THE MOLECULAR TYPING OF HUMAN PAPILLOMAVIRUSES FROM PATIENTS WITH INVASIVE CARCINOMA OF THE CERVIX IN CAPE TOWN

Nicola Susan Brink

A dissertation submitted to the Faculty of Medicine,

University of Cape Town, in partial fulfillment

of the requirements for the degree of

Master of Medicine (Virology)

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University of Cape Town

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Abstract

South Africa has one of the highest incidences of carcinoma of the cervix in the world. The incidence of HPV infection in South African patients with invasive carcinoma of the cervix, as well as the types of HPV involved is unknown.

Biopsies were obtained from consecutive patients with invasive carcinoma of the cervix presenting to the Combined Assessment Clinic at Groote Schuur Hospital, Cape Town.

HPV DNA was detected by Southern blot hybridization in a total of 38/86 patients with invasive carcinoma of the cervix in Cape Town. HPV 16 DNA was detected in a total of 13/86 (15%) patients. Although this is somewhat lower than has been reported in many studies, a geographical variation in the prevalence of HPV 16 in different countries is well documented.

The application of a more sensitive technique, the polymerase chain reaction, increased the detection of HPV 16 DNA to 16/86. The polymerase chain reaction did not, however, significantly increase the detection of HPV 16 DNA

Declaration

I declare that this dissertation is my own work, and has not been submitted for any degree or examination at any other university.

I empower the university to reproduce, for the purpose of research, either the whole, or any portion of the contents in any manner whatsoever.

Signed

Dr Nicola Susan Brink

Nov. 1990

Acknowledgements

I would like to thank the following people:

1.My supervisors, Dr. A-L Williamson and Professor J. Moodie for guidance throughout this project and advice during preparation of this manuscript.

2. Dr C. Dehaeck for her useful collaboration and for collecting the biopsies for this study.

3. Miss J. Johnson for valuable assistance in the laboratory.

4. Mrs L. Alexander for help in typing and preparing the manuscript.

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1. Introduction

Cancer of the cervix is the most common cancer amongst women world-wide and in developing countries it ranks as the most common form of cancer. (Armstrong, 1988). About 500 000 new cases of carcinoma of the cervix are diagnosed world-wide annually.(Shah and Howley, 1990). The pattern in South Africa follows that observed in developing countries. Cervical cancer is the most common cancer among women in South Africa with an incidence of 0,6/1000 among black women aged 15 - 65 years. (Cronje, 1989).

Epidemiological evidence suggests that carcinoma of the cervix is the consequence of a genital infection. In 1842 an Italian physician, Rigoni-Stern, noted that carcinoma of the cervix was rare among nuns and was more frequently found in married women as compared to unmarried women.(Melnick *et al.*, 1989). The association of this disease with certain unique risk factors such as multiple sexual partners and age of first intercourse, suggested that carcinoma of the cervix is possibly caused by a sexually transmitted infectious agent. (Rotkin, 1973).

The aetiology of carcinoma of the cervix has been the subject of intense speculation over the past two decades with possible candidates including non-infectious factors such as spermatozoa and a variety of infectious agents including Trichomonas vaginalis and herpes simplex virus. (Melnick *et al.*, 1989). The role of papillomaviruses as a possible candidate in the aetiology of carcinoma of the cervix was first proposed in 1974 by zur Hausen. (zur Hausen, 1987). The papillomaviruses are a highly heterogeneous group of DNA viruses some of which have been associated with naturally occurring cancers. (Shah and Howley, 1990). This recognition that human papillomavirus (HPV) types are closely linked to certain cancers has focused interest on a specific subgroup of human papillomaviruses found in the genital tract and especially those types associated with carcinoma of the cervix

1.1. The Virus

1.1.1. General.

The papillomaviruses are grouped together with the polyomaviruses to form the papovavirus family. The papillomaviruses are larger than the polyomaviruses measuring 55 nm in diameter. Each virion contains a double-stranded DNA molecule. (Pfister, 1987a). A detailed knowledge of the structure and biological properties of the virus is necessary in understanding the role of HPV in the genesis of cancer.

1.1.2. Classification and nomenclature

Papillomaviruses are classified firstly according to their natural host, with most papillomaviruses having a very restricted host range. Further classification into different types is according to the genetic relatedness of the viral genome.(Coggin and zur Hausen, 1979). If two papillomaviruses show less than 50% hybridization in liquid reassociation kinetics they are defined as different types, so that HPV 6 and HPV 11, which have an overall nucleotide sequence homology of 82% but give a value of 25% in liquid reassociation kinetics, are classified as different types. (Howley, 1990). To date 60 distinct HPV types have been described. (De Villiers, 1989) Subtypes are assigned if a new isolate has more than 50% but less than complete homology with a known HPV type. (Coggin and zur Hausen, 1979).

1.1.3. Genetic Organization

The human papillomaviruses have a circular double stranded DNA genome of approximately 8000 base pairs (bp). All the open reading frames (ORF) are characteristically located on the same DNA strand. (Petterson *et al.*, 1987). Comparison of 10 papillomaviruses whose complete DNA sequences have been determined show that they share a similar genetic arrangement. (Giro and Danos, 1986). The open reading frames involved in cell transformation and DNA replication are called "early" or "E" ORFs and those coding for virion structural proteins are called "late" or "L" ORFs. Between the early and late ORFs there is a non-coding regulatory region of approximately 1000 bp which contains the

regulatory regions. (Campo, 1988). Specific functions have been assigned to some of the individual papillomavirus open reading frames. E1 is thought to be necessary for episomal (plasmid) replication, E2 for regulation of transcription and E4 codes for a late cytoplasmic protein. The functions of E3 and E8 are not yet known. E5, E6 and E7 are involved in transformation. L1 and L2 code for the capsid proteins of the virus. (Shah and Howley, 1990).

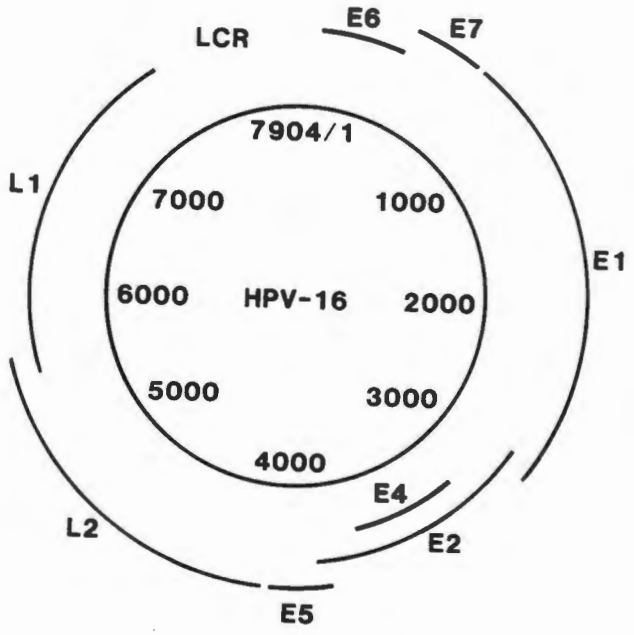

Figure 1. The genomic map of HPV 16. The "early" open reading frames are designated E1 - E7, the "late" open reading frames L1 and L2 and the long control region as the LCR. (Modified from Shah and Howley, 1990).

1.1.4. Biological properties

Papillomaviruses display a high degree of species and tissue specificity. Human papillomaviruses infect the epithelium of the skin and mucous membranes producing epithelial tumours. On this basis they can be divided into cutaneous and mucosal types. These tumours show limited growth and often regress spontaneously. The virus probably enters the skin through microscopic lesions and infects the basal cells. The persisting viral genome either increases the rate of cell proliferation or prolongs the normal lifespan of the cell, both of which lead to hyperplasia and the formation of a wart. (Pfister, 1987b). Epidermal cells are not permissive for DNA replication at the beginning of the differentiation process and the initiation of viral DNA replication only occurs in keratinising cells. Viral replication is therefore linked to the differentiation of the squamous epithelial cell. (Taichman and LaPorta, 1987). The virus may also infect mucosal surfaces. Proliferating cells are exposed at the squamo-columnar junction of the uterine cervix and most of the cervical HPV infections occur at this site. (Pfister, 1987b). Abnormal cellular differentiation, for example in malignancy, is sometimes accompanied by a non-productive infection. This feature is demonstrated in figure 2.

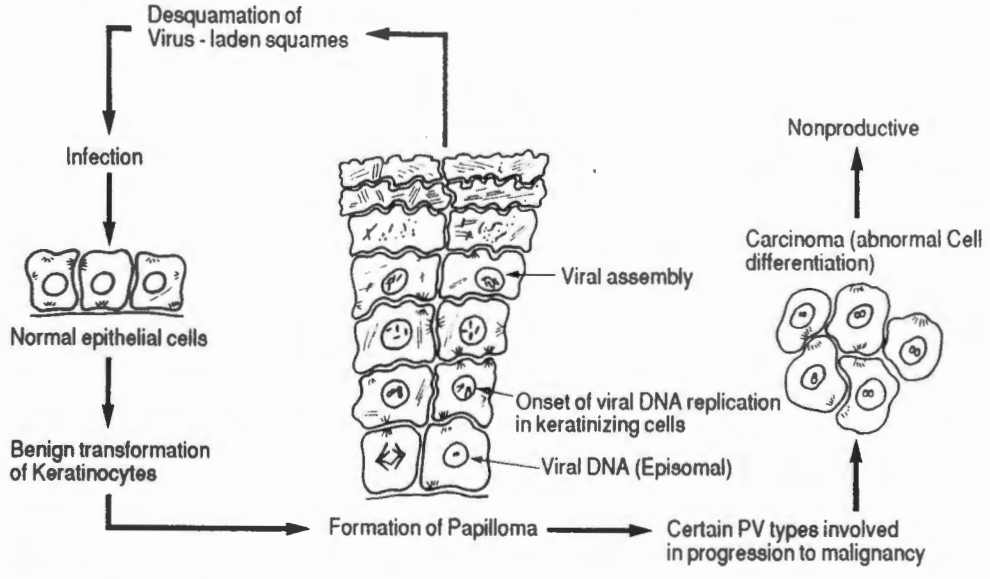

Figure 2. Illustration of the replication cycle of papillomaviruses. (Modified from Taichman and LaPorta, 1987).

