cases and 25% of non-pregnant cases, indicating that HPV 16 showed the most active replication in both groups. In addition, smears contained more than 10 pg viral DNA in 45 % of pregnant women

compared to 20% in the non-pregnant group. The difference in the quantities of viral DNA was not due to different amounts of cellular DNA, as the number of cells obtained in both groups were almost identical.

All the above authors have used methods which are not as sensitive as PCR (Guerrero *et al.*, 1992). Perhaps using the latter method, the prevalence of HPVs might be higher than previously reported. Albeit, HPVs are some of the most common sexually transmitted pathogens to occur in the genital tract during pregnancy and are capable of being transmitted perinatally.

There is currently much interest in developing vaccines against genital HPVs, especially those associated with squamous cell carcinomas (Cason *et al.*, 1993). However a complete understanding of the natural history of HPV infections is required before a successful immunisation strategy can be introduced. Indeed the routes of transmission of other viruses, such as hepatitis B and rubella were known before prevention by vaccination was contemplated (Department of Health, 1992).

This chapter describes a prospective study to investigate the frequency of transmission of HPVs types 6, 11, 16, 18, 31 and 33, from mother to infant. Maternal exfoliated genital cells (during pregnancy) and infant nasopharyngeal (NPA), buccal and genital swabs were analysed for HPV-DNA using the sensitive PCR.

Table 2.1

Detection of HPV types 16, 18, 31, 33, 35 in exfoliated cervical cells of pregnant women.

Authors	No HPV positive no. tested (%)	Hybridisation technique used for HPV detection
Fife *et al.* (1987)	15/234 (6.4%)	Dot, Southern, Reverse
Schneider *et al.* (1987)	26/92 (28%)	Southern
Czegledy *et al.*(1989)	35/101 (34.6%)	Filter *in situ*
Rando *et al.*(1989)	20/110 (18%)	Southern
Sedlacek *et al.* (1989)	16/45 (36%)	Southern
Hording *et al.* (1990)	101/1362 (7%)	Filter *in situ*
Peng *et al.* (1990)	1/45 (2.25%)	Southern
Soares *et al.* (1990)	44/748 (5.95)	ViraPap/Viratype kit
Smith *et al.* (1991)	13/72 (18.1%)	ViraPap/Viratype kit

2.2 Materials and methods

2.2.1 Study group

One hundred and twenty pregnant women attending antenatal clinics at St Thomas' were approached for this study. Informed consent was sought and obtained from 90 (75%) of these women for the collection of specimens from themselves and their infants. Complete mother infant data were present in 69 (77%) of the 90 women (Appendix 1).

In order to increase the probability of obtaining women with a genital HPV infection, women with a previous history of abnormal smears and genital warts were selectively recruited. Thirty one women (group A) aged 16-42 (mean 30.1) had a previous history of abnormal smears (cervical intraepithelial neoplasia [CIN] I-III) and/or genital warts: whilst 38 women (group B) aged 19-38 (mean 29.3) had no history of CIN or genital warts (Appendix 1).

Ethical approval for this study was obtained from the ethical committee of West Lambeth Health Authority.

2.2.2 Sample collection

Cell scrapes from the cervix and posterior vaginal fornix were collected from women with sterile swab between 20 and 38 weeks gestation. These samples were put into 5 mls of distilled water and stored at - 20^0 C until processed for HPV-DNA. Also any evidence of perianal, vulvar, vaginal or cervical condyloma was noted.

Nasopharyngeal aspirates (NPAs) were taken from infants following delivery of the head. Sterile swabs were taken from the mouth and from the shaft of the penis of male infants and labia of female infants 24 h post-delivery. Infants were also examined for clinical HPV infection. All samples

were put into 5 mls of distilled water and stored at -20°C until processed for HPV-DNA. The average length of storage was one week.

2.2.3 DNA extraction

The 5 mls samples were thawed, and then vortexed in order to dislodge the cells from the swab. Prior to DNA extraction epithelial cells of each sample were counted in a Neubauer counting chamber. A mean count of 5.2×10^5 cells were found for the pregnant group, 2×10^5 in buccal swabs and 10^5 in genital swabs of infants.

One ml of sample was placed into a 2 ml Eppendorf tube which was then centrifuged at 3000 revolutions per minute (rpm) for 10 min. The supernatant was discarded and the pellet resuspended in a solution of 200 μl of proteinase K buffer. The latter consisted of, 50 mM KCl, 2.5 mM Mg Cl_2, 10 mM Tris-HCl (pH 8.3), 0.45% NP-40 (Sigma, Poole, UK), 0.45% Tween 20 (Sigma, Poole, UK) and 60μg/ml proteinase K (Sigma, Poole, UK). Samples were incubated at 55^0 C for 4 h, and the protease was heat-inactivated at 95^0 C for 10 min. If a specimen contained blood, the cell pellet was rinsed with 1 ml of TE (10 mM Tris [pH 7.5] and 1 mM EDTA) to lyse red blood cells before the K buffer was added. Subsequently, 10 μl of the homogeneous cell suspension was used for PCR.

2.2.4 HPV-DNA amplification using the Polymerase chain Reaction

Amplification reactions were carried out using the heat-stable thermus acquaticus (Taq) polymerase (Advanced Biotechnologies Ltd.). Reactions contained aliquots of 10 μl of clinical sample in 100 μl of solutions containing 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris (pH 8.3), 200 μM of each dNTP (dATP, dGTP, dCTP, dTTP), 2.5 units Taq polymerase, and 50 pmoles of each

consensus primer MY 09 and MY 11 (Table 2.3). The mixture was overlaid with several drops of paraffin oil to prevent evaporation.

Each reaction was subjected to 40 amplification cycles in a DNA thermal cycler (Perkin-Elmer Cetus Instruments), using thermocycle step parameters of 94^0 C for 30 sec, 55^0 C for 45 sec and 72^0 C for 1 min. Prior to this amplification cycle, 5 min was included at 95^0 C for DNA denaturation. An additional 10 min was included at the final 72^0 C elongation cycle.

2.2.5 HPV detection using polyacrylamide gel

Finally, 18 μl of the amplification product were mixed with 2 ul of loading buffer (30% Ficoll, 0.25% Orange G., 250 mM EDTA) and analysed in 2% (w/v) agarose gels stained with 5 μl of ethidium bromide (10mg/ml). Amplicons were then visualised using ultraviolet light.

The Primers used (MY 09 & MY 11), were designed to amplify approximately 450-bp fragments of DNA from cancer associated and any other genital HPVs. The amplification product spans nucleotides 6722-7170 in HPV-6 and corresponding regions of other genital HPVs.

Control HPV DNAs used in amplifications were DNAs from cell lines containing HPVs (HPV-16: CaSki, or XH1; and HPV-18: HeLa). Water was used as an HPV-negative control.

2.2.6 Detection of different types of HPV using type-specific PCR

Samples determined to be HPV-DNA positive using the consensus primers were typed by PCR using HPV type specific primers as described by van den Brule *et al.*, (1990). Two PCR reactions were performed on each sample; one using primers for HPV types -6, -11, -16 and another using HPV type-18, -31 and -33 primers (Table 2.2). Reactions contained aliquots of 10 μl

of sample in 100 μl of solutions containing 50 mM KCL, 2 mM $MgCl_2$, 10 mM Tris (pH 8.3), 200 uM of each dNTP (dATP, dGTP, dCTP, dTTP), 2.5 units Taq polymerase, and 50 pmoles of each type-specific primers.

Each reaction was subjected to 40 amplification cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments), using thermocycle step parameters of 95^0 C for 30 sec, 55^0 C for 20 sec, and 72^0 C for 20 sec. Prior to this amplification cycle, 5 min was included at 95^0 C for DNA denaturation. An additional 10 min was included at the final 72^0 C elongation cycle.

Amplicons were analysed on a 4% (w/v) agarose gel and individual HPV types were identified by the characteristic molecular weight of the amplicon for each HPV type.

Samples that were HPV positive using consensus primers but negative by type-specific primers -6, -11, -16, -18, -31 and -33 were suspected to contain another of the 22 genital HPV types (HPV-X).

Table 2.2

Specification of oligonucleotides used as primers for HPV detection with PCR

Group specific primers (GP)	Sequence	Approx. size
MY09	-5 CGTCCMARRGGAWACTGATC 3′	450 bp
MY11	+5 GCMCAGGGWCATAAYAATGG3′	
Type specific primers (T-S. P)		
6.1	+5′TAGTGGGCCTATGGCTCGTC 3′	280 bp
6.2	-5′TCCATTAGCCTCCACGGGTG 3′	
11.1	+5′GGAATACATGCGCCATGTGG3′	360 bp
11.2	-5′CGAGCAGACGTCCGTCCTCG 3′	
16.1	+5′TGCTAGTGCTTATGCAGCAA 3′	152 bp
16.2	-5′ ATTTACTGCAACATTGGTAC 3′	
18.1	+5′ AAGGATGCTGCACCGGCTGA 3′	216 bp
18.2	-5′ CACGCACACGCTTGGCAGGT 3′	
31.1	+5′ ATGGTGATGTACACAACACC 3′	514 bp
31.2	-5′ GTAGTTGCAGGACAACTGAC 3′	
33.1	+5′ ATGATAGATGATGTAACGCC 3′	455 bp
33.2	-5′ GCACACTCCATGCGTATCAG 3′	

2.2.7 Beta globin PCR

To verify that sufficient DNA for PCR amplification was present in clinical samples, a beta-globin PCR (Saiki *et al.*, 1986) was also performed.

Samples determined to be HPV-DNA negative using the consensus primers were analysed. Reactions contained aliquots of 10 ul of clinical sample in 100 μl of solutions containing 50 mM KCL, 2 mM $MgCl_2$, 10 mM Tris (pH 8.3), 200 uM of each dNTP (dATP, dGTP, dCTP, dTTP), 2.5 units Taq polymerase, and 50 pmoles of beta globin primers PCO3 and PCO4 (Saiki *et al.*, 1985).

Each reaction was subjected to 40 amplification cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments), using thermocycle step parameters of 95^0 C for 40 sec, 55^0 C for 40 sec and 72^0 C for 50 sec. Prior to this amplification cycle, 5 min was included at 95^0 C for DNA denaturation. An additional 10 min was included at the final 72^0 C elongation cycle.

Amplicons were analysed on a 2% (w/v) agarose gel and were identified by the characteristic molecular weight.

A summary of the methods is shown in figure 2.1.

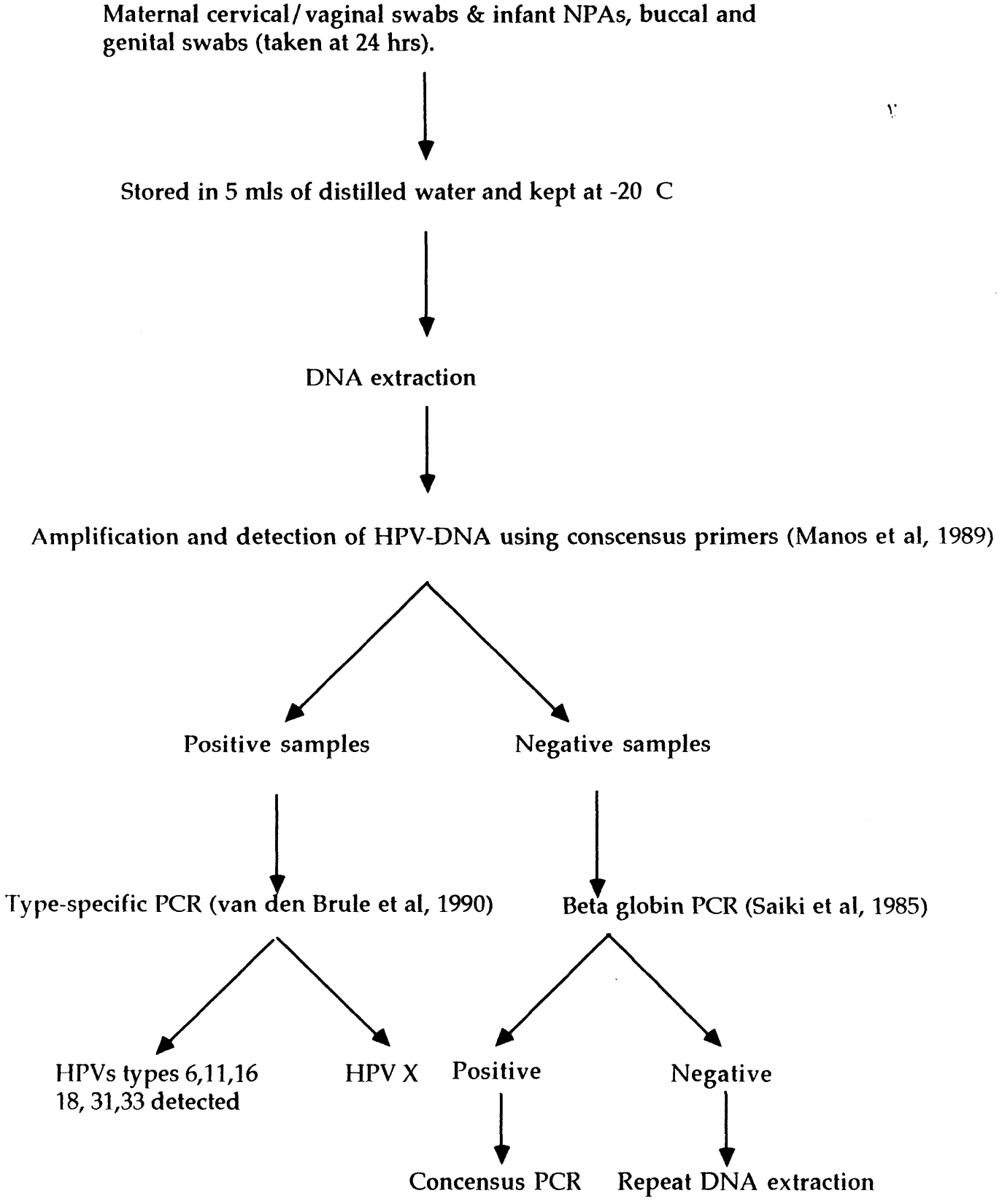

Figure 2.1

Summary of the study population and the methods employed in detecting HPV DNA.

2.2.8 Validation of the PCR

2.2.8.1 Sensitivity

Hela cell lines (contains 10 copies of HPV-18 DNA per genome) were used to test the sensitivity of consensus PCR. Ten serial dilutions of Hela were prepared (50,000 ng - 0.05 pg) and subsequently, 10 μl of each dilution sample was used for PCR using consensus primers. Amplicons were analysed in 2% (w/v) agarose gel stained with ethidium bromide. The lowest concentration of amplicon detected visually was noted.

2.2.8.2 Specificity

The specificity of PCR was determined using cell lines containing HPV 16 (Caski) or HPV 18 (Hela). Using type-specific primers, 10 μl of each of the cell lines were amplified and visualised as previously described. The sizes of the amplified products were noted.

The specificity was also determined by the absence of bands when type specific primers were used in reactions containing HPV of a different type- that is, HPV type 16 specific primers and DNA from Hela (HPV 18), HPV type 18 specific primers and DNA from Caski cells. Furthermore, type-specific primers were used in PCR using cell lines that did not contain HPV (A-413).

To further confirm the specificity of the PCR, Southern blot analysis of the amplified products with appropriate HPV probes labelled with phosphorus-32 were carried out.

2.2.8.3 Southern blot analysis of the type specific PCR product

The DNA from the amplification products of type-specific PCR was transferred from the gel to a nylon membrane (Biotrace, Gelman Sciences) by diffusion blotting in 0.5 N NaOH-1.5 M NaCl overnight. The membrane was

then saturated with 2 x SCC (SCC: [0.15 M NaCl + 0.015 M sodium citrate]). This was incubated for 2 h at 55^0 C in a prehybridisation solution (0.5 M sodium phosphate [pH 7.4], 7% sodium dodecyl sulphate, 1mM EDTA). Hybridisations were performed with HPV-16 specific ^{32}P end-labelled oligonucleotide (5- GCAAACCACCTATAGGGGAACACTGGGGCA -3') at 55^0 C for 16 h. Washes were then performed with 3 x SCC-0.5% sodium dodecyl sulphate at 55^0C. Autoradiography was performed overnight at - 80^0C.

2.3 Statistical Analysis of data

Advice was sought from Ms Kate Tilling (Statistician at St Thomas' Hospital).

A 95% confidence interval was calculated to compare proportions between 2 groups. Kappa statistic was calculated to analyse agreement between types of HPV detected in mothers and their infants.

2.4 Results

2.4.1 Prevalence and type of HPV-DNA detected in pregnant women

Using consensus primers (MY 09, MY 11), a single 450 bp fragment was amplified from the cervical/vaginal swabs of 37 (54%) of the 69 pregnant women indicating that HPV-DNA was present (Plate 2.1). Twenty (65%) of the 37 women were in group A, whilst 17 (45%) were in group B (Table 2.3).

Table 2.3

Prevalence of genital HPVs in pregnant women using consensus primers

	Group A (n=31)	Group B (n=38)	Groups A&B (n=69)
HPV positive	20	17	37
%	65	45	54
HPV negative	11	21	32
%	35	55	46

The 95% confidence interval for the difference in proportions between HPV positive women in groups A and B is (-0.03, 0.431, p=0.1013), indicating that there is no significant difference between the 2 groups.

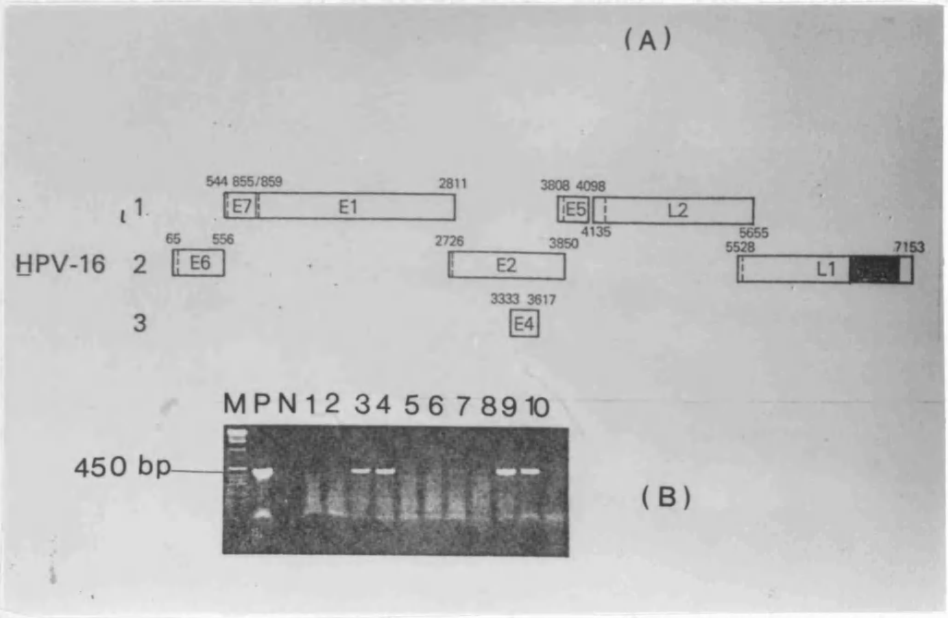

Plate 2.1

A) HPV-16 genome: the shaded area demonstrates the region where primers (MY 09 &MY 11) were designed to amplify.

B) PCR analysis of cervical scrapes DNA from 10 individual women. The 450 base pair fragment of amplified DNA was only present in samples containing HPV-DNA (lanes 3,4,9 and 10).

M; molecular weight ladder, P; positive control (Caski), N; negative control (water).

Using type-specific primers, it was possible to discriminate between several HPV types by different lengths of the PCR amplicon (Plate 2.2). Using this method, HPV 16 DNA was detected in 16 (43%) of the 37 positive samples: 8 (40%) in group A and 8 (47%) in group B (p= 0.666). The prevalence of the other HPV types is shown in Table 2.4.

Table 2.4

Distribution of HPV types detected in maternal genital cells.

	HPV types									
	6	11	16	18	6/11	11/16	16/18	16/33	31/33	X
Group A	0	0	8	2	0	1	3	1	1	4
Group B	0	1	8	0	1	1	2	0	0	4

Group A: women with a previous history of abnormal smears and/or genital warts.

Group B: women with no history of abnormal smears and/or genital warts.

HPV X: unknown HPV type.

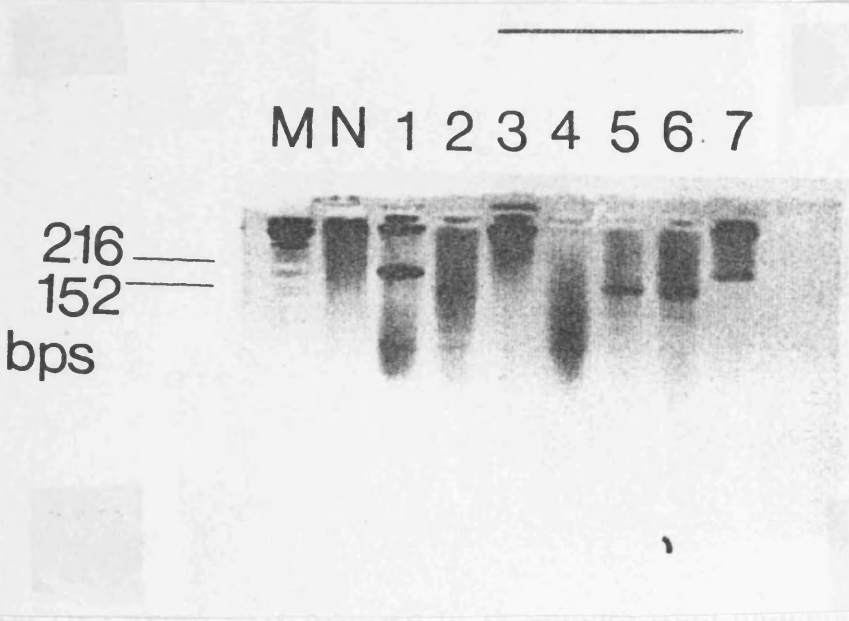

Plate 2.2

Detection of HPV genotypes in cervical/vaginal scrapes of 5 women (HPV DNA positive using consensus primers) by PCR using HPV -16 and -18 type-specific primers. Amplified bands of 152 and 216 base pairs indicate HPV types 16 and 18 respectively.

M; molecular weight ladder, N; negative control, lane 1: Hela cell line (HPV-18), lane 2: Caski cell line (HPV-16), lane 3 & 4: no amplicons corresponding to HPV - 16 or -18 (HPV X- see figure 2.1), lanes 5 & 6: women with HPV-16, lane 7: woman with HPV-18.

2.4.2 HPV-DNA detected in the buccal and genital swabs of infants at 24 h

At 24 h, 19 (27%) of 70 infants (one set of twins) had HPV-DNA at either a buccal and/or genital site. Seven infants had HPV-DNA in buccal samples alone, 6 had HPV-DNA in genital samples alone and 6 had HPV-DNA at both sites.

Sixteen (84%) of 19 HPV-DNA positive infants were delivered to mothers (one set of twins) who were also HPV-DNA positive; 3 (16%) were delivered to 3 mothers that were HPV-DNA negative [(** 95% CI 0.11, 0.54, p=0.002) table 2.5].

Of the 19 positive infants, HPV type 16 was detected in 12 (63%), type 18 in 3 (16%), dual infection with type 16/18 in 3 (16%) and type 11 in 1 (5%). The distribution of the different HPV types between buccal and genital sites is demonstrated in table 2.6.

Identical HPV types were detected in 14 samples from mothers and their infants, including dual HPV infections in 3 mother/infant pairs [(Kappa=0.79) (Table 2.8)].

Beta-globin was detected by PCR in all samples that were HPV-DNA negative, indicating that these samples contained sufficient DNA for analysis.

Appendix 2, gives the data on each mother and infant pair at birth.

** This is 95% CI for difference in proportion of HPV DNA positive mothers having HPV DNA positive babies and HPV DNA negative mothers having HPV DNA positive babies.

Table 2.5

Prevalence of HPV-DNA in maternal genital samples and in buccal and genital samples of their infants.

	Mother (n=69)	
	HPV DNA pos. (n=37)	HPV DNA neg. (n=32)
Infant (n=70)		
HPV DNA +	16*	3
HPV DNA -	22	29

* Set of twins

The proportion of infants of HPV positive mothers who are themselves positive (π_1) to the proportion of infants of negative mothers who are themselves negative (π_2).

$\pi_1 = 16/38$ $\pi_2 = 3/32$

The 95% confidence interval for $\pi_1 - \pi_1$ is (0.11, 0.54).

This implies that there is a significant association between the HPV status of the mother and the infant (p=0.002).

Table 2.6

Distribution of HPV types in buccal and genital swabs of infants (n=19) delivered.

	6	11	6/11	16	18	16/18	31/33	X
i) Buccal	0	0	0	6 (1)	1	0	0	0
ii) Genital	0	1	0	3 (2)	2	0	0	0
iii) Buccal & genital	0	0	0	3	0	3	0	0

Number in brackets imply the number of infants delivered to HPV negative mothers.

Table 2.7

Concordance of HPV genotypes detected in maternal genital samples and their infants' buccal and genital samples.

	Child				
	11	16	16/18	18	Total
Mother					
11	**1**	0	0	0	1
16	0	**9**	0	0	9
16/18	0	0	**3**	2	5
18	0	0	0	**1**	1
Total	1	9	3	3	16

Kappa statistics used to test for agreement between the mother's and infant's virus types.

Kappa= 0.79 (good agreement) [A maximum of 1.00 when agreement is perfect and a value of zero indicates no agreement better than chance]

2.4.3 HPV detected in the NPAs of infants at 24 h

Three of the 30 (10%) infants were positive for HPV-DNA. Using type specific primers HPV type -16 was detected in all 3 samples (Table 2.8).

Table 2.8

Concordance of HPV genotypes detected in maternal genital samples and their infants' nasopharyngeal aspirates.

Mother	Infants	
	NPA	Buccal swab at 24 hours
16/18	16	ND
16	16	ND
18	16	18

16: HPV-16 DNA detected
18: HPV-18 DNA detected
ND: Not detected.

2.4.4 Validity of PCR

2.4.4.1 Sensitivity of consensus PCR

As little as 5 pg of DNA from Hela could be detected (Plate 2.3). As Hela cells contain 10 copies of HPV 18 DNA, this implies that the PCR can detect around 10 copies of HPV- DNA.

2.4.4.2 Specificity

The size of the amplified products were as predicted from the design of the type-specific primers (Plate 2.4), that is 152 bp for HPV 16 (Caski) and 216 bp for HPV 18 (Hela cells).

There were no bands visible when type-specific primers were used in PCR reactions with DNA isolated from cell lines with a different known HPV type and with cell lines that did not contain HPV (A 431- human squamous carcinoma cell line with no HPV, Plate 2.5).