1.2. The Spectrum of Disease caused by HPV

The human papillomaviruses comprise three clincopathological groups - cutaneotropic types found in immunologically normal individuals, cutaneotropic viruses causing multiple warts and dry scaly flat lesions in patients with Epidermodysplasia verruciformis and mucosatropic viruses infecting the genital, oral and respiratory mucosa. (Pfister, 1987a).

1.2.1. Genital HPV infection

The spectrum of clinical manifestations of genital HPV infections includes both benign and malignant lesions of the cervix , vulva and penis. In addition HPV 16 DNA has been demonstrated in normal cervical tissue. (de Villiers *et al.*, 1987; Gergety *et al.*, 1987). Classical condylomata are probably the most frequently recognized clinical manifestation of genital HPV infections. However, various studies have shown an association between HPV and cervical intraepithelial neoplasia (CIN) (de Villiers *et al.*, 1987; Crum *et al.*, 1984) and this association has been the subject of extensive research over the past decade.

1.2.2. Carcinoma of the cervix

The association of HPV with carcinoma of the cervix was first proposed about 18 years ago, but has been difficult to prove its validity as it is neither possible to grow the virus in vitro, nor are there reliable serological tests to detect current or past infection with HPV. (zur Hausen, 1987). The cloning of genomes of papillomaviruses into plasmids for use as molecular probes enabled the identification of specific HPV nucleotide sequences in a proportion of cervical cancers, as well as in cervical cancer cell lines. Although sixty different HPV types have been identified, only HPV types 16, 18, 31, 33, 35, 45, 51, 52, 56 and 58 have been isolated from patients with cervical intraepithelial neoplasia (CIN) or cervical carcinoma. HPV types 6, 11, 30, 34, 40, 42, 43, 44 and 57 were not originally isolated from patients with genital malignancies, but have been found rarely in cases with CIN and cervical carcinoma. (De Villiers, 1989).

HPV 16 DNA was first demonstrated in a biopsy sample from a patient with invasive carcinoma of the cervix by Durst *et al.*, 1983. These workers showed that

there were nucleotide sequences in the biopsy material which hybridized with HPV type 11 DNA under non-stringent conditions. The DNA from this biopsy was not homologous with other HPV types. Initial studies showed that 11/18 cervical cancer samples from German patients contained sequences which hybridized with HPV 16 DNA under conditions of high stringency. (Durst *et al.*, 1983).

In 1984 Boshart and colleagues reported the isolation and partial characterization of a new type of HPV, HPV 18 from a cervical cancer biopsy. HPV 18 DNA was originally detected in 9/36 cervical carcinomas from Africa and Brazil and 2/13 cervical tumours from Germany. In addition HPV 18 DNA was found in cells of the HeLa, KB and C4-1 cell lines, all of which are derived from cervical carcinomas. (Boshart *et al.*, 1984)

In 1986 the molecular cloning and characterization of HPV 33 was reported as an additional potentially oncogenic HPV. (Beaudenon *et al.*, 1986) Other reports have appeared in the literature identifying specific HPV types in association with cervical neoplasia (Kahn *et al.*, 1986; Lorincz *et al.*, 1986; Naghashfar *et al.*, 1987; Nuovo *et al.*, 1988; Shimoda *et al.*, 1988; Yajima *et al.*, 1988 and Lorincz *et al.*, 1989).

In spite of the increasing number of different HPV types associated with carcinoma of the cervix, HPV types 16, 18 and 33 are most commonly associated with malignant change. Some doubt has, however, been cast on the aetiological role of HPV in carcinoma of the cervix by the demonstration of HPV 16 in 84% of women with normal cervical cytology using the more sensitive polymerase chain reaction. (Tidy *et al.*, 1989a). Tidy and his colleagues proposed the existence of a less oncogenic subtype of HPV 16 which they termed HPV type 16b. HPV 16b was thought to contain a 21bp deletion within the upstream regulatory region. It was proposed that this deletion removed a pair of E2 consensus binding sites. The E2 protein requires two binding sites in close proximity to mediate its transactivating function. (Tidy *et al.*,.1989b). The proposed existence of a less oncogenic subtype of the HPV 16 prototype provided a plausible explanation for the demonstration of HPV 16 in normal cervical tissue. However, doubts were cast on the existence of HPV 16b when other workers were unable to reproduce the results of Tidy and his co-workers. On reinvestigation of the original samples it was found that accidental contamination of the original DNA samples with products of an earlier PCR reaction had occurred. The findings were retracted. (Tidy *et al.*, 1989c) .

1.3. Epidemiological evidence : HPV and Carcinoma of the cervix.

In contrast to the experimental evidence linking HPV to carcinoma of the cervix, epidemiological evidence supporting this association is only now accumulating.In the past studies were often numerically small and either included inappropriate controls or were completely lacking controls. A further problem with many comparative studies was the use of different sampling methods for cases and controls. Biopsies were usually analyzed from cervical cancer patients whilst cytological specimens were often used for the control group. To convincingly demonstrate the association of HPV infection with carcinoma of the cervix it would be necessary to prospectively follow a large group of randomly selected women, examined for HPV infection and characterized for other risk factors, and to determine the incidence rate for carcinoma of the cervix. This is obviously not possible because of ethical considerations. (Munoz *et al.*, 1988). An alternative would be to use case/control studies to compare the prevalence of HPV infection in women with cancer of the cervix and women with normal cervices. (Munoz *et al.*, 1988).

A multicenter case/control study in Latin America of 756 cases of invasive carcinoma of the cervix and 1467 randomly selected age-matched controls showed that cervical infection with HPV 16 or 18 was strongly associated with cervical cancer. The presence of HPV DNA was detected using filter *in situ* hybridization. Seven hundred and fifty nine cases of invasive cancer of the cervix and 1466 randomly selected age matched controls were included in the study. Sixty-two percent of the cases had HPV 16/18 DNA compared to 32 percent of randomly selected controls. (Reeves *et al.*, 1989).

A further study suggested that the use of primer directed enzymatic amplification of specific target DNA sequences (the polymerase chain reaction) may be a useful epidemiological tool in investigating cervical HPV infection. In this study the authors demonstrated that 36/38 women with cytological abnormalities were infected with HPV 16. However 7/10 women with no cytological abnormality were also infected with HPV type 16, or a combination of HPV types 16 and 11, which suggests that HPV infection may be more common than previously suspected. (Young *et al.*, 1989). The polymerase chain reaction may be used for large scale epidemiological studies in the future.

1.4. Papillomaviruses and Malignancy

Papillomaviruses have been implicated in the development of squamous cell carcinomas in man and animals, but proving a causal relationship between a virus and a human cancer may be extremely difficult. Firstly, there may be a long latent period between the viral infection and the development of a cancer, and secondly, there is often a low prevalence of cancer amongst infected individuals. (Henderson, 1989).

The role of papillomaviruses in the development of genital malignancies has been extensively investigated. The papillomaviruses are a highly heterogeneous group of viruses, but despite the multiplicity of HPV types only a few types have been associated with naturally occurring cancers. (Campo, 1988). This observation has led to the hypothesis that some HPV have a "more malignant" potential than others. However, no obvious differences have been found in the genomic organization of the "more malignant" types. Comparison of 10 papillomaviruses whose complete sequences have been determined show that they share a similar genetic plan. (Giro and Danos, 1986). Alternatively it has been speculated that the greater malignant potential could be due to more subtle genetic differences or a greater propensity of the target cell for malignant transformation, or both. This is best illustrated by bovine papillomavirus 2 (BPV 2) which is strongly associated with neoplastic transformation in the bladder of cattle, but produces only benign proliferations in the skin and esophagus. (Jarret *et al.*, 1984).

1.4.1. Cellular transformation

Experimental work using bovine papillomavirus (BPV) has shown that both BPV-1 or its DNA can transform mouse fibroblasts in culture and that the morphologically transformed cells are tumorigenic for nude mice. (Dvoretzky *et al.*, 1980). The transforming activity has been localized to a fragment containing 69% of the bovine papillomavirus genome (Campo, 1988). Sequence analysis by Chen and his co-workers showed that this fragment contains the "early" ORFs and the non-coding regulatory region. (Chen *et al.*, 1982). The transforming function of the papillomaviruses has thus been clearly demonstrated, but the role of the virus in the progression to malignancy and the maintenance of the malignant state is less clear. (Campo, 1988). It is possible that papillomaviruses may prime the epithelium for

malignant change, but progression to the full malignant phenotype is dependent on co-factors (see below).

1.4.2. The physical state of the viral DNA

Early studies showed that the physical state of the viral DNA within the host cell is probably important in the development of papillomavirus associated malignancies. An analysis of the physical state of the viral DNA in cervical carcinoma tissue and derived cell lines, showed that the viral DNA is usually integrated into the host cell genome.(Durst *et al.*, 1985). It has been generally accepted that integration of HPV DNA usually results in interruption of the E1 or E2 ORFs of the viral genome with preferential retention of the LCR/E6/E7 region (see figure 1). E6 and E7 are thought to be essential in some stages of HPV induced cellular transformation. These genes may also be necessary for maintenance of the transformed phenotype. The long control region (LCR) contains promoter elements for early genes, a transcriptional enhancer and possibly the origin of replication of the virus. Therefore preferential retention of the LCR may be important in the development of the malignant phenotype either by ensuring continuous expression of the E6/E7 genes or by *cis* or *trans* activation of cellular oncogenes. (Choo *et al.*, 1987). However, recent studies of four clones of integrated HPV DNA showed that the interruptions of the viral genome occurred not only at the E1/E2, but also at L1/L2, indicating that there is no site specific for integration in the viral sequence. These investigators confirmed the retention of the E6/E7 and the LCR. (Wagatsukura *et al.*, 1990).

Controversy over the importance of the physical state of the viral DNA in the host cell has arisen from a recent report from Japan. These workers demonstrated that not only integrated but also episomal HPV DNA alone can be demonstrated in invasive carcinoma of the cervix. (Matsukura *et al.*, 1989). In addition, integrated HPV viral sequences have also been found in normal tissue and low grade neoplastic tissue indicating the importance of additional co-factors in the development of malignancy. (Schneider-Maunoury *et al.*, 1987). The importance of the physical state of the viral DNA within the host cell and the role of integrated viral DNA in the eventual progression to malignancy has therefore not been precisely defined.

1.4.3. Role of cellular oncogenes

With the discovery that viral DNA may be integrated into the host cell genome in cervical cancer it has been postulated that cell transformation may occur by insertional mutagenesis with consequent activation of cellular oncogenes. The insertion of a foreign genetic element near to cellular oncogenes controlling growth could affect their expression and lead to uncontrolled cell growth. (Campo, 1988). In several different carcinoma cell lines the HPV DNA sequences have been localized to regions containing cellular oncogenes such as c-*src*, c-*raf* and c-*myc*. (Durst *et al.*,1987). In addition, amplification and rearrangements of the cellular oncogenes c-*myc* and c-Ha-*ras* have been observed in cervical cancers harbouring HPV genomes. (Campo, 1988). A study by Riou *et al.*,1987 examined biopsies from patients with invasive carcinoma of the cervix for the expression of c-*myc* proto-oncogene. Biopsies were obtained from 72 patients with untreated invasive carcinoma of the cervix. The expression of c-*myc* was analysed by Northern blot and slot blot hybridization of total RNA. C-*myc* over expression was associated with a poorer prognosis and an increased incidence of early relapse. Riou and his colleagues therefore proposed that analysis of c-*myc* RNA in biopsies from patients with cervical carcinoma could possibly identify patients at greater risk for early recurrence. (Riou *et al.*, 1987).

1.4.4. Role of HPV gene products

As mentioned earlier the E6/E7 genes appear to be necessary for transformation. The E7 gene product possibly plays an important role in this process as the E7 protein of HPV is capable of binding to the retinoblastoma (RB) gene protein. The retinoblastoma gene polypeptide is absent in retinoblastoma tumours of the eye and it has been postulated that this polypeptide may play a critical role in limiting the proliferation of certain cells. The binding of the E7 protein to the retinoblastoma gene polypeptide product may therefore be associated with increased cell proliferation and oncogenesis and may play a necessary role in multistep tumorigenesis. (Marx, 1989). Further work on the importance of specific viral gene products showed that the presence of anti-E7 antibodies in the sera from patients correlated with the presence of cervical malignancy. Jochmus-Kudielka and her co-workers tested sera from patients with cervical cancer, patients with HPV associated lesions and patients attending hospital for reasons unrelated to HPV

infection for the presence of antibodies to HPV type 16 E4 and E7 proteins. The importance of the E7 protein in the development of cervical malignancy is highlighted above. The E4 protein was chosen because it was found to be most prominent in HPV induced skin warts. A significant association was demonstrated between the presence of antibodies to HPV E7 and the presence of cervical carcinoma. (Jochmus-Kudliekla *et al.*, 1989).

Differences between the oncogenic and non-oncogenic HPV types have been the subject of intense investigation. Gage and her co-workers demonstrated that the retinoblastoma (RB) protein preferentially binds to the phosphorylated form of HPV 6b E7, which comprises only a minor fraction of the total E7 expressed in a transfected cell line. The E7 gene product of HPV 16 was shown to bind more extensively to the RB protein. These characteristics may help to explain the difference in the oncogenic potential of the oncogenic and non-oncogenic types of genital papillomaviruses. (Gage *et al.*, 1990).

1.4.5. Role of Cofactors

The role of co-factors in the full expression of the malignant phenotype has been repeatedly emphasized. As mentioned above HPV 16 DNA can be demonstrated in normal tissue indicating the importance in additional factors in the progression to malignancy. A number of co-factors have been proposed in the development of cervical malignancy.

Hormones appear to act as tumour promoting agents in their ability to enhance the incidence of cancer. Durst *et al.*, 1989 demonstrated that hydrocortisone had a marked enhancing effect on Kirsten murine sarcoma virus (Ki-MSV) induced transformation in HPV 16 immortalized cells. (Durst *et al.*, 1989).

Smoking is an important risk factor in the development of genital cancer. Nicotine may give rise to the powerful carcinogens (N-nitrosomines) which have been demonstrated in vaginal fluids. (Pfister, 1987b).

In 1982 zur Hausen proposed that genital cancer results from a "promoting" papillomavirus infection in combination with a variety of initiating events. The initiating event may be in the form of a HSV infection, but other cofactors such as smoking and hormonal influences may be important in genital cancer development

in papillomavirus infected tissue. The genetic predisposition of the host may also be important in the full expression of the malignant phenotype. (zur Hausen, 1982). He recently re-emphasized the importance of co-factors in the development of genital malignancy at the VIII International Congress of Virology in Berlin in August 1990.

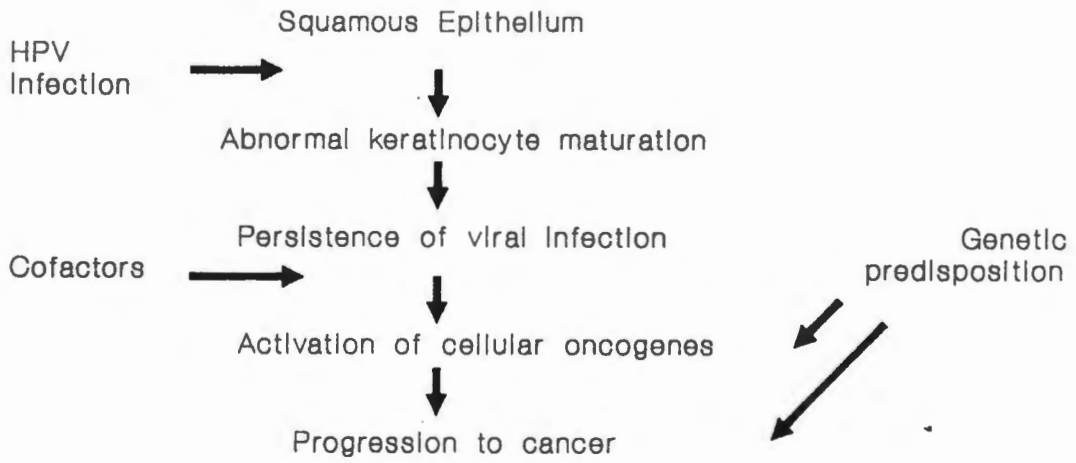

Figure 3: Model of synergism between a "promoting" papillomavirus infection and "initiating" events (cofactors) eg. smoking with the progression to malignancy. (Modified from zur Hausen, 1982).

1.5. Diagnosis of HPV infection

The diagnosis of HPV infection and research into the development of diagnostic tests are difficult as it is not possible to grow the virus *in vitro*, nor are there reliable serological tests. In addition, there are no satisfactory animal models for genital cancer. The human keratinocyte is the natural host for many HPV types, but keratinocytes are fastidious cells and are difficult to grow. Laboratory studies are therefore time consuming and require expertise.

1.5.1. Cytology and Histology.

Cells pathognomonic of HPV infection are known as koilocytes. These cells have an abnormal hyperchromatic nuclei surrounded by a zone of transparent cytoplasm. These light microscopic findings may provide useful information on the presence of HPV , but no information is obtained on the type of HPV.

1.5.2. Electron Microscopy

The success of electron microscopy in the diagnosis of HPV infection depends on the demonstration of viral particles. As viral expression and assembly takes place only in terminally differentiated cells, undifferentiated malignant cells do not usually contain HPV virions. (Caussy *et al.*, 1988). This technique is therefore not suitable for the detection of HPV infections in cervical cancer cells. A further drawback is that the morphology of the different HPV types is identical and no information is therefore obtained on the type of HPV present. (Caussy *et al.*, 1988).

1.5.3. Immunocytochemistry

Immunologic methods can be used to detect the presence of papillomavirus antigens by the peroxidase-anti-peroxidase method using primary antibodies to disrupted papillomavirus raised in rabbits. A major disadvantage of this method is that the commercially available antisera react with the group specific antigens of HPV and do not provide any information on the type of HPV present. (Roman and Fife, 1989).

1.5.4. Nucleic acid hybridization

DNA hybridization is currently the only technique which types HPV. Several modifications of this technique are currently available.

Southern Blot Hybridization

Southern blot hybridization is considered the "gold standard". Total cellular DNA is extracted and digested with a restriction endonuclease. The initial enzyme used for HPV typing is usually *Pst*I. After digestion with this enzyme the specific *Pst*I fragments are separated on an agarose gel. To detect the specific HPV fragments the DNA must be denatured, neutralized and transferred onto a nylon membrane by the process of Southern blotting. The nylon membrane is then probed with cloned HPV DNA. Disadvantages of this technique include the fact that it is extremely time consuming and requires relatively large amounts of DNA (10 ug) to achieve the desired sensitivity. Advantages include its sensitivity and specificity. In addition, it also provides suggestive information on the physical state of the viral DNA within the host cell, that is whether the viral DNA is present in an extrachromosomal or episomal form, or has integrated into the host cell chromosome as often occurs with malignancy. (Roman and Fife, 1989).

Dot blot hybridization

In this procedure the total cellular DNA is extracted from the biopsy, but it is not digested with a restriction endonuclease but applied directly to a nylon membrane. Dot blot hybridization has the advantage of being a relatively rapid procedure. It also requires less DNA than Southern blot hybridization. This technique must be adequately controlled to avoid false positive results.

Reverse blot hybridization.

In this technique the cellular DNA is radiolabeled and hybridized with known HPV types. The cloned HPV types are digested with the appropriate restriction endonuclease and the fragments are separated by gel electrophoresis. The DNA is then transferred onto a nylon membrane and probed with radiolabelled cellular DNA. (De Villiers *et al.*, 1986).

Tissue *in situ* hybridization

Tissue *In situ* hybridization has the advantage of providing information on the localization of the HPV genomes in the specimen. The DNA is not isolated from the specimen, but the tissue sections are probed directly. This technique can be used for paraffin sections and allow retrospective studies to be performed. (Beckman *et al.*, 1985) .

Filter *in situ* hybridization

In this technique the cells are applied directly to a filter and disrupted on the filter. The DNA is then denatured and neutralized and hybridized with a cloned HPV probe. An advantage of this technique is that it allows detection of DNA in material from scrapings and smears. However, as with dot blot hybridization, this technique is also prone to false positive results. (De Villiers *et al.*, 1987).

1.5.5. The polymerase chain reaction.

The polymerase chain reaction is a new and extremely powerful tool in the field of molecular biology. The technique selectively amplifies a specific target sequence of DNA. The amplification primers used in the reaction are oligonucleotides which flank the sequence of interest. The DNA extracted from the specimen is denatured and then allowed to anneal with the primers. In the presence of a DNA polymerase a new strand of DNA will be synthesized using the oligonucleotide as a primer. Repeated cycles of denaturing, annealing and primer extension allows the specific target sequence to be amplified. After amplification is completed the presence of the amplified target sequence should be confirmed. Knowledge of the target sequence makes it possible to predict the size of the amplified product. The size of the amplified product can be estimated by comparing it to a molecular weight marker on an agarose gel. The identity of the amplified sequence should be confirmed by hybridization. (Roman and Fife, 1989; Eisenstein, 1990).