Confirmation of HPV type by southern blot using an HPV-16 specific oligonucleotide probe indicated that PCR alone was specific and sensitive enough to detect all HPV-DNA present, as no further positive samples were detected (Plate 2.6).

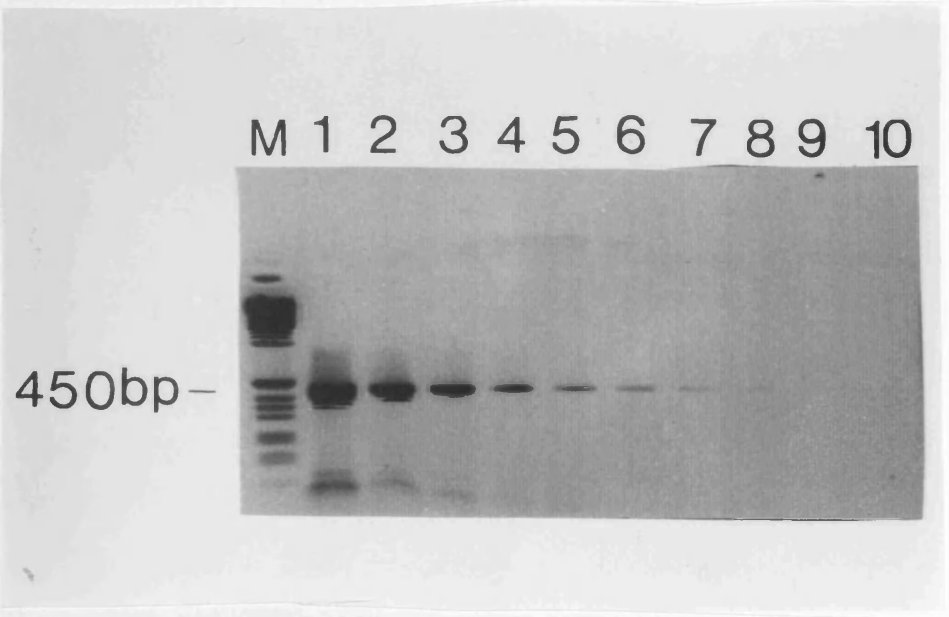

Plate 2.3

Sensitivity of PCR after 40 cycles of amplification with consensus primers and different concentration of DNA from Hela cells (50,000 ng - 0.05 pg).

Hela cell lines (contains 10 copies of HPV-18 DNA per genome) were used to test the sensitivity of consensus PCR. Ten serial dilutions (lanes 1 to 10) of Hela were prepared. Amplicons were analysed in 2% (w/v) agarose gel stained with ethidium bromide. The lowest concentration of amplicon detected visually was noted (lane 8, 5 pg)- corresponding to 10 copies of HPV.

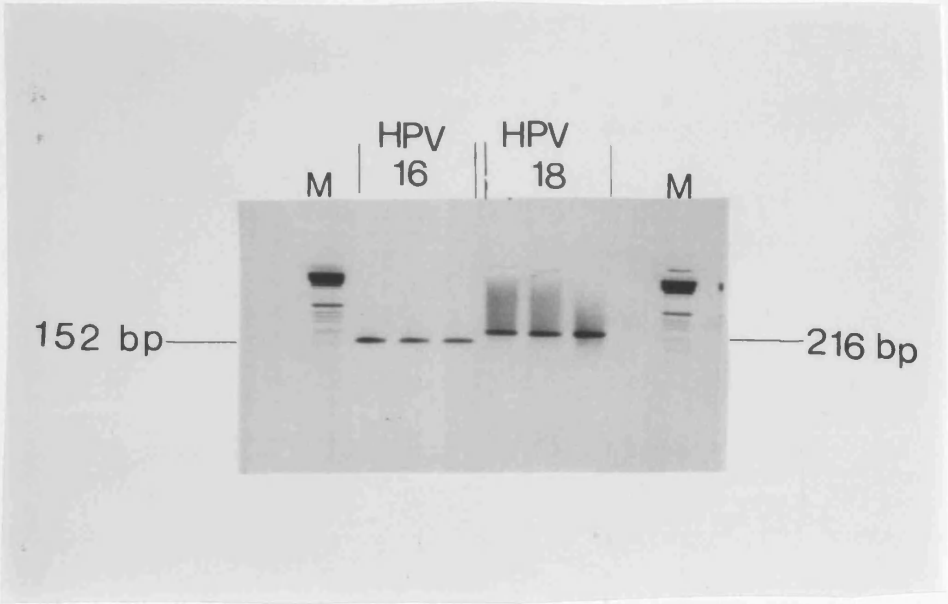

Plate 2.4

Demonstration of the specificity of PCR technique using primers for HPV-16 and HPV-18 to amplify Caski and Hela cell lines respectively. The size of the amplified products were as predicted from the design of the primers- that is 152 for Caski cells and HPV 16 primers (lanes 1,2 and 3) and 216 for Hela cells and HPV 18 primers (lanes 4, 5 and 6).

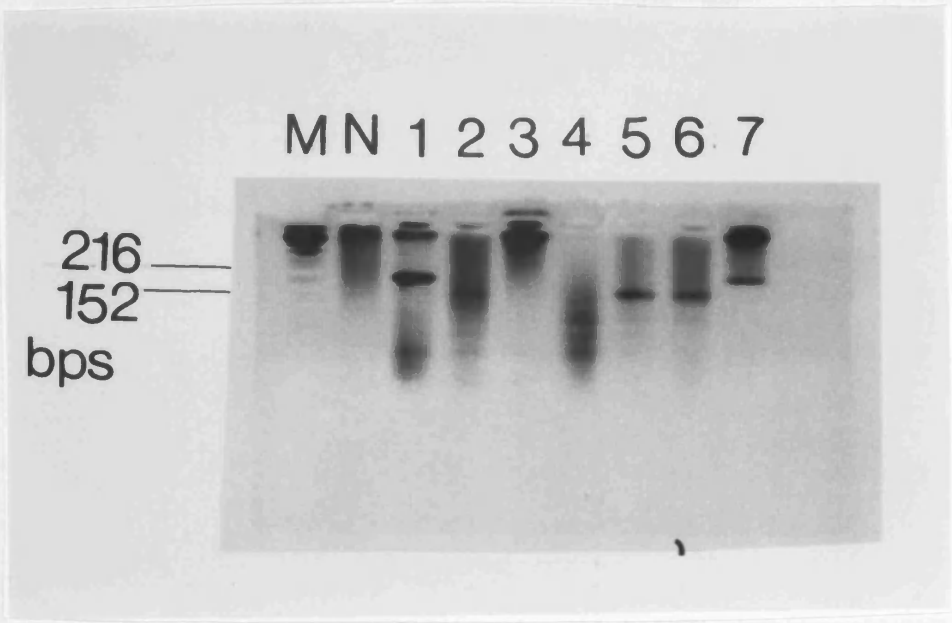

Plate 2.5

Specificity of the HPV 16 and 18 primers. Using HPV-16 type specific primers the 152 bp fragment of amplified DNA was only present in samples that contained HPV 16 (Caski: lanes 2, 5 and 6). No amplified product was detectable in lanes 3 (Hela) and 4 (A-413). Similarly using HPV-18 specific primers, the 216 bp fragment of DNA was only present in samples that contained HPV18 (Hela: lanes 1 and 7).

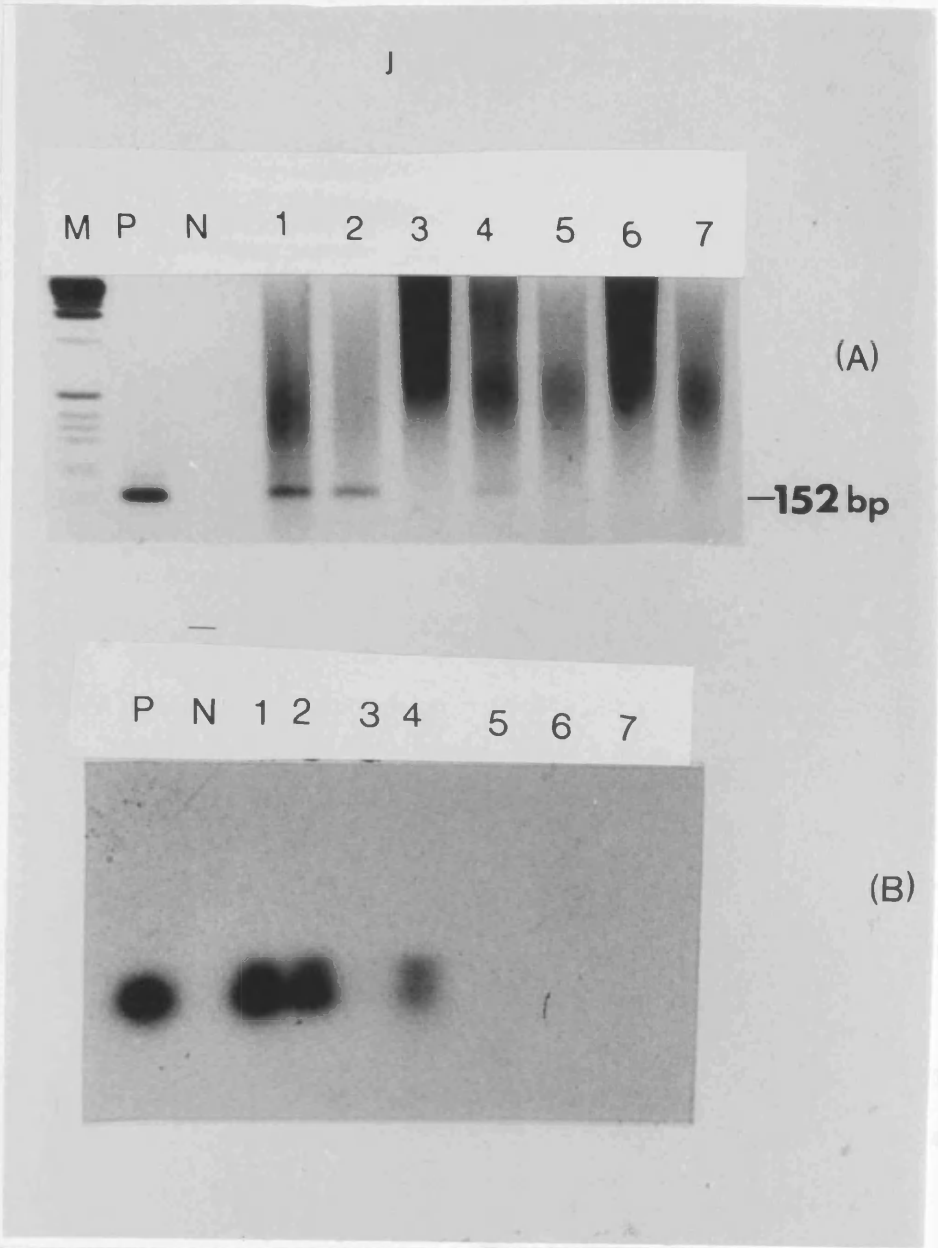

Plate 2.6

A) Amplification of DNA from maternal genital samples using HPV-16 type specific primers. M: marker; P: Caski cell line; N: distilled water; lanes 1, 2 and 4: HPV-16 positive samples.

B) Southern blot of amplified PCR products from gel confirming specificity for HPV type 16 DNA. This confirms that the PCR used is sensitive enough to detect all HPV-DNA present as no further positive samples were detected when an HPV-16 specific oligonucleotide probe was used.

2.5 Discussion

2.5.1 Prevalence of genital HPVs in pregnant women

Several previous authors have commented about the high prevalence of genital HPVs in pregnant women and using hybridisation techniques the maximum rate has been quoted as 36% (Sedlacek *et al.*, 1989).

In the current study, 37 (54%) of 69 pregnant women had detectable genital HPVs. This higher detection rate compared to previous studies is not surprising for two reasons. Firstly, the more sensitive PCR technique was used and secondly a high risk population were specifically selected for this study.

Using PCR, Fredericks *et al.*, (1993) also detected genital HPVs in 11 (37%) of 30 women six weeks after delivery. The difference in the detection rates between the latter author and our study probably reflect the period at which genital samples were acquired, as genital HPVs show a high regression rate after delivery (Rando *et al.*, 1989).

It is therefore apparent that detection rate of genital HPVs varies from one study to the other according to i) the techniques employed, ii) the period at which samples were acquired and iii) the population studied.

Albeit, pregnancy seems to be a period of transient immunosuppression and is a risk factor for viral infection or to an increased replication of persisting virus (La Vecchia *et al.*, 1986a). Similarly, renal transplant recipients treated with immunosuppressives have an increased incidence of genital HPV infection and subsequent genital neoplasia (Halpert *et al.*, 1986).

The prevalence of genital HPV infection appears to vary in different age groups. Using filter *in situ* hybridisation De Villiers *et al.* (1987) reported that the highest HPV rate was in the age group 20-25 years (13%) with a steady decline thereafter. In another study, HPV detection rate was 11% in the 15-54

age group compared to 3.2% in women older than 54 years (De Villiers, 1992). The mean age of women in the current study was 29 years, whilst the HPV detection rate was 54%. This higher rate compared to the studies conducted by De Villiers *et al.*, (1987, 1992) may be explained in two ways. Firstly, we used PCR, a more sensitive technique than that used by De Villiers. Secondly our study population consisted of pregnant women with a previous history of genital warts and/or CIN.

Despite the increased sensitivity provided by PCR amplification methods, sampling errors will continue to be a problem. This was highlighted in 2 previous studies (Pao *et al.*, 1990, Schneider *et al.*, 1992). When PCR was used to detect the prevalence of HPV in cervicovaginal cells of women with normal cervical cytology and in women with CIN, HPV-DNA was detected in 43 (42.2%) of the 102 normal individuals and in all 12 CIN patients ((Pao *et al.*, 1990). Five days later, an additional 9 positive results were detected from individuals who were initially HPV negative. Conversely, 26 (24.3%) of 107 HPV positive individuals were HPV-negative when sampled a second time. In another study, cervical smears of 21 cytologically negative women were reviewed monthly with HPV 16 type-specific primers and 28.6% were positive at the first visit compared with 66.7% who were ever positive (Schneider *et al.*, 1992). Thus, single point detection rates of cervical HPV infections may be insufficient for assessing the true prevalence of disease.

In the current study, samples were only obtained once antenatally. Whilst it would have been valuable to have repeated this to check for variations in the prevalence of HPVs, due to anxiety expressed by some pregnant women this was not carried out. The commonest concerns were that a repeat swab may provoke pre-term labour or per vaginal bleeding. Albeit, as far as possible swabs were taken post-natally (Chapter 4).

HPV-16 was detected in 8 (40%) of 20 and 8 (47%) of 17 HPV positive

women in groups A and B respectively. Two previous studies have also reported a higher prevalence of HPV -16 in pregnant women (Schneider *et al.*, 1987; Hording *et al.*, 1990). Schneider *et al.* (1987) reported that 42.3% of pregnant women had detectable HPV 16 in their genital tracts.

Eight (22%) samples that were HPV positive using consensus primers but negative by type-specific primers -6, -11, -16, -18, -31 and -33 were suspected to contain another of the 22 genital HPV types (HPV-X).

2.5.2 HPV detected in the buccal and genital sites of infants at 24 h

Sixteen (one set of twins) infants born to 37 mothers were HPV positive at 24 h at either their buccal or genital sites, a detection rate of 43%.

Three infants were delivered to HPV negative mothers. This can be due to at least three possible reasons: 1) failure in detecting HPV-DNA in mothers as it is well recognised that women who are HPV-negative on the first examination may prove to be HPV-positive (Pao *et al.*, 1990, Schneider *et al.*, 1992) 2) infants may have acquired HPV infection within 24 h postnatally from a variety of sources including breast milk, from siblings, *via* kissing or even by exposure to contaminated fomites (Bergeron *et al.*, 1990), 3) HPV transmission from the mother well in advance of the delivery (e.g., transplacental route).

Identical HPV types were detected in samples from 14 mothers and their infants, including dual HPV infections in three mother-infant pairs. Thus HPV types -16 and -18 may frequently be transmitted from mother to infant.

Patients (group A) were selected to obtain a high prevalence of genital HPV infection in order to demonstrate perinatal transmission and may not be representative of the general population. Despite this, it is noteworthy that women (group B) without a history of genital warts and/or cervical abnormalities had a high prevalence of genital HPV and were also capable of

perinatal transmission.

Recently, a report by Favre *et al.*, (1998), adds to the debate regarding HPV transmission. Epidermodysplasia verruciformis (EV) is a rare skin disease, characterized by an abnormal genetic susceptibility to infection by a group of related HPVs. The presence of the same EV types in skin lesions, cervical scrapes, amniotic fluid and placenta despite their absence from maternal peripheral blood in a patient with EV provides evidence consistent with vertical transmission. However, the approach used identifies only viral DNA and does not demonstrate viral infection.

Equally, in the current chapter, it may be argued that HPV DNA and not infection has been demonstrated.

Two recent studies also demonstrated a high perinatal transmission of HPVs (Puranen *et al.*, 1998, Tseng *et al.*, 1998). In the latter study, the overall frequency of HPV transmission from HPV 16/18 positive mothers to the infants was 40 per cent.

Infection with viruses such as rubella during the prenatal and neonatal period can lead to immunological tolerance (Mims, 1987). Whether acquisition of HPV or some form of immunological tolerance to HPV during the perinatal period predisposes to an increased risk of cervical intraepithelial neoplasia in later life deserves consideration.

The perinatal acquisition of HPVs has implications for the introduction of prophylactic vaccines, especially at a time when a high prevalence is detected amongst the pregnant population. Hepatitis-B vaccines are of value in the prevention of the HBs Ag carrier state in the newborn infants of mothers who are chronic carriers (Wong *et al.*, 1984).

2.5.3 HPV detected in the nasopharyngeal aspirates (NPA) of infants at delivery

It was assumed that by collecting NPAs immediately following delivery of the head, it would be less likely that samples would become contaminated.

HPV-DNA was demonstrated in NPAs of 3 (10%) of 30 infants born to HPV-DNA positive mothers: HPV-16 was detected in all 3 positive NPAs. Subsequent buccal swabs taken at 24h, demonstrated that HPV had persisted in only one of the three infants. In this case, the HPV type detected was concordant with the maternal HPV type but different to the NPA (Table 2.6). The mother may have harboured dual infection i.e. HPV types 16 and 18 but only one type was detected antenatally (Mc Cance *et al.* 1985).

In another small prospective study, Sedlacek *et al.*, (1989) also demonstrated that HPVs were frequently transmitted at birth, as HPV-DNA was detected by southern blot in NPAs from 11/23 infants (48%) born to HPV-DNA positive mothers. However, the latter author only typed HPV-DNA in maternal genital samples and not that found in infants and therefore could not demonstrate concordance.

2.5.4 General discussion

Three different laboratories were used to analyse samples. One laboratory was used specifically for the extraction of DNA from clinical samples. Samples were separated from one another and covered with a sterile gauze when opening and closing tubes, hence minimizing possible aerosol contamination. Disposable plugged pipette tips were used throughout and gloves were frequently changed during sample preparation. A second laboratory was used to prepare PCR reagents. The addition of the clinical samples to the PCR reagents were carried out in a third separate laboratory. Appropriate negative control (distilled water:1 per 5 clinical samples)

amplicons were interspersed amongst the clinical amplicons. To minimize sample-to-sample contamination only a maximum of 5 clinical samples were analysed at each step.

Consensus primers described by Manos *et al.*, (1989) were used as they have the ability to amplify a wide range of HPV genotypes. The most effective consensus regions are located within the highly conserved regions of the L1 ORFs of HPVs (De Villiers, 1989). Methods employed in this chapter may be comparable to larger studies that have used consensus and type-specific primers (Aznar *et al.*, 1993; Kuypers *et al.* 1993; Munoz et al. 1992).

The specificity of the PCR was confirmed by a) detection of an amplified band consistent in size with that predicted from the design of the oligonucleotide primers, b) the absence of amplification of HPV from cells containing another HPV type or just cellular DNA, c) southern blotting using HPV 16 specific oligonucleotide probes.

The sensitivity of the consensus PCR was about 10 genome copies of HPV in a given sample: this sensitivity is similar to previous findings (van den Brule *et al.* 1989; Snijders *et al.* 1990).

Although it is probable that HPV DNA was acquired during passage through an infected birth canal, transmission *in utero* or postnatally cannot be excluded. These infants have been reviewed at six weeks and six months to determine whether persistence of HPV DNA remains.

Chapter 3

Perinatal papillomavirus infection: a study to determine whether intact amniotic membranes protect against prenatal infection.

Chapter 3 Perinatal papillomavirus infection: a study to determine whether intact amniotic membranes protect against prenatal infection.

3.0 Objective:

To determine whether the amniotic membrane protects the fetus in women with genital HPV infections.

3.1 Background

In the previous chapter, 16 (43%) infants delivered vaginally to 37 HPV positive mothers were HPV positive. However, the relative contribution of intrapartum and intrauterine routes of transmission was not determined.

Although it is likely that perinatal acquisition of HPVs occurs through an infected birth canal, one case of juvenile laryngeal papillomatosis has been reported following an elective caesarean section, suggesting that infection may also be acquired *in utero* (Shah *et al.*, 1986).

To address this issue, amniotic fluids from women with an intact amniotic sac and cervical/vaginal swabs from pregnant women were analysed for HPV-DNA using the PCR. Buccal and genital swabs from those infants born to HPV-DNA positive mothers were also assessed.

3.2 Method and Materials

3.2.1 Study group

Twenty five consenting pregnant women undergoing elective caesarean section or amniocentesis were recruited (Appendix 3) . The women were between 19 and 41 years of age (mean 28.7). Twenty one had an elective

caesarean section and 4 had amniocentesis for karyotyping at 15 weeks gestation (3 for maternal age and 1 for choroid plexus cysts on ultra sound).

Eight further amniotic samples were collected and analysed by Dr. R Nath (Appendix 3 B). In this group, the mean age was 33.8 years and there was no previous history of vaginal warts and/or CIN.

Ethical approval for this study was obtained from the Ethics Committee of West Lambeth Health Authority.

3.2.2 Sample collection

Cell scrapes from the cervix and posterior vaginal fornix were collected with sterile cotton swabs.

One ml of amniotic fluid was obtained during amniocentesis.

At elective caesarean section, a 1-2 cm transverse uterine incision was made in the lower uterine segment above the reflected bladder through the myometrium. The myometrium was split digitally making sure that the amniotic sac remained intact. One ml of amniotic fluid was collected by passing a sterile 21 gauge needle directly into the amniotic sac. Samples were discarded in cases where the amniotic sac had ruptured prior to collection, thereby avoiding contamination.

Clinically, there was no evidence of premature rupture of the amniotic sac and hence no risk of ascending genital infection. Swabs were taken from the mouth and from the shaft of the penis of the male infants and the labia of the female infants 24 hours after delivery as described previously. The samples were placed in 5 mls distilled water and stored at - 20^0 C until processed for HPV-DNA.

3.2.3 HPV-DNA amplification and typing using the PCR

DNA was extracted from samples as previously described. Following

DNA extraction, 10 μl of the homogeneous cell suspension was used for PCR. The first 25 samples were initially tested for HPV DNA by PCR using consensus primers (Manos *et al.*, 1989). Samples determined to be HPV-DNA positive by PCR were typed using HPV type 6, 11, 16 and 18 specific primers (van den Brule *et al.*, 1990). To verify that each sample contained sufficient DNA for PCR amplification, a beta-globin PCR reaction was also performed (Saiki *et al.*, 1986). All standard precautions were taken to prevent and detect contamination of PCR reactions (Muir *et al.*, 1993).

In the remaining eight samples, nested PCRs using *Taq* polymerase were used to amplify HPV DNA (Kaye *et al.*, 1996)

3.3 Results

A single 450 bp fragment was amplified from the cervical/vaginal swabs of 4 of the 25 of women indicating that HPV-DNA was present, but amniotic fluids from all 25 women were HPV-DNA negative. Using type-specific primers; HPV -16 was demonstrated in 3 and HPV -18 in 1 cervical/vaginal swabs.

The buccal and genital swabs of the 4 infants born to the HPV positive mothers were negative for HPV-DNA.

Beta globin was detected by PCR in all samples that were HPV-DNA negative, indicating that these samples contained sufficient DNA for analysis and that there were no inhibitors of PCR in amniotic fluid (Plate 3.1).

Using the nested PCR, six of the eight women had HPV DNA in their cervical swabs. However, the amniotic fluid was negative for HPV DNA in the 8 samples.

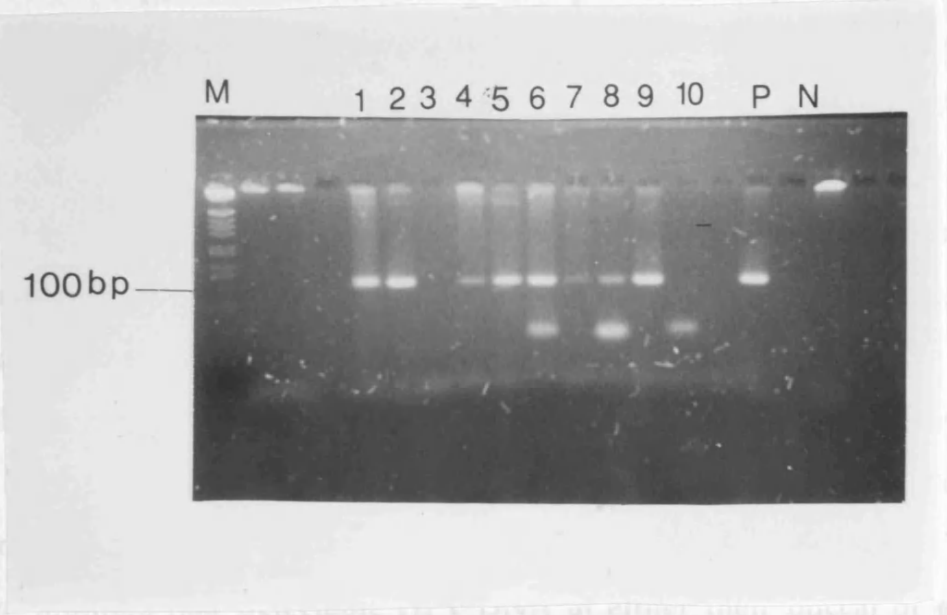

Plate 3.1

PCR products after gel electrophoresis and ethidium staining using beta-globin primers PCO3 and PCO4 as an internal reaction control. The amplicons (100 bp) demonstrate that enough DNA was present for HPV amplification lanes: 4, 5, 7, 8, 9, 10, 11 and 12. M: marker; lane 16: distilled water; lane 15: Caski cell line.