2. Aim of the study

Cancer of the cervix is the most common cancer among women in South Africa, with an incidence of 0,6/1000 among black women. (Cronje, 1989) No information is currently available on the frequency and types of human papillomaviruses found in patients with invasive carcinoma of the cervix in South Africa. A study by Williamson *et al.*, 1989 showed that biopsies from 69% of patients with cervical intraepithelial neoplasia grade 3 (CIN 3) contained HPV DNA by Southern blot hybridization. (Williamson *et al.*, 1989) This confirmed the presence of human papillomavirus infection in patients with premalignant genital lesions in our geographical region.

The aim of this study was therefore to determine:

1.The frequency of HPV infection in patients with invasive carcinoma of the cervix in patients attending the Gynaecological Oncology Clinic at Groote Schuur Hospital.

2. The types of HPV present in these patients.

3. Methods

3.1. Study population and sample collection

Biopsies were obtained from 93 consecutive patients with invasive carcinoma of the cervix attending the Combined Assessment Clinic at Groote Schuur hospital. This clinic is run by members of the Radiotherapy and Gynaecology departments, as well as nursing staff, and provides a multidisciplinary approach to patients with genital malignancies. Punch biopsies were taken from the center of the cervical lesion by an experienced gynaecologist with a special interest in oncology. Each biopsy was divided in half. Half the biopsy was preserved in formalin for histology and the other half was transported without delay to the virology laboratory and stored at -70^{0}C for DNA extraction.

Biopsies from 75 patients were histologically classified as squamous carcinoma with invasion. A further 6 were classified as adenocarcinomas, 3 as adenosquamous carcinomas and 1 as an undifferentiated carcinoma. In 8 patients the histopathology results were not available.

3.2. Preparation of biopsy DNA

3.2.1. DNA Extraction

Just prior to processing, the biopsies were thawed and a small amount of ground glass was added to each biopsy. The biopsies were ground in a mortar and pestle and suspended in 4ml of lysis buffer containing 100mM Tris, 100mM NaCl, 5mM EDTA (pH 7,5) and 1% SDS w/v and transferred to a conical polypropylene tube. (Falcon, New Jersey, USA). (Crum *et al.*, 1985). Proteinase K (Boehringer-Mannheim, FRG) was added to a final concentration of 100 ug/ml of the mixture which then was incubated for at least 3 hours at 56^{0}C. The DNA was extracted twice with phenol. One milliliter of ultrapure water (BDH Ltd., England) was added to the interface of the first extraction which was re-extracted with phenol, and the aqueous phases were pooled prior to the second phenol extraction. (Williamson *et al.*, 1989). The DNA was then extracted once with chloroform: isoamyl alcohol (24:1). After the chloroform extraction the aqueous phase containing the DNA was transferred into microfuge tubes (Eppendorf, FRG) and

3M CH_3COONa was added to a final concentration of 0,3M CH_3COONa. Two volumes of 100% ethanol were added and the microfuge tubes were placed on dry ice for 15 minutes. The DNA was recovered by centrifugation in a Sigma 201M microfuge at 10 000 rpm for 15 minutes. The pellets were washed with 500ul of 70% alcohol, centrifuged at 10 000rpm for 15 minutes, dried in a dessicator, resuspended in 10 ul of ultra-pure water and stored at 4^0C.

3.2.2. Estimation of the amount of DNA

Ten microliters of each sample of biopsy DNA was subjected to electrophoresis in a 1% agarose gel at 80 volts for.1 hour. A *Hind* III digest of 1ug of lambda phage DNA was included in each gel. The gel was then stained with ethidium bromide at a concentration of 0,5ug/ml of water and viewed on an ultraviolet transilluminator. The bands of DNA in each lane were visually evaluated. (Berger and Kimmel, 1987).

3.3. Restriction endonuclease digestion and agarose gel electrophoresis

Four to eight micrograms of biopsy DNA were utilised in each reaction. One unit of the restriction enzyme *PstI* together with the appropriate buffer (Boehringer Mannheim, FRG) was added for each estimated microgram of sample DNA. Reaction mixtures were incubated for 1 hour at 37^0C prior to termination with 100mM NaEDTA (ph 8), 1% SDS, 0,1% bromophenol blue and 0,1% xylene cyanol.

One per cent agarose (SeaKem, FMC Bioproducts, USA) was dissolved in Tris-acetate buffer (0,04M Tris-acetate and 0,001M EDTA). and poured at 56^0C into appropriately sized molds. The gels were placed in submarine electrophoresis tanks with Tris-acetate buffer. Samples digested with *PstI* were loaded into each well. Lambda DNA digested with *PstI* was loaded into the first and last well. The DNA fragments were separated at a constant voltage of 80 volts for 4 hours or overnight at 30 volts.

Gels were stained with the fluorescent dye, ethidium bromide, at a concentration of 0,5ug/ml of water and visualized on an ultraviolet transilluminator. (Maniatis, 1982).

3.4. Photography

After staining with ethidium bromide the agarose gel was photographed on an ultraviolet transilluminator. Two photographs were taken, one with a ruler aligned vertically and one with a ruler aligned horizontally to facilitate later interpretation of fragment size.

3.5. Comparison of methods used for transfer of DNA fragments

In order to optimize transfer of DNA fragments onto a nylon membrane, (Hybond N+, Amersham, UK), an experiment comparing Smith and Summers Southern blotting with classical Southern blotting and electroblotting was performed. Plasmid (pBR 322) DNA containing cloned HPV 16 DNA was quantitated using the method described for biopsy DNA and digested with the restriction endonuclease *Pst*I.. Serial tenfold dilutions from 1ug to 1 pg of the digested cloned HPV 16 DNA were made. Equivalent amounts of DNA were subjected to electrophoresis on three 1% agarose gels. The following methods of DNA transfer to a nylon membrane were compared:

3.5.1. Smith and Summers Southern blotting

After electrophoresis one of the agarose gels was placed in 0,25M HCl and gently agitated for 20 minutes. The gel was then rinsed in distilled water and placed in 0,5M NaOH and 1,5M NaCl for 20 minutes to denature the DNA fragments. After rinsing, the gel was neutralized in 1M $CH_3COOHNH_4$ and 0,02M NaOH for 40 minutes. The agarose gel was then placed onto a solid surface. A nylon membrane exactly the size of the gel was placed on top of the gel. Care was taken to avoid trapping bubbles beneath the membrane. Three sheets of Whatman 3mm filter paper were placed on top of the nylon membrane and a stack of paper towels was placed on top of the filter paper. The filter paper and stack of paper towels were weighted to ensure even transfer of the DNA fragments.

3.5.2. Classical Southern blotting:

After electrophoresis a second agarose gel was placed in 0.25M HCl for 20 minutes. The gel was then rinsed in distilled water and a tray with a platform covered with a wick made from 3 sheets of Whatman 3MM filter paper was filled with 0,4M NaOH. The agarose gel was placed on the wick and a sheet of the nylon membrane exactly the size of the gel was placed on top of the gel. Care was taken to avoid trapping bubbles beneath the membrane. Three sheets of Whatman 3MM filter paper, a stack of absorbent paper towels and weights were placed on top of the nylon membrane. Transfer was allowed to proceed over night.

3.5.3. Electroblotting

After electrophoresis DNA in the third agarose gel was denatured and neutralized as described for the Southern blotting procedure. The agarose gel was then placed in 25mM Tris, pH 7.4 for 10 minutes. A sponge saturated with 25mM Tris and piece of blotting paper were placed on top of a graphite anode. The nylon membrane was placed on top of the blotting paper, followed by the agarose gel, another piece of blotting paper and a second sponge saturated with 25mM Tris. A graphite cathode was placed on top of the sponge. Electrophoretic transfer was then allowed to proceed over 4 hours at a constant voltage of 100 volts. (Rybicki - personal communication).

The DNA fragments on all three of the nylon membranes were fixed by placing each membrane on two to three pieces of blotting paper soaked in 0,4M NaOH for twenty minutes.

3.6. Standardization of conditions used to suppress background radioactivity

An experiment was performed to determine the optimal conditions for prehybridization. Dilutions of cloned HPV 16 were made as described above. Two prehybridization solutions were compared:

1. The nylon membranes were prehybridized overnight in 6xSSC (0,9M NaCl and 0,9M sodium citrate), 30% formamide v/v, 0.02% salmon sperm w/v (Sigma, St. Louis) and 0,5% w/v fat-free milk powder at 42^0C (Williamson *et al*, 1989).

2. The nylon membranes were prehybridized in a 5% solution of commercial blocking reagent w/v (Boehringer Mannheim, FRG) dissolved in 6xSSC with 30% formamide v/v and 10% Dextran Sulphate w/v at 42^0C overnight. Samples were processed using this method.

3.7. Plasmids

Plasmids containing cloned HPV 16 and 18 DNA were provided by Dr E-M de Villiers (German Cancer Research Centre, Heidelberg, FRG), plasmid containing HPV 31 and 35 were provided by Dr A.T. Lorincz (Bethesda Research Laboratories, Gaithersberg) and plasmid containing HPV 33 was provided by Dr. G. Orth (Pasteur Institute, Paris).

3.7.1. Transformation of competent (DK-1) cells with HPV DNA

Competent cells (DK-1 cells, an *E. Coli* strain) were kindly prepared by Karen Pratt in the Molecular Biology Unit, Department of Medical Microbiology, University of Cape Town.

Ten microliters of each HPV plasmid, containing less than 40ng of DNA was used. The amount of plasmid DNA was calculated from the concentration value supplied by the laboratory donating the plasmid. This was added to 200ul of DK-1 cells with a transforming efficiency of $5x10^6$ transformants per microgram of DNA. The mixture was placed on ice for 30 minutes. The tubes were then transferred to a

water-bath and heat shocked for 2 minutes at 42^0C. One milliliter of Luria broth was added to each tube and the mixture was incubated for 1 - 3 hours. Two hundred microliters of this transformation mixture was spread onto Luria agar plates containing ampicillin. The plates were allowed to dry and then inverted and incubated at 37^0C overnight.

The following day a single colony was selected and inoculated into 5 ml of Luria broth and incubated overnight. (Maniatis, 1982).