3.4 Discussion

In chapter 2, 37 (54%) of 69 pregnant women had detectable genital HPVs. In the current study, only 10 (30%) of the 33 women attending amniocentesis and elective caesarean section had detectable genital HPVs. The lower detection rate in the latter group may be the result of a fewer number of women with a previous cervical abnormality. Also, despite the increased sensitivity provided by PCR amplification methods, sampling errors will continue to be a problem. This was highlighted in 2 previous studies (Pao *et al.*, 1990, Schneider *et al.*, 1992).

Although it may be argued that two different types of PCRs were used in the current study, the sensitivities (10 genome copies for the consensus and five for the nested PCR) were similar.

Although HPV-DNA was not detected in the buccal and genital swabs of the 4 infants born by elective caesarean section to HPV-DNA positive mothers, previously 16 (43%) infants delivered vaginally to 37 HPV-DNA positive mothers had detectable HPV-DNA at either their buccal or genital sites (Chapter 2).

Two previous studies have commented about HPVs and amniotic fluid (Sedlacek *et al.*, 1989; Smith *et al.*, 1991). There are however serious flaws in both studies.

Using hybridisation techniques, HPV-DNA was detected in amniotic fluids of women following artificial rupture of membranes (Sedlacek *et al.* 1989). However, as amniotic fluid was collected *via* the birth canal, it is likely to have been contaminated by vaginal secretions. In the same study, 4 women with intact membranes who had caesarean sections were found to have no detectable HPV in the amniotic fluid, although one woman was positive in her cervical/vaginal swab.

Smith *et al.*, (1991) reported an infant delivered by caesarean section who

had demonstrable buccal HPV infection. However, the indication for delivery by caesarean section was chorioamnionitis, and it is likely that this patient had amniotic leakage prior to delivery.

There has also been a single case report in which an infant delivered to a mother with genital warts had perianal warts at delivery (Tang *et al.*, 1978). This was in an infant delivered following rupture of membranes for 24 hours prior to delivery. Although the author in this case implied that HPV infection in the neonate was by transplacental haemotogenous route, this is unlikely. The interval between exposure to HPVs and development of genital warts requires a longer incubation period (Oriel, 1972) and as HPV infection does not extend below the *stratum basale* there is no viraemic phase. An ascending infection through an unperceived tear in the amniotic membrane is the most likely explanation.

All amniotic fluids in our study were collected transabdominally as described in the methods, thus minimising contamination. As HPVs may also be acquired *via* contaminated fomites (Bergeron *et al.*,1990) and contamination from the abdominal drapes, patients were excluded if the amniotic sac had ruptured prior to sample collection.

It would be valuable to select a larger cohort of genital HPV positive women antenatally and then collect amniotic fluids at elective caesarean section in order to confirm these initial findings. This is obviously labour intensive for 2 reasons. Firstly, a large number of HPV positive patients would have to be recruited. This problem can be overcome by specifically selecting women that have had abnormal smears and or genital warts (Chapter 2). Secondly, only 15% (caesarean section rate at St Thomas') of the selected genital HPV positive women will have an elective caesarean section and are eligible for amniotic fluid collection.

Nevertheless, these findings suggest that whilst HPV-DNA is detectable in the genital tract of pregnant women and may be acquired by infants at delivery, there is no evidence of intrauterine infection as long as there is no rupture of membranes.

Chapter 4

Persistence of human papillomaviruses at six weeks and six months.

Chapter 4 Persistence of human papillomaviruses at six weeks and six months.

4.0 Objectives

i) To determine whether perinatally acquired HPVs persists to 6 weeks and 6 months.

ii) To evaluate the prevalence of HPV-DNA in the genital tract of women in the postpartum period.

iii) To assess maternal breast milk and buccal swabs for HPV-DNA: demonstrating other routes of acquisition by infants.

4.1 Background

In a recent study using PCR, genital samples from women and buccal swabs from their infants (only obtained 6 weeks postpartum) were analysed for HPV-DNA (Fredericks *et al.* 1993). Eight HPV positive infants were delivered to 11 HPV positive mothers, a transmission rate of 73%. The latter author then concluded that HPV acquired from mothers had persisted for at least 6 weeks, even though samples were not collected at birth.

In this chapter, infants previously seen at 24 h were reviewed again at 6 weeks and 6 months. As before, samples were obtained from mothers and infants and analysed for HPV-DNA using PCR.

The detection rate of maternal genital HPV infection six weeks post partum was also compared to the antenatal period, as it is recognised that HPVs show a high regression rate after delivery.

As (Chapter 2) 3 HPV positive infants were delivered to 3 genital HPV negative mothers, other routes of acquisition of HPVs by infants were sought.

4.2 Materials and methods

4.2.1 Study group & sample collection

Of the 70 infants previously seen at 24h, 49 (70%) and 19 (27%) attended for follow up at six weeks and at six months respectively (Appendix 4).

On both occasions, sterile swabs were taken from the mouth and from the shaft of the penis of the male infants and the labia and mons pubis of the female infants. Infants were also examined for HPV infection in buccal mucosa and genitals.

Sterile swabs from the cervix and posterior vaginal fornix were also taken from 31/48 (65%) mothers at six weeks and analysed for HPV-DNA. Some women already had cervical smears taken by their General Practitioner and felt a repeat examination was unnecessary. Others had started menstruating, whilst some were reluctant to have a speculum examination so soon following delivery.

Mothers (n=15) who were breast feeding at six weeks, were asked to provide a sample. Sterile universal containers were given to individual women and samples collected. Buccal swabs were taken from the same women (Figure 4).

All samples (maternal and infant) were put separately into 5 mls of distilled water in sterile universal containers and stored at - 20^0 C until processed for HPV-DNA using PCR.

4.2.2 Methods

4.2.2.1 Consensus and type-specific PCR.

The DNA was extracted as previously described in page 62. All samples were tested for HPV-DNA by PCR using general primers (Manos *et al.*, 1989).

Samples found positive using the consensus primers were then typed for HPVs 6, 11, 16, 18, 31 and 33 using type specific primers (van den Brule *et al.*, 1990). To confirm that sufficient DNA was present for amplification a B- globin was carried out on all samples (Saiki *et al.*, 1986). All standard precautions were taken to prevent and detect contamination of PCR reaction (Muir *et al.*, 1993).

4.3 Results

4.3.1 HPV DNA status of infants after 6 weeks

Of the 16 infants who were HPV positive (delivered to HPV positive women) at 24 h, 7 (44%) remained HPV DNA positive (all 7 had HPV DNA at buccal site). Five infants who were HPV DNA positive at 24 h remained HPV-16 positive; 1 who was HPV-18 DNA positive remained HPV-18 positive and 1 who was HPV 16/18 positive was now HPV 16 positive. That is, 7 (19%) infants born to 37 HPV positive mothers had HPV infection which persisted at 6 weeks (appendix 4).

Four infants (not previously HPV positive at birth) acquired HPV positivity from an undetermined source. Three who were HPV-16 positive at six weeks were HPV-DNA negative at birth. However, the latter infants were delivered to mothers that harboured HPV-DNA in their genital tracts. The other infant, who was HPV negative at birth and born to an HPV negative mother, had HPV -18 DNA detectable at 6 weeks .

Figure 4.1
Summary of the study population and sample collection.

4.3.2 HPV DNA status of infants after 6 months

Samples were only available from 19 infants, 9 of which had been HPV-DNA positive at birth. At 6 months, all samples were HPV-DNA negative.

The concordance and type of HPV-DNA detected in the genital tract of mothers (antenatally) and their infants at birth, six weeks and six months is shown in appendix 4. Those infants not shown in the table were HPV-DNA negative at six weeks and six months.

4.3.3 HPV DNA status of mothers at six weeks

Compared to the antenatal period (37/69: 54%), only 12/31 (39%) of the women had detectable genital HPV-DNA. This comparison was not significant ($p>0.5$).

All 15 maternal breast milk samples and buccal swabs were HPV-DNA negative. Five (33%) of these women had detectable HPV-DNA in their genital tract: 3 had HPV-16, whilst 2 had HPV-X. One infant born to an HPV positive mother had detectable HPV-DNA at her buccal site at 6 weeks.

Beta-globin was detected by PCR in all samples that were HPV-DNA negative indicating that these samples contained sufficient DNA for analysis.

4.4 Discussion

HPV-DNA was transmitted to 46% of infants at 24 h born to mothers infected with the same genital HPV type (Chapter 2). Six weeks later, 7/37 (19%) of infants born to HPV positive mothers had HPV infections. At 6 month, none of the infants had detectable HPV-DNA at either their external genitalia or buccal sites.

Four infants that were HPV negative at 24 h, were HPV positive at six weeks. These infants may have been infected from another source such as contaminated fomites, clothing (Bergeron *et al* 1990), kisses or breast milk. The latter two situations are unlikely as maternal buccal swabs and breast milks were all negative for HPV-DNA. Alternatively, these 4 anomalous observations may be explained by the commonly experienced problem of sampling (Schneider *et al.* 1992), which are inevitable since HPV infections are frequently multifocal (McCance *et al.* 1985).

At six weeks, the detection of HPV DNA in samples from infants does not imply infection. Indeed, the absence of HPV DNA at six months favours contamination rather than infection. Alternatively, the virus was persisting at internal sites which was ethically unacceptable to sample.

Intrapartum transmission of HPV and infection is suggested by the association between genital warts in the mother and the development of laryngeal papillomas in the child during the first 2 years of life (Quick *et al.*, 1980). The importance of vaginal contact is highlighted by observations that laryngeal papillomatosis is almost always associated with vaginal delivery (108/109 deliveries) rather than caesarean section (Shah *et al.*, 1986).

In order to determine vertical transmission and persistence, Puranen *et al.*, (1996) studied children from ages 0.3 to 11.6 years old born to mothers followed up for genital HPVs. They concluded that perinatally acquired HPV infection might persist in the oral cavity for years without any significant

clinical lesions. However, a detailed sequence analysis is needed to confirm the identity of the child's and mother's HPV type.

Evidence of HPV infections in 229 children were recently investigated by examining the incidence of seropositivity to eukaryote-derived HPV-16 L1 and L2 capsid proteins expressed in insect cells *via* recombinant baculoviruses (Cason *et al*. 1994a, b). Under comparable conditions seropositivity rates were higher when L2 proteins were used, indeed 83.1% of children were seropositive when tested for Ig of all classes. Thus when children's sera for IgM antibodies (indicative of recent or persistent infections) to the L2 protein were investigated, between 20 to 60% of children were IgM positive to L2 protein within 3 years of birth. These data suggest that early exposure to HPVs is a common event and may reflect perinatal acquisition of virus.

Overall however, there was a biphasic distribution of IgM positivity with peak incidences occurring between 2 to 5 years and another peak at 16 years. The first peak may reflect HPV antibodies induced as a result of early and/or perinatal HPV infections, whereas the second peak may be due to exposure to HPVs as a result of increasing social activity or even early sexual encounters.

In conclusion, HPV infections acquired at birth may persist in the buccal cavity or external genitalia for six weeks, but by six months there was no evidence of HPV-DNA at these sites (a larger number of infants need to be studied at six months to confirm this). Data from serological investigations also confirmed that HPV infections may be acquired early in life and that they are recognised by the immune system.

Chapter 5

Viral load as a determinant for transmission of HPV type 16 from mother to infant.

.Chapter 5 Viral load as a determinant for transmission of HPV type 16 from mother to child.

5.0 Objective

i) To determine whether maternal genital HPV load is a factor favouring perinatal transmission.

5.1 Background

Most HIV-infected children acquire their infection through mother to child transmission (Newell & Peckham 1993) and currently in Europe, rates vary between 15-20%. There is some evidence that maternal viral load in vaginal secretions, as well as serum, can be associated with an increased risk of vertical transmission (Puel *et al.*, 1992). Indeed therapies, such as AZT (Zidovudine) are aimed at reducing maternal viral load by reducing viral replication (Archoan *et al.*, 1989).

Previously (chapter 2) at 24 h, 16 HPV-DNA positive infants were delivered to 37 HPV-DNA positive women, a transmission rate of 43%. Based on the fact that a high maternal viral load may favour vertical transmission of HIV, a study was conducted to investigate whether this applied to genital HPVs.

5.2 Materials and methods

5.2.1 Study group

To determine whether maternal viral load may favour transmission, 15 samples belonging to women already known to have genital HPV -16 (chapter 2), with infants that were HPV DNA positive and negative at 24 h were specifically chosen (Appendix 5). These samples had previously been analysed by consensus and type specific primers (chapter 2) and subsequently

stored in the freezer at - 20 ^{0}C till further use.

Of these 15 HPV-16 DNA positive women, 8 had previously given birth to infants (n=9; one pair of twins) who were positive for HPV-16 DNA at 24 h, whilst 7 gave birth to infants (n=7) that did not have detectable HPV-DNA at their buccal or genital sites (appendix 5).

5.2.2 Quantitation

Two specific primers from within the E7 open reading frame (ORF) of HPV-16 were used to amplify the region between nucleotides 557 and 859. Primer E7-N (5'-TAATCATGCATGGAGATACACC-3') was located between nucleotides 557 to 578 and primer E7-C (5'-ATTATGGTTTCTGAGAACAGAT-3') between nucleotides 839 to 859. PCR amplification was carried out in a 100μl volume containing 1 μM of each primer, 50 mM KCl, 10 mM Tris-HCL pH 8.3, 1 mM Mg Cl_2, 200 μM of all 4 dNTPs and 2 units of Taq DNA polymerase (Advanced Biotechnologies Ltd.). Amplification was performed in an Omnigene Thermal Cycler (Hybaid Ltd.). The first cycle was at 94^0 C for 5 min followed by subsequent cycles each of 30 sec at 94^{0}C, 30 sec at 55^0 C and 35 sec at 72^0 C. An extension step of 10 min at 72^0 C was included as a final cycle. One hundred nanogram of pAT-16 DNA were subjected to varying number of cycles in order to determine the course of PCR amplification.

Positive controls used in PCR quantitation assays included: a pAT/HPV-16 construct which contains the entire HPV-16 sequence cloned in at the Bam H1 site (located within the L1 ORF at nucleotide 6151) and XH1, a cell line which is derived from a cervical carcinoma and contains approximately 100 HPV-16 genome copies per cell (Han, 1991).

After amplification, 20 ul of each reaction was electrophoresed in a 2% (w/v) agarose gel with ethidium bromide and visualised under ultraviolet

light.

Negative images of gels were obtained using an Imager Gel Documentation System (Appligene Ltd.) and then scanned with a laser densitometer (LKB 2222-010 ultrascan XL. LKB Bromma Ltd.) to determine the area of individual bands which were expressed in arbitrary units.

5.3 Statistical analysis

Unpaired students t-tests were used to assist interpretation of data.

5.4 Results

5.4.1 Quantitation of HPV-16 PCR products

When different number of cycles were assessed, it was found that between 10 to 40 cycles there was a linear accumulation of product; at greater numbers of cycles the efficiency of amplification decreased (Figure 5.1). Amplification for 30 cycles was thus used in all subsequent quantitative PCR reactions.

To enable estimation of viral load in terms of copy number in the clinical samples, a known number of copies of HPV 16 genome purified from XH1 cell-line were amplified (5.2) and a standard curve was constructed from the peak areas obtained (Figure 5.3). This method was shown to be reproducible: when 3 clinical samples were amplified and scanned two times, for one sample the readings were identical (0.6 Units), the second varied by 6.8% (2.9 & 2.7 units) and A third varied by only 6.1% (3.1& 3.3 Units). A typical densitometric scan is shown in Figure 5.4.

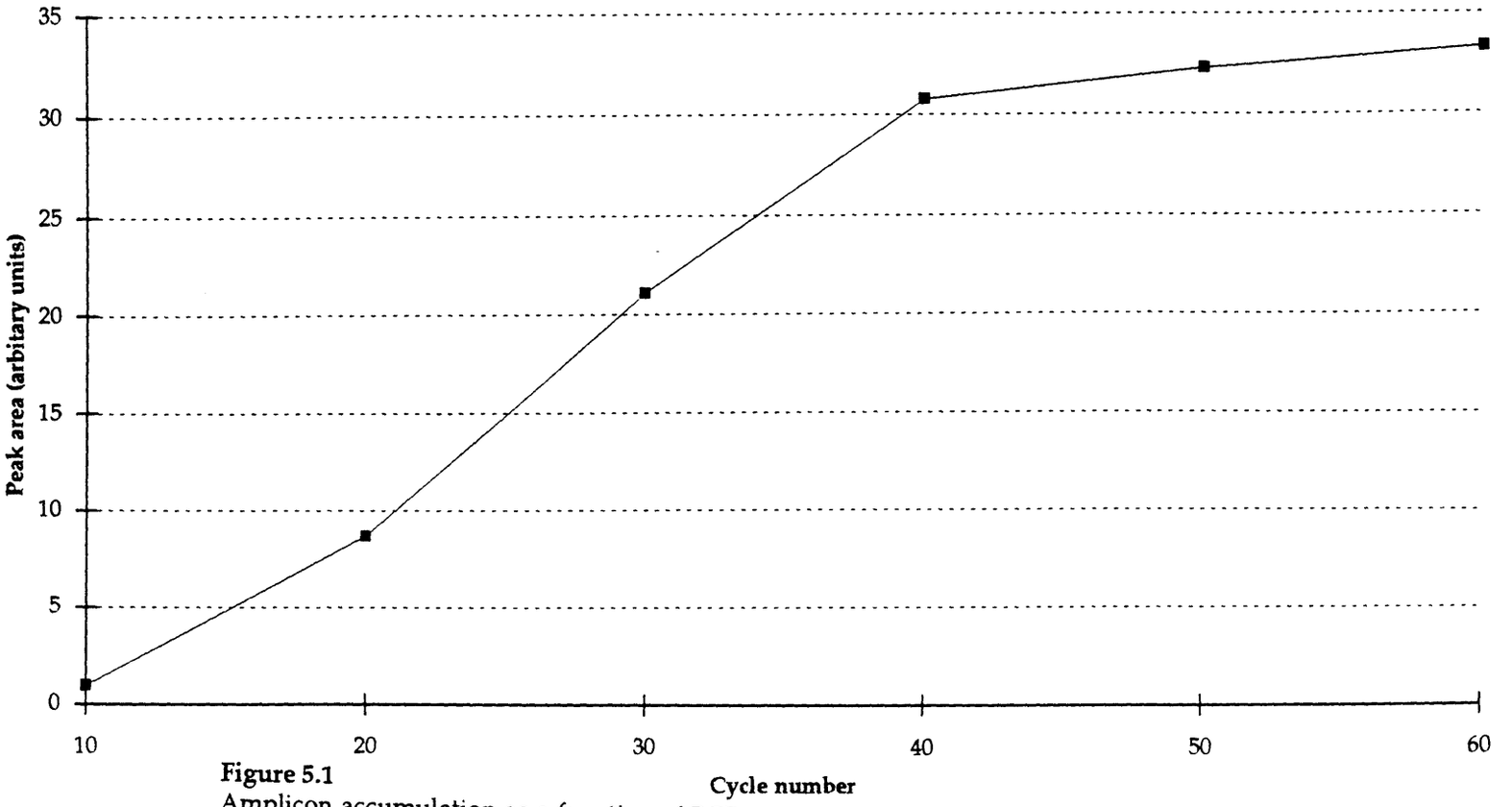

Figure 5.1
Amplicon accumulation as a function of PCR cycle number: 100 ng of a pAT/HPV-16 construct were subjected to varying numbers of cycles in order to determine the range of linear amplification.

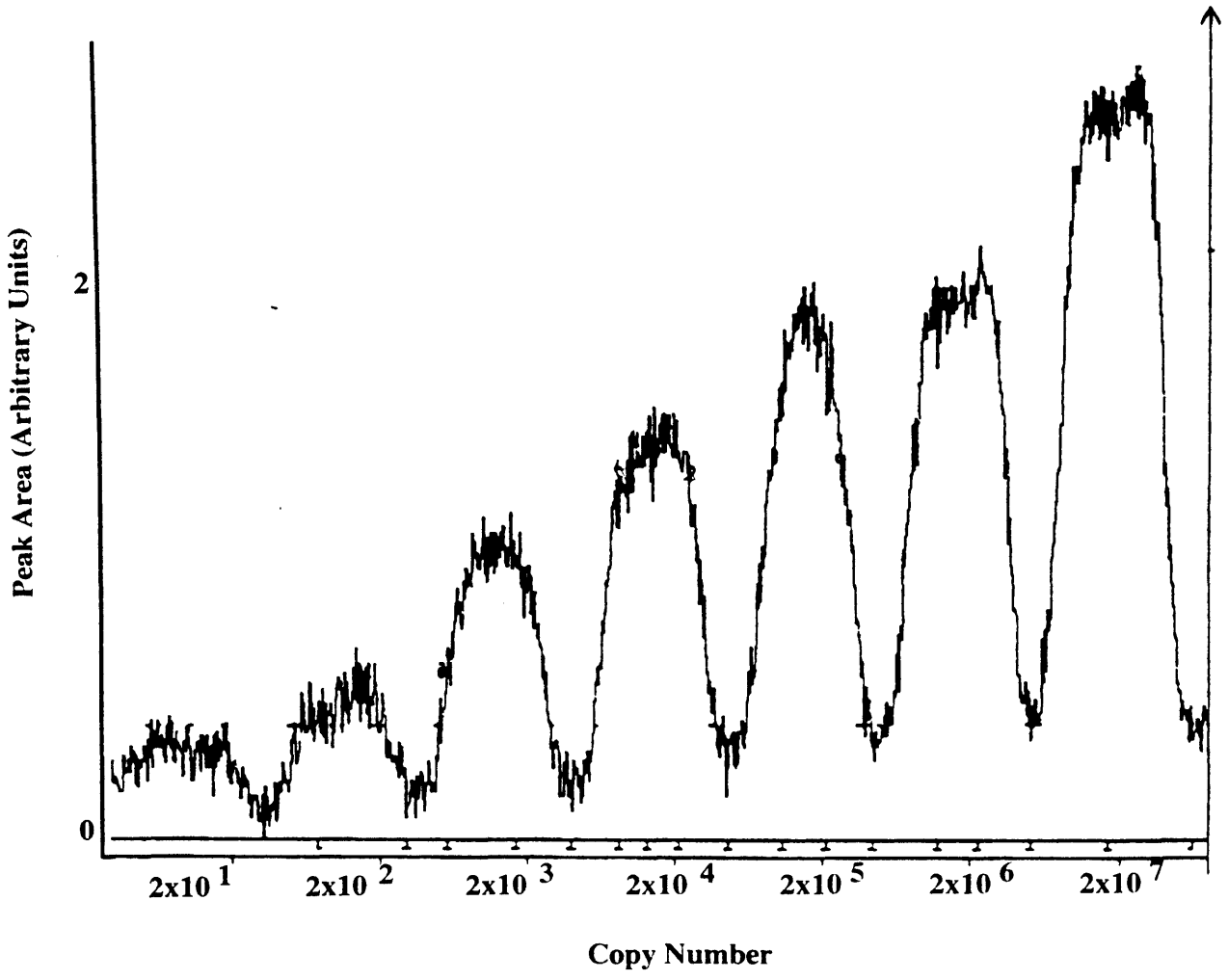

Figure 5.2
Densitometric scan of XH1 PCR products showing increase in peak areas with increasing copy number.

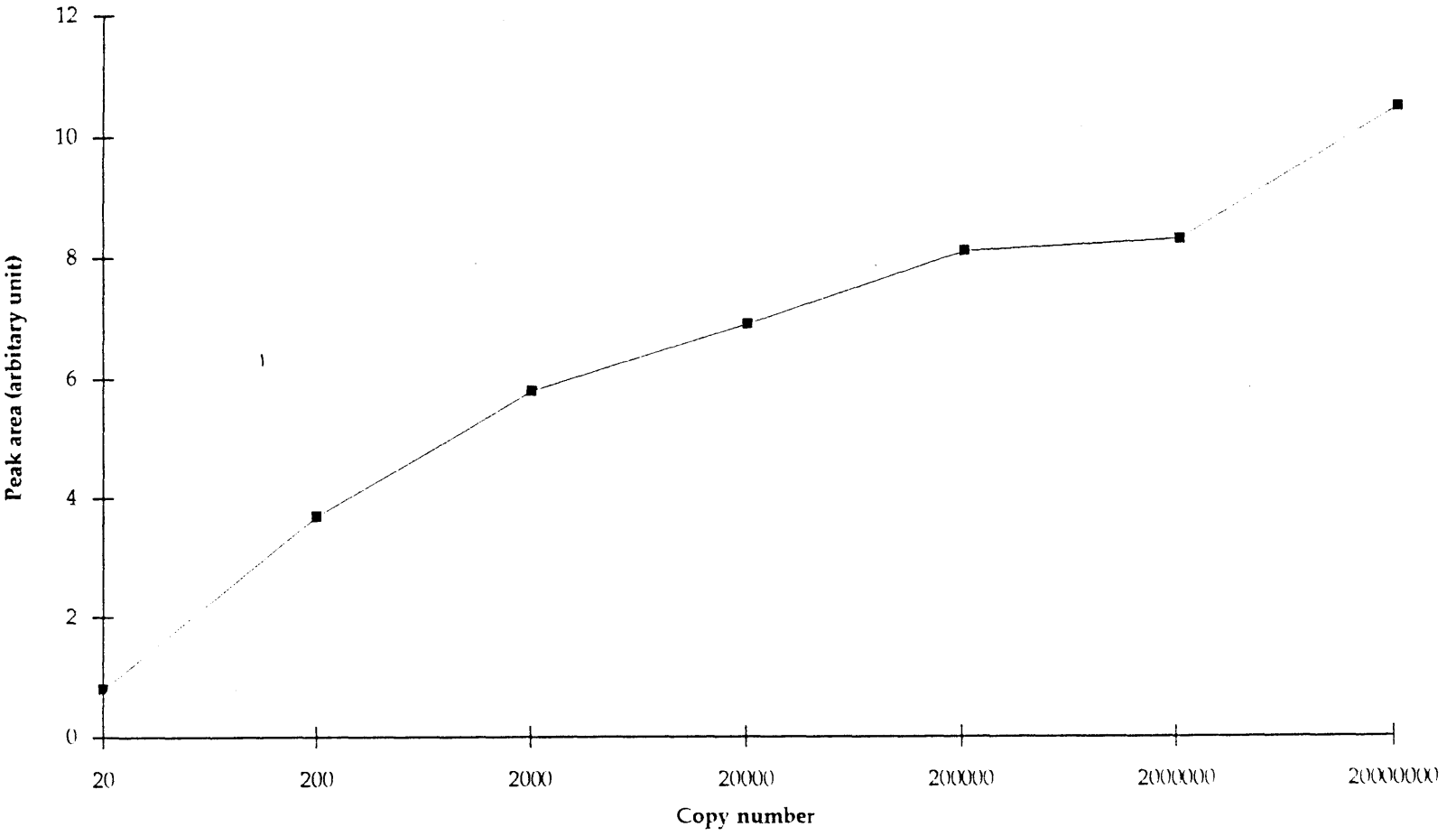

Figure 5.3 Standard curve for estimation of HPV-16 DNA in maternal samples. Following PCR and densitometric analyses of dilutions of DNA from XH1cell line, peak areas were plotted against copy number.