3.7.2. Plasmid mini-preparations

Three milliliters of the above *E. Coli* culture was centrifuged in microfuge tubes. A minipreparation kit (Diagen, FRG) was used according to the manufacturers instructions. Briefly, the bacterial pellet was resuspended in a buffer containing RNase A (100 mg/ml) in 50mM Tris/HCl and 10mM EDTA, pH 8. Three hundred microliters of a second buffer containing 200mM NaOH and 0,1% SDS w/v was added and the mixture was incubated at room temperature for five minutes. Three hundred microliters of a third buffer containing 2,55M CH_3COOK was then added, the solution was mixed gently and centrifuged in microfuge tubes for 15 minutes. The supernatant was removed. An anion exchange resin (Quigen mini-column, Diagen, FRG) was equilibrated with 1 ml of a buffer containing 750mM NaCl, 50mM MOPS (3-N-morpholino-propanesulphonic acid) and 15% ethanol v/v, pH 7. The supernatant containing the plasmid DNA was added to the column. Impurities were eluted from the column using a buffer containing 1,0M NaCl, 50mM MOPS and 15% ethanol v/v, pH 7.0. The plasmid DNA was eluted with a buffer containing 1,2M NaCl, 50mM MOPS and 15% ethanol, pH 8.

The plasmid DNA was precipitated as described for the processing of the biopsy DNA and dissolved in 50ul of TE buffer (10mM Tris/HCl and 1mM EDTA).

Ten microliters of the plasmid DNA solution were digested with the restriction endonuclease *Pst*I. The plasmid DNA fragments together with a known quantity of a DNA molecular weight marker (lambda phage DNA digested with the restriction endonuclease *Pst*I) were separated by electrophoresis on a 1% agarose gel. The agarose gel was stained with ethidium bromide at a concentration of 0,5ug/ml of water and viewed on an ultraviolet transilluminator. The size of the fragments

obtained after digestion of the plasmid DNA and separation of the fragments by electrophoresis was determined.

3.8. Preparation of the HPV DNA probe.

3.8.1. Preparation of HPV specific fragments

HPV 16 plasmid DNA was incubated with the appropriate volume of the restriction enzyme*Bam* HI and restriction buffer to excise the HPV specific fragment. Plasmid DNA and the HPV 16 specific fragment were separated by electrophoresis at a constant voltage of 80 volts for 1 hour. The piece of agarose gel containing the HPV fragment was excised, cut into fine pieces and extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1). The DNA was recovered by ethanol salt precipitation. HPV DNA fragments were prepared in the same way for HPV types 18, 31, 33 and 35 using the appropriate restriction endonuclease to excise the HPV specific fragment. (*Bam*HI for HPV 35, *Eco*RI for HPV 18 and 31 and *Bgl*II for HPV 33). The concentration of the HPV specific fragment was estimated using the method described above for quantitation of biopsy DNA.

3.8.2. ^{32}P Labeling

The HPV DNA specific fragments were labelled with ^{32}P by random priming. (Multiprime DNA labeling system, Amersham, U.K.) The kit was used according to the manufacturers instructions. Twenty-five nanograms of the appropriate HPV specific DNA fragment was denatured by heating to 95 - 100^{0}C for 2 minutes and then chilled on ice. Ten microliters of a buffer supplied by the manufacturers containing unlabeled nucleotides (dATP, dTTP and dGTP) was added to the DNA solution followed by 5 ul of a solution containing random sequence hexamers. The volume was adjusted to 50ul by the addition of deionized water. Five microliters of radiolabelled (^{32}P) nucleotide (dCTP) and 2ul DNA polymerase were then added. The reaction was then incubated at room temperature for at least 3 hours.

3.8.3. Removal of unincorporated label by Sephadex G-50 chromotography

The reaction mixture was layered onto the surface a column of Sephadex G-50. (Pharmacia, Sweden). Fractions of 80ul were eluted from the column using a buffer containing 150mM NaCl, 10mM EDTA, 0,1% SDS (w/v) and 50mM Tris. The radioactivity of the eluted fractions was determined using a hand-held Geiger counter and the fractions containing labelled DNA were pooled. The DNA was then denatured by heating. (Maniatis, 1982).

3.9. Hybridization and posthybridization washes.

The pooled radiolabelled probe was added to the prehybridization buffer and hybridized to the biopsy DNA bound to the nylon membrane for 24 - 48 hours at 42^0C.

Unbound radiolabelled HPV probe was removed from the nylon membrane by 1 wash of 2xSSC with 0,1% SDS w/v followed by 2 washes of 0,5xSSC with 0,1% SDS w/v at room temperature.

3.10. Autoradiography and estimation of DNA fragment size

The nylon membranes were wrapped in a plastic folder and placed on a piece of X-ray film (Curix RPI, Bayer, South Africa) in an X-ray cassette with two image intensifying screens. This was stored at -70^0C for 1 - 3 weeks. The X-ray films were developed using Ilford Phenisol x-ray developer and fixed with Amfix high speed fixer (Maybaker, Port Elizabeth).

The distance travelled by the DNA fragments detected by hybridization was measured and the molecular weight was calculated using a computer programme "Gel".

3.11. The Polymerase Chain Reaction for the detection of HPV 16

The polymerase chain reaction was performed using a DNA amplification reagent kit (GeneAmp, Perkin Elmer Cetus, Norwalk, USA) according to the manufacturers instructions. Great care was taken to avoid DNA contamination. Reactions were performed in capped 0,5ml polypropylene microfuge tubes. A clinical specimen in which HPV 16 had been detected with Southern blot hybridization was used as a positive control.

Primers with the following sequences were used for PCR:

Primer 1 5'AGGCCAACTAAATGTCAC (Tidy *et al.*, 1989)

Primer 2 5'GGTGCATAAAATGTCTGC (A-L Williamson - personal communication from Seedorf *et al.*, 1985)

A mixture of the polymerase chain reaction components was made containing 0,6ug of each primer (2ul of each primer) and 0,2mM (1ul) each the nucleotide solutions dATP, dGTP, dCTP and dTTP supplied by the manufacturer per reaction. Reaction buffer containing a final concentration of 10mM Tris-HCl, 50mM KCl, 1,5mM $MgCl_2$ and 0,001% gelatin w/v was added. A recombinant *Taq* DNA polymerase "Amplitaq" (Perkin Elmer Cetus, Norwalk, USA) was used at a concentration of 2,5 units/assay. Biopsy DNA (0,6ug) was added and the final volume was adjusted to 50ul/reaction with distilled water.

A thermal cycler (Techne PHC-2) was used. The DNA was first denatured at 94^0C for 4 minutes. This was followed by 35 cycles of denaturation (1 minute at 94^0C), annealing (2 minutes at 45^0C) and extension (3 minutes at 72^0C). After completion of the 35th cycle of amplification, extension was completed at 72^0C for 7 minutes.

Fifteen microliters of the amplified reaction mix was then subjected to electrophoresis on a 3% agarose gel together with molecular weight markers (DNA molecular weight marker V1 - Boehringer Mannheim, FRG). Ethidium bromide was incorporated into the gel at a final concentration of 0,5ug/ml. The distance travelled by the amplified PCR product was compared to the distance travelled by the molecular weight marker.

To confirm the specificity of the amplified product, the DNA fragments were transferred onto a nylon membrane (Hybond N+, Amersham, UK) using the Smith and Summers modification of Southern blotting and treated with a commercial blocking reagent (Boehringer Mannheim, FRG) dissolved in 6XSSC with 30% formamide v/v at 42^0C overnight.

A HPV type 16 probe consisting of amplified plasmid was labelled with ^{32}P.(Multiprime DNA labeling system, Amersham, UK). Preparation of the DNA probe, hybridisation, post-hybridisation washes and autoradiography were performed as described above.

4. Results

4.1. Determination of optimal conditions for transfer of DNA fragments to a nylon membrane

The lowest dilution of plasmid detected using electroblotting and Smith and Summers Southern blotting was 1ng compared to 10ng for classical Southern blotting.

4.2. Standardization of conditions used to suppress background radioactivity

The result of pre-treating nylon membranes a prehybridization solution containing a 5% solution of a commercial blocking agent (Boehringer Mannheim, FRG) dissolved in 6xSSC with 30% formamide v/v and 10% Dextran sulphate w/v is shown in figure 9a. There is a marked reduction in background radioactivity when compared to the nylon membrane treated with a prehybridization solution containing 6xSSC, 30% formamide, 0.02% salmon sperm w/v and 0,5% v/w fat-free milk powder. (figure 9b).

4.3. Southern blot hybridization

A total of 86/93 biopsies were processed by the method described using the Smith and Summers modification of Southern blotting. Each specimen was probed with cloned HPV types 16, 18, 31, 33 and 35. (See table 1). Autoradiographs with no detectable HPV DNA after 3 weeks were considered to be negative.

4.3.1. HPV 16 probe.

A typical pattern was seen in 13/86 of the biopsies after digestion with the restriction endonuclease *Pst*I. This pattern is illustrated in lane 3 of figure 4 and lanes 1 and 2 of figure 5b. Biopsy DNA from a further 3 cases (cases 7, 27 and 91) hybridized with the cloned HPV 16 probe, however the pattern obtained differed from that of the typical HPV 16 pattern. (See figure 4, lane 1).

4.3.2. HPV 18 probe

Two out of a total of 86 biopsies showed a typical pattern with the HPV 18 probe. (See table 1). Figure 7 shows a photograph of an autoradiograph with biopsy DNA digested with *Pst*I. Two fragments of 6337 bp and 555 bp are visible.

4.3.3. HPV 31 probe

Biopsy DNA from patient 73, a 44 year old coloured women showed a pattern consistent with HPV 31.

4.3.4. HPV 33 probe

Biopsy DNA from 1 patient hybridized with a cloned HPV 33 probe with a pattern consistent with HPV 33.

4.3.5. HPV 35 probe

No hybridization consistent with the pattern of HPV 35 was obtained after *Pst*1 digestion of biopsy DNA

4.3.6. Unclassified patterns

In 18/86 patients the biopsy DNA obtained cross-hybridized with one or more of the cloned HPV DNA probes but the pattern obtained was variable. Biopsy DNA from 11 patients cross-hybridized with the cloned HPV 16 probe, 3 cross-hybridized with the HPV 33 probe and 1 with the HPV 31 probe. A further 3 biopsies cross- hybridized with a combination of HPV DNA probes - 1 with HPV 16 and 31, 1 with HPV 16 and 18 and 1 with HPV 16 and 35. Lane 10 of figure 6 shows biopsy DNA from patient 14 cross-hybridizing with the HPV 33 probe.