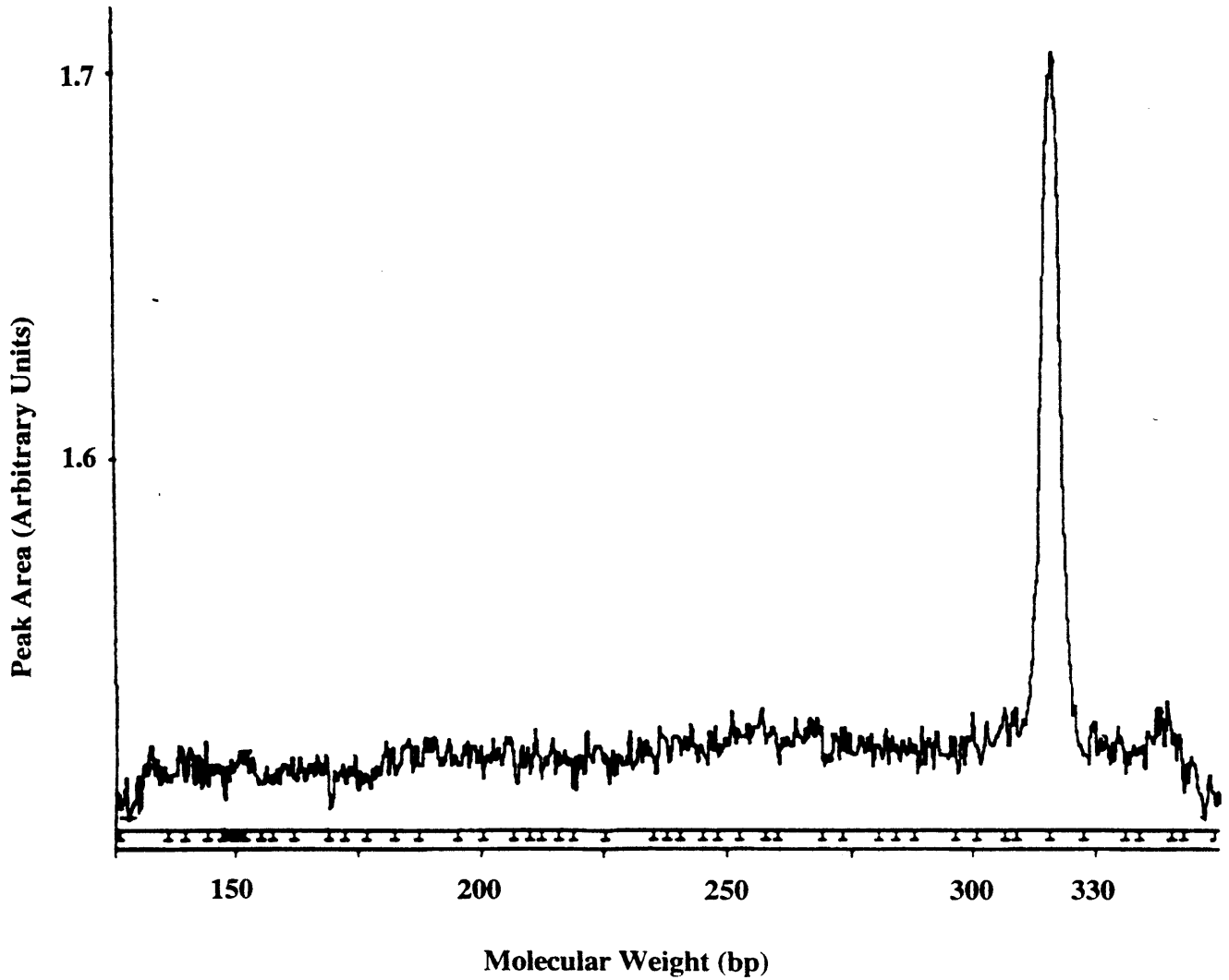

Figure 5.4 Typical densitometric scan of PCR products. Negative gel images were scanned with a laser densitometer and peak areas used to estimate HPV-16 copy number.

5.4.2 Maternal HPV-16 Viral load

The 8 women who transmitted HPV-16 to their infants had significantly ($p<0.05$) higher viral loads (mean +/- standard deviations: 4.35 +/- 2.8 Units/PCR sample) than those 7 women who did not transmit the virus (1.83 +/- 1.12 units, $p=0.044$: Table 5.1).

In terms of copy number of HPV -16 genomes, those mothers who transmitted the virus ranged from 35 to 5 x 10^6 copies /PCR sample (629,886 +/- 1,765,883) whereas those women who did not transmit the virus had between 17 to 195 HPV-16 copies (70.8 +/- 65.25 copies: $p=0.35$). Whilst there was no clear cut off value between those women who did and did not transmit virus, all women with a viral load of 4.0 Units (325 copies) or greater (n=4) transmitted infection to their infants, whereas those with areas of less than 1.6 Units (22 copies: n=3) failed to infect their infants (Table 5.1). One woman (patient 7) with a relatively low viral load (1.6) units gave birth to twins both of whom were HPV-16 positive.

To ensure that these results did not reflect differing efficiencies of sample collection a quantitative assessment of the PCR products of the B-globin gene amplification was performed. There was no significant difference ($p>0.10$) in the quantity of B-globin amplicons between those who did (0,96+/- 0.13 Units) and did not (0.97 +/- 0.22 Units) transmit HPV-16 ($p=0.91$).

Table 5.1 PCR results and estimated viral copy number amongst HPV-16 positive women.

Samples	B-globin PCR	Consensus PCR	HPV-16 PCR	Transmission at 24 h	B-globin Peak area*	HPV-16 Peak area*	Approx. Copy area**
1	+	+	+	T	0.8	9.2	5 x10
2	+	+	+	T	1.0	7.1	35,000
3	+	+	+	T	0.9	6.2	3,500
4	+	+	+	T	0.8	4.0	325
5	+	+	+	T	0.9	3.1	150
6	+	+	+	T	1.1	2.0	49
7	+	+	+	T	1.1	1.6	35
8	+	+	+	T	1.1	1.6	35
9	+	+	+	N	1.3	3.3	95
10	+	+	+	N	0.9	2.7	98
11	+	+	+	N	1.2	2.7	98
12	+	+	+	N	0.8	2.0	49
13	+	+	+	N	0.7	0.9	22
14	+	+	+	N	0.8	0.6	17
15	+	+	+	N	1.1	0.6	17

T: Mothers transmitting HPV16 N: Mothers not transmitted HPV16 *arbitary units.
**Approx. No. of HPV-16 genome copies present in 10μl of maternal sample

5.5 Discussion

When viral load was estimated from the quantity of PCR product, it followed that mothers who transmit HPV-16 to their children have a higher genital viral load than non-transmitters. These findings were not an artifact of sample collection or amplification efficiency, since when the same samples were tested for β-globin by PCR there were no difference between the quantities of β-globin amplified amongst mothers who transmitted HPV-16 compared to those that did not.

There are indeed other methods of quantifying PCR products. These include hybridisation with radioactive (Kellog *et al.*, 1990) or non-radioative probes (Zipeto *et al.*, 1993), limiting dilution of the sample followed by PCR (Simmonds *et al.*, 1990) and modified PCR assays using an internal standard (Fox *et al.*, 1992). However, the simple technique of densitometric scanning of negative photographic images of HPV-16 amplicons in agarose gels used in this study was found to be sensitive, fast and consistent method of estimating PCR products. It is assumed that the amount of HPV in the original sample is a reflection of the maternal viral load.

Thus genital HPV load, whilst an important determinant, is not the sole factor in determining transmission from mother to child. This is analogous to vertical transmission of HIV, as other factors such as mode of delivery and viral characteristics may also be important.

Chapter 6

Other studies (same group), conclusions and recommendations

Chapter 6 Other studies, conclusions and recommendations

6.1 Other Studies

It may be argued that the HPV DNA detected in infants at 24 h and at 6 weeks could be due to contamination rather than infection. This may be a valid point as HPV DNA was not detected at 6 months.

Using the same maternal-infant samples previously collected and analysed by Pakarian (chapters 2 and 4), Biswas *et al.*, (St Thomas' Hospital) aimed to distinguish between contamination and infection.

A selected group of children were seen at about 2 years of age. All of these children had HPV-16 positive buccal swabs and had previously been delivered to HPV-16 positive mothers. The children were investigated for the presence of transcriptionally active HPV-16 infections as evidenced by detecting HPV-16 early region messenger RNA (E-RNA) by nested reverse transcription PCR (RT-PCR).

Four of the 10 (40%) children' samples were positive for HPV-16 mRNA. This result (unpublished data) demonstrates that vertical transmission of HPV-16 DNA from mother to infant results in transcriptionally active infection in children at the age of 2 years. The RT-PCR used above had a low analytic sensitivity (about 2,000 copies of HPV-16 mRNA) as compared to the PCR (less than 10 HPV-16 DNA copies) and may therefore represent an under-estimate of the true prevalence of active infections among HPV-16 DNA positive children.

The above study by Biswas *et al.*, does support infection rather than contamination. It would therefore be valuable to analyse all the infant samples at 24 h (70 samples), 6 weeks (49 samples) and 6 months (19 samples) using the RT-PCR.

The development of laryngeal papillomas in infants that are delivered

to mothers with genital warts also supports the idea of infection rather than contamination (Mounts & Shah, 1984).

Further evidence suggesting infection comes from several case reports of children born with anogenital condylomata (Tang *et al.*, 1978, Rogo & Nyansera, 1989, Cohen *et al.*, 1990).

To extend these observations over a greater age range of children HPV - 16 L1 and L2 proteins were expressed in insect cells *via* recombinant baculoviruses and sera from another group of 229 children were examined to determine at what age IgM antibodies to HPV were acquired (Cason *et al.*, 1995). There was a bimodal distribution of IgM seropositivity which peaked between 2 and 5 and 13 and 16 years of age, suggesting that two distinct modes of transmission may occur. This serological study concluded that HPV may be transmitted from mother to infant and result in persistent infections. The increased seropositivity rates at around 16 years of age, may demonstrate a time commonly associated with the onset of sexual activity.

Several recent studies have also demonstrated that cancer associated HPVs are transmitted at birth (Cason *et al.*, 1995, Puranen *et al.*, 1996, Tseng *et al*, 1998). A review of the literature, demonstrated that the perinatal transmission of HPV 16/18 DNA from HPV-positive mothers to their infants occurs in 38-73% of the births. Watts *et al.*, (1998), however, reported a low vertical transmission rate.

The differences in detection rates may be due to several factors including the sensitivity of the PCR, the types of primers used, methods used in collecting samples and the women population selected for the study.

The differences in sensitivity and types of primers used are further highlighted by analysing the results of Pakarian *et al.*, (1994) and Cason *et al.*, (1995). In both studies, different primers were used on identical samples. Cason *et al.*, (1995) compared to Pakarian *et al.*, (1994) detected a higher

proportion of pregnant women with HPV (80% v.s. 54%). The former author also detected a higher proportion of HPV DNA in infants at 24 h (73% v.s. 43%), at 6 weeks (79.5% v.s. 19%) and at six months (83.3% v.s. 0%).

The differences in detection rates is partly explained by the fact that the primers used by Cason *et al.*, (1995), could detect 2 to 5 HPV- 16 genome copies. The primers used by Pakarian *et al.*, (1994) could detect 10 HPV-16 genome copies. The other explanation could be due to false positive results. This is unlikely as in both studies stringent precautions were taken.

Recently Kay *et al* ., (1996) [samples previously collected by Pakarian] demonstrated persistence and a maternal source of infectivity at 6 weeks and 2 years. This author sequenced a 521 bp segment of the upstream regulatory region (URR) of HPV-16 DNA isolated from 13 maternal samples and from samples taken from their infants.

Maternal samples were collected as previously described. Swabs were also taken from buccal mucosa and genitals of infants at six weeks; only buccal swabs were taken at 2 years of age.

Infants were considered HPV-16 positive if E5 DNA was detected at buccal and/or at genital sites by both PCR and Southern blot; buccal samples from children were used for sequencing (Kay *et al.*, 1996).

In summary, using E5 primers, HPV -16 DNA was detected in all maternal cervical samples. At six weeks, 11 of the thirteen infants had demonstrable HPV -16 DNA either at their buccal and/or genital sites. HPV-16 DNA was detected in 9 of the thirteen infants at 2 years (these infants were HPV -16 DNA positive at six weeks). The 6 weeks samples from two infants were negative for HPV -16 DNA in PCR using E5 or URR primers. These 2 infants had demonstrable HPV -16 DNA at 2 years.

In 2 mother/infant sets identical HPV -16 variants were detected in maternal and infant samples. The authors conclude that in these 2 instances

the mother was the source of persistent infant HPV -16 infections.

In 3 mother/infant sets both unique and common nucleotide changes in matched maternal and infant samples were noted. Common nucleotide changes in matched maternal and infant samples again implies a maternal source of infant infections.

Three mothers had variant URR sequences but their infants had prototypic or different variants at 6 weeks or 2 years of age. Re-infection in the infant from an unknown source or that all 3 samples were from subjects with heterogeneous HPV-16 infections with different variants predominating at different sample sites may reflect the data.

The study by Kay *et al.* (1996), demonstrates persistence at 2 years and two sources of infant HPV-16 infections: firstly, the mother, and secondly, an as yet unidentified source(s).