Biopsy DNA which did not hybridize with any of the probes used was further investigated for the presence of HPV 16 DNA using the polymerase chain reaction. Another 7 biopsies with insufficient DNA for processing by Southern blot hybridization were also investigated using this technique.

Figure 8 shows the detection of a 229bp amplified fragment with a PCR generated HPV 16 fragment labelled with ^{32}P. The positive control (lane 2) was biopsy DNA from one of the patients with carcinoma of the cervix in which HPV 16 was detected by Southern blot hybridization. In lanes 8, 11 and 13 samples containing amplified DNA hybridizing with the HPV 16 probe can be seen. The signal in lane 11 is extremely faint, but became clearer after the autoradiograph was exposed for a longer time. In some of the specimens a lower molecular weight product cross-hybridized with the probe. (lane 17) This was thought to be an artifact caused by primer oligomerization. Amplification of the 229 bp fragment which hybridized with a HPV 16 probe was obtained in 3/48 biopsies with no detectable HPV DNA by Southern blot hybridization and in 2/7 of the biopsies with insufficient DNA for Southern blot hybridization.

Table 1

Detection of HPV 16, 18, 31, 33 and 35 in patients with invasive carcinoma of the cervix in Cape Town

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
1	60	Black	Squamous		HPV 16	
2	42	Black	Squamous	Unclass.		
3	44	Black	Squamous			
4	50	Coloured	Squamous	HPV 33		
5	77	Black	Squamous	HPV 16		Extra bands
6	46	Coloured	Squamous			
7	42	Black	Adeno-sq	HPV 16 variant		Extra bands
8	66	Black	Adenoca			
9	76	Coloured	Squamous	HPV 16		
10	73	White	Squamous			
11	68	Black	Squamous			
12	38	Coloured	Squamous			
13	78	Coloured	Squamous	Unclass.		

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
14	35	Black	Undiff.	Unclass.		
15	63	Black	Squamous		HPV 16	
16	50	Coloured	Squamous			
17	56	Coloured	Squamous	Unclass.		
18	63	White	Squamous	HPV 16		
19	67	Coloured	Squamous			
20	60	Coloured	Squamous			
21	45	Black	Squamous			
22	40	Black	Squamous	HPV 16		
23	29	Coloured	Adeno	HPV 18		
24	41	Black	Squamous			
25	66	White	Squamous	HPV 16		Extra bands
26	44	White	Squamous			
27	56	Coloured	Squamous	HPV16 variant		
28	46	Coloured	Squamous	HPV 16		Extra bands
29	41	Coloured	Squamous			

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
30	47	Coloured	Adenoca			
31	66	Coloured	Squamous	Unclass.		
32	54	Coloured	Squamous			
33	50	Black	Squamous			
34	36	Coloured	Adeno-sq	HPV 16		Extra bands
35	55	Black	Squamous	Unclass.		
36	57	Coloured	Squamous			
37	28	Coloured	Squamous	Unclass.		
38	34	Coloured	Squamous			
39	34	Coloured	Squamous			
40	47	Coloured	Squamous	Unclass.		
41	42	Black	Squamous			
42	42	Coloured	Squamous			
43	77	Coloured	Squamous			
44	68	White	Squamous	Unclass.		
45	50	Black	Adenoca			
46	30	Black	Adenoca			

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
47	42	Black	Squamous			
48	69	Coloured	Squamous	Unclass.		
49	60	Black	Squamous			
50	57	Coloured	Squamous			
51	52	Coloured	Not available	Unclass.		
52	28	White	Squamous	Unclass.		
53	50	Black	Squamous			
54	27	Black	Squamous	HPV 18		
55	44	Coloured	Squamous	HPV 16		Extra bands
56	57	Coloured	Squamous			
57	58	Coloured	Squamous			
58	59	Black	Adeno-sq	Unclass.		
59	43	Black	Squamous	Unclass.		
60	56	White	Squamous	Unclass.		
61	53	Coloured	Squamous			
62	35	Black	Squamous			
63	30	Black	Squamous	Unclass.		

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
64	40	Black	Squamous			
65	54	Black	Squamous	HPV 16		
66	48	Coloured	Squamous			
67	51	Coloured	Squamous		HPV 16	
68	56	Coloured	Squamous			
69	39	Coloured	Adenoca			
70	57	Black	Adenoca			
71	53	Coloured	Squamous			
72	48	Black	Squamous			
73	44	Coloured	Squamous	HPV 31		
74	48	Black	Squamous	Unclass.		
75	58	Coloured	Squamous			
76	55	Coloured	Squamous		HPV 16	
77	48	Black	Squamous			
78	58	Black	Squamous			
79	53	Black	Squamous			
80	48	Coloured	Squamous		HPV 16	
81	43	Coloured	Squamous			

Patient No.	Age	Race	Histopath	HPV Probe	HPV 16 PCR	Comment
82	52	Coloured	Squamous	HPV 16		
83	38	Coloured	Not available			
84	67	Coloured	Squamous			
85	N/A	N/A	Not available	Unclass.		
86	N/A	N/A	Not available			
87	42	Coloured	Squamous	HPV 16		
88	44	Coloured	Squamous			
89	31	Coloured	Squamous	HPV 16		Extra bands
90	N/A	N/A	Not available			
91	N/A	N/A	Not available	HPV 16 variant		
92	N/A	N/A	Not available			
93	N/A	N/A	Not available	HPV 16		Extra bands

5. Discussion

5.1. Quantitation of biopsy DNA.

The quantity of DNA recovered from each biopsy was estimated by the intensity of the ethidium bromide staining when compared to a known amount of *Hin*d III digested lambda DNA fragments subjected to electrophoresis in the same 1% agarose gel. This method provides a crude estimation of the amount of DNA obtained from each biopsy. Shearing of DNA that inevitably occurs during preparation allows the biopsy DNA to migrate into the 1% agarose gel and enables rough quantitation of the biopsy DNA.

5.2. Determination of the optimal conditions for transfer of DNA fragments to the nylon membrane.

There was a tenfold reduction of sensitivity when classical Southern blotting was compared to Smith and Summers Southern blotting and electroblotting. As transfer of DNA fragments appeared to be less efficient in classical Southern blotting, it was decided to use the Smith and Summers modification of Southern blotting to process the clinical specimens.

5.3. Optimization of conditions to suppress background radioactivity

This experiment clearly demonstrated that a 5% solution of a commercial blocking agent (Boehringer Mannheim, FRG) allowed adequate detection of the HPV specific fragments with minimal non-specific binding of ^{32}P to the nylon membrane. (See figure 9).

5.4. Classification of HPV types by Southern blot hybridization

The HPV types were classified by the *Pst*I fragment lengths. (see appendix). The *Pst*I fragment sizes were calculated using the computer programme "Gel" by measuring the distance travelled by the HPV DNA fragment and comparing this to the distance travelled by the molecular weight marker. This method utilizes the fact that the reciprocal of the mobility plotted against fragment length is linear. A least square analysis is used. (Schaffer and Sederoff, 1981).

A variety of *Pst*I fragments may be observed in the autoradiograph demonstrated in figure 4. The HPV 16 pattern is demonstrated in lane 3. The pattern observed correlates with the fragments obtained from *Pst*I digestion of HPV 16. (Baker, 1987). In lane 1 of figure 4 is a possible variant of HPV 16. Fragment A is slightly smaller (2700 bp) when compared to the HPV 16 prototype fragment of 2800 bp and fragment D was slightly larger (1200 bp) than the HPV 16 prototype fragment of 1000 bp. Fragments B and C were identical in size to the HPV 16 prototype fragments. Interestingly 3% of the biopsies investigated by Southern blot contained this HPV 16 "vatiant".

In figure 6 a *Pst*I digest of biopsy DNA conforming with the HPV 33 prototype can be seen in lane 1. (see appendix). Biopsy DNA in lanes 2, 4 and 6 contain HPV 16 cross-hybridizing with the cloned HPV 33 probe. (see below).

HPV 18 DNA was detected in biopsies obtained from 2 patients. Figure 7 shows the detection of HPV 18 in a 29 year old female with an adenocarcinoma of the cervix. The presence of HPV 18 was further confirmed by the sequencing of a PCR generated fragment by Susan Dennis from the Department of Microbiology, University of Cape Town. (Williamson - personal communication).

Twenty-one per cent (18/86) of biopsies from patients with invasive carcinoma of the cervix contained "unclassified" HPV DNA. Biopsy DNA from these patients cross-hybridized with one, or more of the cloned HPV DNA probes but the patterns obtained did not match that of HPV types 16, 18, 31, 33 or 35. Most of these (11/18) biopsies cross-hybridized with the HPV 16 probe, 3 cross-hybridized with the HPV 33 probe and 1 with HPV 31 probe. A further 3 biopsies cross-hybridized with more than one of the HPV DNA probes - 1 with HPV 16 and 31, 1 with HPV

16 and 18 and 1 with HPV 16 and 35. Most of the unclassified types appeared to be distinct from each other. Figure 5a shows a biopsy containing an unclassified HPV type. In this example the biopsy hybridized with cloned HPV 16 under conditions of moderate stringency, generating 2 fragments of 2818 bp and 1776 bp respectively. These are identical to the molecular weights of fragments A and B of the HPV 16 prototype. Although this biopsy was included as an unclassified HPV type, it could possibly be an integrated form of HPV 16 (see below). Another unclassified HPV type is demonstrated in lane 10 of figure 6. The biopsy DNA cross-hybridized with the cloned HPV 33 probe generating a single fragment.

Table 2 summarizes the HPV types detected in this study.

Table 2

HPV types in biopsies obtained from 86 patients with carcinoma of the cervix detected by Southern blot hybridization

HPV PROBE	NUMBER POSITIVE/TOTAL	PERCENTAGE POSITIVE
HPV 16	13/86	15%
HPV 16 variant	3/86	4%
HPV 18	2/86	2%
HPV 31	1/86	1%
HPV 33	1/86	1%
HPV 35	0/86	
Unclassified	18/86	21%
TOTAL	38/86	44%

HPV 16 was detected in only 15% of biopsies obtained from patients with invasive carcinoma of the cervix in Cape Town. This is somewhat lower than many of the studies. However, comparison with a study by Williamson *et al.*, 1989 on the molecular typing in patients with CIN 3 in Cape Town showed a similar detection rate of HPV infection, although significantly less HPV type 33 was detected in patients with carcinoma of the cervix in Cape Town. (Williamson *et al.*, 1989) The number of unclassified HPV types detected in patients with carcinoma of the cervix was also lower. (see Table 4 below)

Table 4

Comparison of the prevalence of HPV infection in biopsies obtained from patients with CIN 3 and invasive carcinoma of the cervix in Cape Town.

HPV Type	Number positive (%)	
	CIN 3*	Carcinoma of the cervix
HPV type 16	16/98 (16%)	13/86 (15%)
HPV type 16 "variant"	6/98 (6%)	3/86 (4%)
HPV type 18	1/98 (1%)	2/86 (2%)
HPV type 31	2/98 (2%)	1/86 (1%)
HPV type 33	8/98 (8%)	1/86 (1%)
HPV 35	0/98	0/86
Unclassified	35/98 (36%)	18/86 (21%)
TOTAL	68/98 (69%)	38/86 (44%)

*Williamson *et al.*, 1989

5.6. Sensitivity of detection of HPV DNA.

The lower prevalence of HPV 16 in our patients with genital malignancies was of some concern to us. The study from Germany by Durst *et al.*, 1983 showed that 61% of patients with invasive carcinoma of the cervix had HPV 16 DNA detected by Southern blot hybridization. (Durst *et al.*, 1983). Although Fukushima *et al.*, 1985 showed that only 11% of biopsies from patients with invasive squamous carcinoma of the cervix contained HPV 16 DNA, their study was numerically small consisting of a total of 11 patients. (Fukushima *et al.*, 1985). Other studies reported 40% or more of biopsies containing HPV 16 DNA. (Durst *et al.*, 1983; McCance *et al.*, 1985 and Choo *et al.*, 1987).

Of importance, however, was the fact that the study by Williamson *et al.*, 1989, on the molecular typing of HPV in patients with cervical intraepithelial neoplasia grade 3 in Cape Town showed a similar detection rate of HPV infection using HPV 16, 18, 31, 33 and 35 probes, although significantly less HPV 33 was detected in patients with carcinoma of the cervix in Cape Town. (Williamson *et al.*, 1989). This similarity in the prevalence of HPV infection in patients with CIN 3 and carcinoma of the cervix in Cape Town suggest a truly low prevalence of HPV 16, 18, 31, 33 and 35 in this region.

This low prevalence was further substantiated by the use of the polymerase chain reaction for the detection of HPV 16 in biopsies from patients in this study who had no detectable HPV DNA on Southern blot hybridization, or where there was insufficient DNA for Southern blot hybridization. The primers used were designed for selective DNA amplification of the upstream regulatory region, as this region together with the E6 and E7 regions are almost always retained when HPV DNA integrates in malignant tumours. (Tidy *et al.*, 1989b). Three of the biopsies with no detectable HPV DNA on Southern blot hybridization contained HPV 16 DNA using the polymerase chain reaction. Assuming that in all the samples with detectable HPV 16 DNA by Southern blot hybridization selective DNA amplification of HPV 16 would also be possible, the use of the polymerase chain reaction has enhanced the sensitivity to a total of 16/86 (19%) containing HPV 16 DNA. This also assumes that no mixed infection existed in the other biopsies with detectable HPV DNA. In a further two biopsies in which selective DNA amplification of HPV 16

was achieved, using the polymerase chain reaction, the amount of DNA for detection using Southern blotting was judged to be insufficient.

The results obtained from the use of the polymerase chain reaction in this study, together with the similarity of the results obtained by Williamson *et al.*, 1989 in their study on cervical intraepithelial neoplasia in Cape Town, indicate that the low prevalence of HPV 16 in this region is not due to an inadequate detection system by Southern blot hybridization.

The polymerase chain reaction is theoretically capable of detecting a single virus genome in 10^5 cells. It is therefore at least 10^5 fold more sensitive than Southern blotting which is capable of detecting one copy of viral DNA per cell. (Young *et al.*, 1989). The exquisite sensitivity of this technique is also one of it's major drawbacks. Great care must be taken to avoid contamination of samples with subsequent false positive results. (Tidy *et al.*, 1989c). Contamination did not occur in this study. No amplification was observed in the negative controls containing no DNA. In addition the biopsies in which selective DNA amplification occurred were evenly distributed throughout the study.

5.7. Typing of human papillomaviruses in carcinoma of the cervix

An ever-increasing number of different human papillomavirus types are being described in association with carcinoma of the cervix. (Durst *et al.*, 1983; Boshart *et al.*, 1984; Beaudenon *et al.*, 1986). A total of 24 different HPV types have been detected in the genital tract. (De Villiers *et al.*, 1989). In this study only 5 of these types were sought using homologous cloned HPV DNA probes.

HPV 16 was the first HPV type to be described in association with carcinoma of the cervix and many studies to date have reported the prevalence of HPV 16. (Durst *et al.*, 1983; McCance *et al.*, 1985; Choo *et al.*, 1987 and Fukushima *et al.*, 1985). However, in this study most of the biopsies in which HPV DNA was detected contained "unclassified" HPV DNA. Interestingly the study by Williamson *et al.*, 1989 also demonstrated a significant proportion of "unclassified" HPV types (35/98 - 36%). These could represent new HPV types and further characterization may reveal novel HPV types of importance in the development of cervical neoplasia in this geographical region.

These "unclassified" HPV types highlight some of the difficulties experienced in classifying HPV types in biopsies obtained from patients with malignancy of the cervix. These difficulties may be due to:

5.7.1. Integration of the viral DNA into the host cell chromosome.

If integration of the HPV DNA into the host cell chromosome occurs, interpretation of the results obtained by Southern blot analysis is difficult. This difficulty is illustrated in figure 5a which shows biopsy DNA hybridizing strongly with the 2818 bp and 1776 bp fragments of a cloned HPV 16 probe. This could possibly represent the integration of part of the HPV 16 genome with interruption of some of the HPV genes so that certain *Pst*I fragments usually generated by the digestion of episomal HPV DNA are lost.

5.7.2. 2.Cross-hybridization of non-homologous HPV probes.

The cross-hybridization by related HPV strains with a non-homologous probe may result in a variation in the intensity of the bands generated after restriction endonuclease digestion, Southern blot hybridization and detection with a ^{32}P labelled cloned HPV probe. Individual bands may not be of sufficient homology to allow detection by this method. Definitive identification of related strains using a non-homologous probe is therefore extremely difficult. This is illustrated in figure 6. Lane 1 shows an HPV 33 prototype. In lanes 4 and 6 strong cross- hybridization with the HPV 33 probe can be seen with fragments A and C of HPV 16. Fragment A of HPV 16 contains part of the E1a ORF, the E1b ORF, the E2 ORF and the E4 ORF. (see appendix). The E1 ORF proteins are highly conserved over the COOH-terminal but less conserved over the NH_2 terminal portion. (Baker, 1987). The E2 proteins are strongly homologous in the NH_2 and COOH terminal domains. (Baker, 1987). Homology between the E1 and E2 sequences probably permit the strong cross-hybridization seen in fragment A. Fragment C of HPV 16 contains part of the LI ORF and part of the L2 ORF. Although the L1 ORF encodes the major capsid protein of the papillomaviruses which is the most highly conserved of the papillomavirus proteins, the L2 proteins have been shown to be well conserved only at the

NH_2 domain. Homology of the L2 proteins is probably responsible for the strong cross- hybridization of fragment C of HPV 16 with the cloned HPV 33 probe. Fragment B and D of the *Pst*I digest of HPV 16 show less intense cross-hybridization with the HPV 33 probe. Fragment B contains the E6/E7/LCR and small portions of the L1 and E1a ORFs. Both the E6 and the E7 ORFs only show limited homology at the amino acid sequence level. (Baker, 1978). This would explain the less intense cross-hybridization. Fragment D contains the E4 and E5 ORF. The E4 proteins are only weakly homologous at the amino acid sequence level, possibly explaining the poorer cross- hybridization. (Baker, 1987). This highlights some the difficulties of typing HPV with non-homologous probes

5.8. The physical state of the viral DNA in the host cell

Previous researchers have emphasized the importance of the physical state of the viral DNA within the host cell in the progression to malignancy. Durst *et al.*, 1985 showed that the viral DNA in biopsies obtained from patients with cervical cancer and in cell lines derived from cervical cancer cells is usually integrated into the host cell chromosome. (Durst *et al.*, 1985). This has been disputed by the findings of Matsukura *et al.*, 1989 who demonstrated that not only integrated, but also episomal HPV DNA alone can be demonstrated in patients with invasive carcinoma of the cervix. (Matsukura *et al.*, 1989).

In our study we found the following:

5.8.1. Integrated HPV DNA

The unpredictable fragment size that may result from integration of HPV into the host cell chromosome makes interpretation of results obtained using Southern blot hybridization difficult as fragments normally generated by *Pst*I digestion of episomal HPV DNA are no longer present. This is illustrated in figure 5a which shows biopsy DNA cross-hybridizing with a cloned HPV 16 probe. The fragments of 2818bp and 1776bp correspond to fragments A and B of HPV 16 respectively. Fragment B contains the E6/E7/LCR responsible for cell transformation by HPV. (Chen *et al.*, 1982). There is a possibility that this biopsy contains an integrated HPV 16, although it was included as an "unclassified" type. Similar findings were documented by Choo *et al.*, 1987 who investigated biopsy material from 6 patients with cancer of the cervix. The DNA samples were analyzed by *Pst*I cleavage. All of the 6 samples had distinct *Pst*I Southern blot patterns. Each biopsy had one or more of the HPV 16 prototype bands missing. The 1776 bp fragment (fragment B) was, however, invariably retained in all of the biopsies. (Choo *et al.*, 1987).

5.8.2. Episomal HPV DNA

HPV 16 DNA in an predominantly episomal form can be seen in lane 3 of figure 4. All of the fragments generated from digestion with *Pst*I are clearly visible. No additional bands are visible.

5.8.3. Combined episomal and integrated HPV DNA

A possible combination of integrated and episomal HPV 16 is illustrated in lane 2 of figure 5b. The conventional fragments obtained after *Pst*I digestion are visible together with additional bands of 4000 bp and 3200bp respectively. Possible explanations for these additional bands include a partial digestion of the biopsy DNA by the restriction endonuclease, integration of the viral DNA into the host cell chromosome or a mixed infection with a combination of HPV 16 and a second HPV type. Analysis of the genetic map of HPV showed that a partial digest would not yield fragments with molecular weights the same as the additional bands seen in lane 2 of figure 5b. (see appendix) In addition fragment C (1549 bp) is far less intense than fragment B (1776 bp) despite the similarities of the molecular weights of these fragments. Fragment B contains the E6 and E7 ORF together with the LCR. It has generally been accepted that integration is usually accompanied with the preferential retention of the E6/E7/LCR region as E6 and E7 are important transforming genes of HPV. (Choo *et al.*, 1987). The greater intensity of fragment B could therefore be explained by the localization of the E6/E7LCR in this region. This feature together with the presence of additional bands strongly suggests that this biopsy DNA contains both integrated and episomal HPV 16 DNA.The presence of both integrated and episomal DNA in biopsies from patients with invasive carcinoma of the cervix has also been demonstrated by Matsukura *et al.*, 1989. These investigators showed that 24/34 biopsies investigated had only episomal HPV 16 DNA, 8 had integrated HPV 16 DNA and 2 had a combination of integrated and episomal HPV DNA. (Matsukura *et al.*, 1989).