In order to demonstrate persistence and a maternal source of infection, 13 maternal samples that had previously been shown to have HPV -16 DNA (Pakarian *et al.* 1996) were specifically chosen by Kay *et al.* (1996).

It is interesting to note that in the latter study, using the same samples as Pakarian *et al.*, (1994), there was a higher rate of transmission and persistence.

This can partly be explained by the fact that nested PCRs were used to produce material for sequencing. This method was specifically chosen as samples had previously been analysed previously in 14 different PCRs, and there was only a limited amounts of target remaining. Secondly, samples from children contained low quantities of HPV-16 DNA, which were not always detected by PCR using HPV consensus primers but were detectable when more sensitive PCRs based on primers in the E5, E6 or E7 ORFs were used (Cason *et al.*, 1995, Cavuslu *et al.*, 1996).

The study by Kay *et al* , (1996) demonstrate persistence of HPVs up to

the age of 2 years.

6.2 Conclusions

Using the polymerase chain reaction, we have demonstrated that HPV acquired at birth may persist in the buccal cavity or external genitalia for up to 2 years. Using HPV consensus primers (MY 09, MY11) HPV DNA was not demonstrable at 6 months (Pakarian *et al.*,, 1994). However, using the same samples but E5 primers, Cason *et al.*, (1995) were able to detect HPV DNA in children at 6 months. The sensitivity of the E 5 primers was quoted as two to five HPV-16 genome copies by the latter author. Using consensus primers the sensitivity was ten genome copies.

The results suggest that perinatal acquisition is *via* the birth canal as HPVs are not detected in amniotic fluid. However, only 10 of 32 women recruited had detectable genital HPVs. We can only conclude that among infected women with uninfected infants, HPV is not detected in amniotic fluid. A larger population of women with high incidence of genital HPV may be needed to adequately answer this question.

We have also shown that mothers who transmit HPV 16 to their infants have a higher load of virus than non transmitters.

6.3 Future studies

The issue of contamination versus infection needs further assessment.

The physical state of the virus (integrated or episomal) is also being investigated to determine whether this is a significant factor in perinatal transmission. It is believed that integration of HPV DNA into cellular DNA results in disruption or a deletion in the E1 or E2 open reading frames (ORFs). For detection of integration, two specific primers from the E2 ORF of the HPV-16 genome are being used.

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Appendix

Maternal No.	Age	Previous Cytology	Smoker	parity
1	22	normal	N	P
2	23	abnormal	?	P
3	24	HPV	Y	M
4	37	HPV	Y	M
5	36	normal	N	M
6	36	abnormal	N	M
7	32	normal	N	P
8	31	normal	X	P
9	36	normal	N	M
10	30	normal	N	M
11	32	HPV	N	M
12	28	normal	X	M
13	33	HPV	X	P
14	28	normal	N	P
15	37	normal	N	P
16	26	normal	X	M
17	34	abnormal	X	M
18	31	normal	X	M
19	35	normal	Y	M
20	26	HPV	N	P
21	34	abnormal	N	M
22	37	abnormal	N	M
23	26	normal	Y	P
24	35	normal	N	M
25	21	normal	N	P
26	26	abnormal	Y	P
27	31	normal	Y	P
28	28	abn/HPV	N	M
29	24	normal	Y	P
30	29	abnormal	X	P
31	35	abnormal	Y	M
32	28	abnormal	X	M
33	19	normal	X	P
34	31	normal	Y	P
35	21	normal	X	P
36	23	normal	N	P
37	26	normal	Y	M
38	28	abnormal	Y	M
39	35	normal	Y	M
40	34	abnormal	N	M
41	29	abn/HPV	X	M
42	36	normal	Y	M
43	29	abnormal	N	P
44	21	normal	N	P
45	19	normal	N	P
46	30	abnormal	Y	M
47	27	abnormal	?	M

48	38	normal	Y	M
49	28	normal	X	M
50	34	normal	N	M
51	32	abnormal	?	M
52	30	normal	N	M
53	25	abnormal	X	M
54	21	normal	Y	P
55	34	normal	N	M
56	31	abnormal	?	M
57	30	normal	Y	M
58	32	normal	N	M
59	16	HPV	Y	P
60	42	HPV	Y	M
61	32	abnormal	?	M
62	38	normal	?	M
63	22	normal	N	P
64	30	abnormal	Y	M
65	33	abnormal	Y	M
66	28	abnormal	N	M
67	34	normal	N	M
68	35	normal	N	M
69 (twins)	28	abnormal	N	P

Maternal No.	Age	Previous Cytology	Smoker	parity	Antenatal cervical HPV DNA & type	HPV DNA @ 24 h in infants & types
1	22	normal	N	P	negative	positive, 16
2	23	abnormal	?	P	negative	negative
3	24	HPV	Y	M	positive, 16	positive, 16
4	37	HPV	Y	M	positive,16	positive, 16
5	36	normal	N	M	positive,16	negative
6	36	abnormal	N	M	positive,16	positive,16
7	32	normal	N	P	positive,16	negative
8	31	normal	X	P	positive, 11	positive 11
9	36	normal	N	M	negative	negative
10	30	normal	N	M	positive, 16	positive, 16
11	32	HPV	N	M	positive, 31/33	negative
12	28	normal	X	M	positive, 16	negative
13	33	HPV	X	P	positive, 16	positive, 16
14	28	normal	N	P	negative	negative
15	37	normal	N	P	positive, 16	positive, 16
16	26	normal	X	M	positive, 16	negative
17	34	abnormal	X	M	positive, 16/33	negative
18	31	normal	X	M	positive, X	negative
19	35	normal	Y	M	negative	positive, 19
20	26	HPV	N	P	positive, 16	positive, 16
21	34	abnormal	N	M	positive, X	negative
22	37	abnormal	N	M	negative	negative
23	26	normal	Y	P	positive, 16	negative
24	35	normal	N	M	negative	positive, 16
25	21	normal	N	P	positive X	negative
26	26	abnormal	Y	P	positive, 16	positive, 16
27	31	normal	Y	P	negative	negative
28	28	abn/HPV	N	M	positive, 16	positive, 16
29	24	normal	Y	P	positive, 11/16	negative
30	29	abnormal	X	P	positive, 11/16	negative

31	35	abnormal	Y	M	positive, 16/18	positive, 18
32	28	abnormal	X	M	negative	negative
33	19	normal	X	P	positive, 16/18	positive,18
34	31	normal	Y	P	negative	negative
35	21	normal	X	P	negative	negative
36	23	normal	N	P	negative	negative
37	26	normal	Y	M	positive, 16/18	negative
38	28	abnormal	Y	M	negative	negative
39	35	normal	Y	M	negative	negative
40	34	abnormal	N	M	positive, 18	positive, 18
41	29	abn/HPV	X	M	positive, 16	negative
42	36	normal	Y	M	positive, 16	negative
43	29	abnormal	N	P	negative	negative
44	21	normal	N	P	negative	negative
45	19	normal	N	P	positive, 6/11	negative
46	30	abnormal	Y	M	positive, 16/18	positive, 16/18
47	27	abnormal	?	M	positive, 18	negative
48	38	normal	Y	M	negative	negative
49	28	normal	X	M	positive, X	negative
50	34	normal	N	M	positive, X	negative
51	32	abnormal	?	M	positive, X	negative
52	30	normal	N	M	negative	negative
53	25	abnormal	X	M	positive, X	negative
54	21	normal	Y	P	negative	negative
55	34	normal	N	M	negative	negative
56	31	abnormal	?	M	positive, X	negative
57	30	normal	Y	M	negative	negative
58	32	normal	N	M	negative	negative
59	16	HPV	Y	P	negative	negative
60	42	HPV	Y	M	negative	negative
61	32	abnormal	?	M	negative	negative

62	38	normal	?	M	negative	negative
63	22	normal	N	P	negative	negative
64	30	abnormal	Y	M	negative	negative
65	33	abnormal	Y	M	negative	negative
66	28	abnormal	N	M	negative	negative
67	34	normal	N	M	negative	negative
68	35	normal	N	M	negative	negative
69 (twins)	28	abnormal	N	P	positive, 16/18	positive, 16/18
						positive, 16/18

Maternal No.	age	HPV DNA in maternal swabs	Amniotic fluid
1 (Amnio)	41		Negative
2	19		Negative
3	34		Negative
4	22		Negative
5	31		Negative
6	27	positive	Negative
7	25		Negative
8 (amnio)	39		Negative
9	28		Negative
10	28		Negative
11	29		Negative
12	26	positive	Negative
13	20		Negative
14	32		Negative
15	23		Negative
16	24	positive	Negative
17	24		Negative
18	31		Negative
19	27		Negative
20 (amnio)	37		Negative
21	29	positive	Negative
22	32		Negative
23 (amnio)	26		Negative
24	38		Negative
25	26		Negative

Maternal No.	age	HPV DNA in maternal swabs	Amniotic fluid
1	37	positive	Negative
2	32	positive	Negative
3	36	positive	Negative
4	39	positive	Negative
5	28		Negative
6	31	positive	Negative
7	36		Negative
8	31	positive	Negative

Maternal No.	Antenatal cervical HPV DNA & type	HPV DNA @ 24 h in infants & types	HPV @ 6/52 in infants	HPV@ 6/12 in infants	maternal breast milk/buccal swab @ 6/52	cervical HPV 6/52
1	negative	positive, 16	not available	not available		
2	negative	negative	not detected	not detected	negative/negative	positive
3	positive, 16	positive, 16	16	not detected	negative/negative	positive
4	positive,16	positive, 16	16	not detected	negative/negative	positive
5	positive,16	negative	not detected	not available		negative
6	positive,16	positive,16	16	not available		
7	positive,16	negative	16	not available		
8	positive, 11	positive 11	not deteted	not available		
9	negative	negative	not available	not available		
10	positive, 16	positive, 16	not detected	not detected	negative/negative	positive
11	positive, 31/33	negative	not detected	not available		positive
12	positive, 16	negative	not available	not available		
13	positive, 16	positive, 16	not available	not detected		negative
14	negative	negative	not detected	not available	negative/negative	positive
15	positive, 16	positive, 16	not available	not available		
16	positive, 16	negative	not detected	not available		negative
17	positive, 16/33	negative	not detected	not available		positive
18	positive, X	negative	not detected	not available		positive
19	negative	positive, 16	16	not available		negative
20	positive, 16	positive, 16	16	not detected		positive
21	positive, X	negative	not available	not available	negative/negative	
22	negative	negative	not detected	not available	negative/negative	negative
23	positive, 16	negative	16	not available		negative
24	negative	positive, 16	not detected	not available		negative
25	positive X	negative	not detected	not available	negative/negative	negative
26	positive, 16	positive, 16	not detected	not detected		negative
27	negative	negative	not detected	not available	negative/negative	negative
28	positive, 16	positive, 16	not detected	not available		negative
29	positive, 11/16	negative	not detected	not available		negative
30	positive, 11/16	negative	not available	not available		
31	positive, 16/18	positive, 18	not detected	not available		positive
32	negative	negative	not available	not available		
33	positive, 16/18	positive,18	not detected	not available		negative
34	negative	negative	not detected	not available		negative
35	negative	negative	not detected	not available		positive
36	negative	negative	not detected	not available	negative/negative	positive
37	positive, 16/18	negative	16	not available		
38	negative	negative	not available	not available		
39	negative	negative	not detected	not available		negative
40	positive, 18	positive, 18	18	not detected		negative
41	positive, 16	negative	not detected	not available		
42	positive, 16	negative	not available	not available		
43	negative	negative	not detected	not available		negative

44	negative	negative	18	not detected		
45	positive, 6/11	negative	not available	not available		
46	positive, 16/18	positive, 16/18	not detected	not available		negative
47	positive, 18	negative	not available	not available		
48	negative	negative	not available	not available		
49	positive, X	negative	not available	not detected	negative/negative	
50	positive, X	negative	not detected	not available		
51	positive, X	negative	not available	not available		
52	negative	negative	not available	not available		
53	positive, X	negative	not available	not available		
54	negative	negative	not available	not available		
55	negative	negative	not available	not available		
56	positive, X	negative	not available	not detected		
57	negative	negative	not detected	not available		
58	negative	negative	not detected	not detected	negative/negative	
59	negative	negative	not detected	not available		
60	negative	negative	not detected	not detected	negative/negative	negative
61	negative	negative	not detected	not detected	negative/negative	
62	negative	negative	not detected	not available		
63	negative	negative	not detecetd	not available		
64	negative	negative	not detected	not detected	negative/negative	
65	negative	negative	not available	not available		
66	negative	negative	not detected	not available		
67	negative	negative	not detected	not detected		
68	negative	negative	not detected	not detected		
69 (twins)	positive, 16/18	positive, 16/18	16	not detected		
		positive, 16/18	not detected	not detected		

Maternal No	Age	Previous cytology	Cervical HPV DNA and type	HPV DNA in infants at 24 h & type
3	24	HPV	positive (16)	positive (16)
4	37	HPV	positive (16)	positive (16)
5	36	normal	positive (16)	negative
6	36	abnormal	positive (16)	positive (16)
7	32	normal	positive, 16	negative
10	39	normal	positive, 16	positive, 16
12	28	normal	positive (16)	negative
13	33	HPV	positive (16)	positive (16)
15	37	normal	positive, 16	positive, 16
16	26	normal	positive,16	negative
23	26	normal	positive (16)	negative
26	26	abnormal	positive (16)	positive (16)
41	34	abnormal	positive (16)	negative
42	36	normal	positive (16)	negative
69 (twins)	28	abnormal	positive (16)	positive (16,18)
				positive (16,18)