Another far less likely explanation for the additional bands present in this biopsy is a mixed infection of different HPV types with the additional bands representing a second type of HPV infection cross-hybridizing with the HPV 16 probe under conditions of moderate stringency due to shared homology.

Further investigation of the physical state of the viral DNA within the cell (that is whether the viral DNA is present in an episomal or integrated form) could include two-dimensional electrophoresis to separate linear and circular DNA, followed by Southern blotting and hybridization with a cloned HPV probe. The episomal HPV DNA is present in a circular form and the integrated HPV DNA is linear. (Durst *et al.*, 1985).

5.9. The prognostic significance detection of HPV DNA in biopsies from patients with invasive carcinoma of the cervix.

Data obtained from the molecular typing of human papillomaviruses has enabled them to be classified into "high risk" types, for example HPV 16 and HPV 18, and "low risk" types, for example HPV 6 and 11. This division is not, however, absolute, as HPV 16 is not always associated with malignant lesions and has been demonstrated in a number of women whose prognosis is unknown. (Toon *et al.*, 1986). Although HPV 6 and 11 are classified as "low risk" viruses, they have occasionally been associated with malignant cervical disease. (Reeves *et al.*, 1987). It would, therefore be incorrect to believe that individuals infected with "low risk" HPV types have no risk of developing cervical cancer.

Biopsies from 2 patients were shown to contain HPV 18 DNA (one of these patients had an adenocarcinoma of the cervix). Of interest was the fact that both of these patients were relatively young (29 and 27 years). In contrast to this the median age of patients with HPV 16 associated tumours in our study was 50 years.

The clinical presentation and the course of the disease can vary greatly in patients with invasive carcinoma of the cervix. Some patients appear to have an unusually rapid progression of disease. A study by Barnes *et al.*, 1988 evaluated the prognostic significance of HPV type 18 in biopsies from patients with invasive carcinoma of the cervix. They obtained biopsies from patients whose ages ranged from 27-80 years. Tumour histology among this group included squamous

carcinomas, adenosquamous carcinomas and adenocarcinomas. Comparison of the tumour grade with the HPV type showed that 4/6 HPV 18 associated tumours were poorly differentiated (one of these patients had an adenocarcinoma). In addition, the median age of the patients in their study with HPV 18 associated tumours was 37 years, which was 12 years younger than the group of patients with HPV 16 associated tumours. These observations suggest that HPV 18 may be associated with a more aggressive form of cervical neoplasia. (Barnes *et al.*, 1988). Although biopsies from only two of our patients had detectable HPV 18 DNA, both of these patients were relatively young (29 and 27 years). One of these patients had an adenocarcinoma of the cervix.

A further study on the role of HPV 18 in the development of cervical neoplasia postulated that HPV 18 might be involved in rapid progression of cervical intraepithelial neoplasia to invasive carcinoma of the cervix. Kurman *et al.*, 1988 documented a particularly low prevalence of HPV 18 infection in patients with all grades of cervical intraepithelial neoplasia. These authors showed that HPV 16 accounted for 41% and HPV 18 for 22% of HPV infections in biopsies obtained from patients with invasive squamous carcinomas in their study. However, HPV 16 was found in 37% of all grades of CIN but HPV 18 was present in only 3% of patients with CIN. This deficit of HPV 18 infection in CIN compared to cervical cancer could represent the rapid progression of HPV 18 through the intraepithelial stage of neoplasia. (Kurman *et al.*, 1988).

5.10. The role of HPV typing in South Africa

South Africa has one of the highest incidences of cancer of the cervix in the world with an incidence of 0,6/1000 among black woman aged 15 - 65 years. (Cronje, 1989). At this stage a full scale typing programme in South Africa is not indicated and preference should be given to cytological screening. In an editorial in the South African Medical Journal, Cronje points out that despite the high incidence of cancer of the cervix in South Africa cytological services have been abandoned in certain public health clinics due to lack of funds. He appeals for a nation- wide screening programme for cervical cancer using cervical cytology. (Cronje, 1989). Cervical cytology is an inexpensive and relatively effective screening technique and should have priority in any cervical cancer screening programme. We could not justify the

expense of routine typing of HPV when certain clinics do not do routine cytological screening.

At present the typing of HPV is still an expensive technique that requires expertise and a Virology laboratory with a special interest in this particular field. A large scale typing programme would have to be rapid, inexpensive and provide both sensitive and specific information on the type of HPV present in a particular sample. Ideally patient material should also be obtained by a relatively non-invasive technique such as cytological sampling. (Roman and Fife, 1989). The current "gold standard" in the molecular typing of HPV is Southern blot hybridization. This is a slow and expensive procedure requiring relatively large amounts of DNA which are usually only obtainable by cervical biopsy. This technique is therefore not ideal for determining the prevalence of different types of HPV infection in the general population.

Caution must be exercised in the interpretation of the results obtained from the typing of HPV as certain "high risk" HPV types have been found in normal cervical tissue and the clinical interpretation of these results is uncertain. (Young *et al.*, 1989). It is therefore probably more appropriate to treat the patient according to the degree of cervical intraepithelial neoplasia rather that the type of HPV infection.

The typing of HPV in Cape Town with currently available cloned HPV probes would be of limited value as many of our patients have "unclassified" HPV types. Further characterization of these "unclassified" HPV types would be of great value to determine the prevalent strains of HPV in our region.

Although a large scale typing programme is not indicated at present, especially in the South African situation, the molecular typing of human papillomaviruses in patients with malignant and pre-malignant cervical disease as well as in women with no apparent cervical pathology provides valuable information. Cancer of the cervix is the second most common cancer amongst women world-wide. (Armstrong, 1987). This disease therefore contributes significantly to the morbidity and mortality of women world-wide. A thorough understanding of the nature of the disease and its causal agent(s) is necessary for any control programme

6. Conclusions

HPV DNA was detected in a total of 44% of patients with invasive carcinoma of the cervix in Cape Town by Southern blot hybridization. HPV 16 DNA was detected in a total of 16/86 (19%) patients by Southern blot hybridization or the polymerase chain reaction. This is somewhat less than what has been reported in many studies. (Durst *et al.*, 1983; McCance *et al.*, 1985). However, the geographical variation in the prevalence of HPV 16 infection has been well documented with the some areas reporting only 11% of patients with invasive carcinoma of the cervix with detectable HPV 16 DNA detectable by Southern blot hybridization. (Fukushima *et al.*, 1985)

Biopsies from patients with invasive carcinoma of the cervix in Cape Town showed a similar detection rate of HPV DNA when compared to a study on cervical intraepithelial neoplasia grade 3 in Cape Town. (Williamson *et al.*, 1989). This, together with the fact that the application of a more sensitive technique, the polymerase chain reaction, did not significantly increase our detection rate of HPV 16 DNA in biopsies obtained from patients with invasive carcinoma of the cervix, indicates a truly low prevalence of HPV 16 in this region.

Further characterization of the "unclassified" HPV types detected in this study will determine whether these are novel HPV types which are important in the development of genital neoplasia in our geographical region

7. Figures

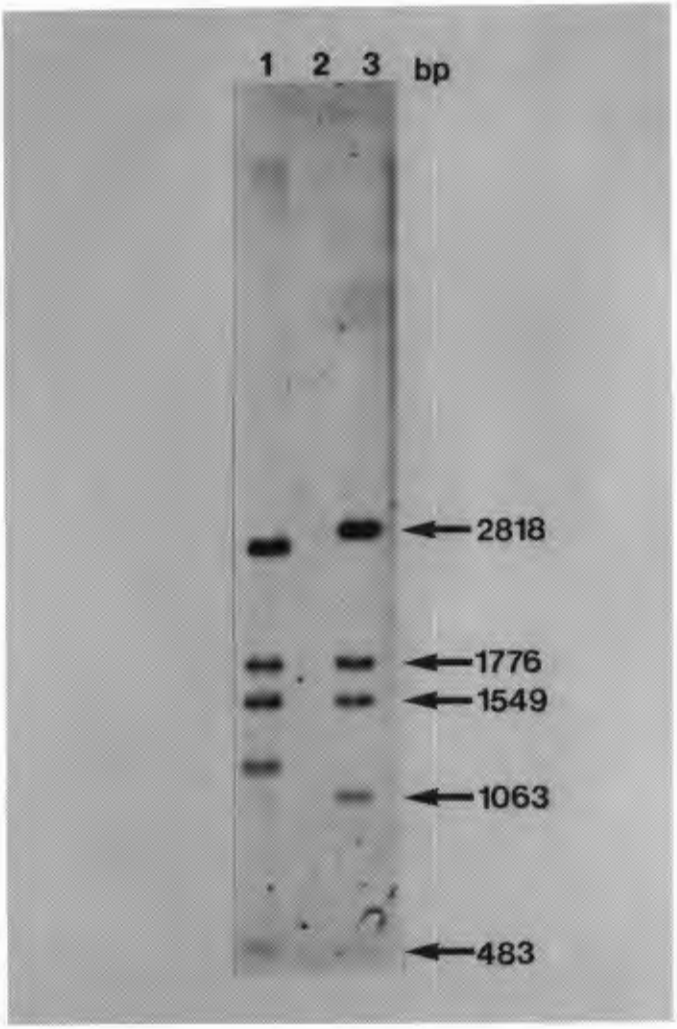

Figure 4: Autoradiograph demonstrating the presence of a HPV 16 prototype and a HPV 16 "variant".

Autoradiograph of agarose gel electrophoresis fractionated *Pst*I fragments of total DNA from biopsies obtained from patients with carcinoma of the cervix. The biopsy DNA was hybridized with cloned HPV 16 DNA under conditions of moderate stringency.

Lane 1: HPV 16 "variant"

Lane 2: Carcinoma of the cervix DNA negative for HPV 16

Lane 3: HPV 16 prototype

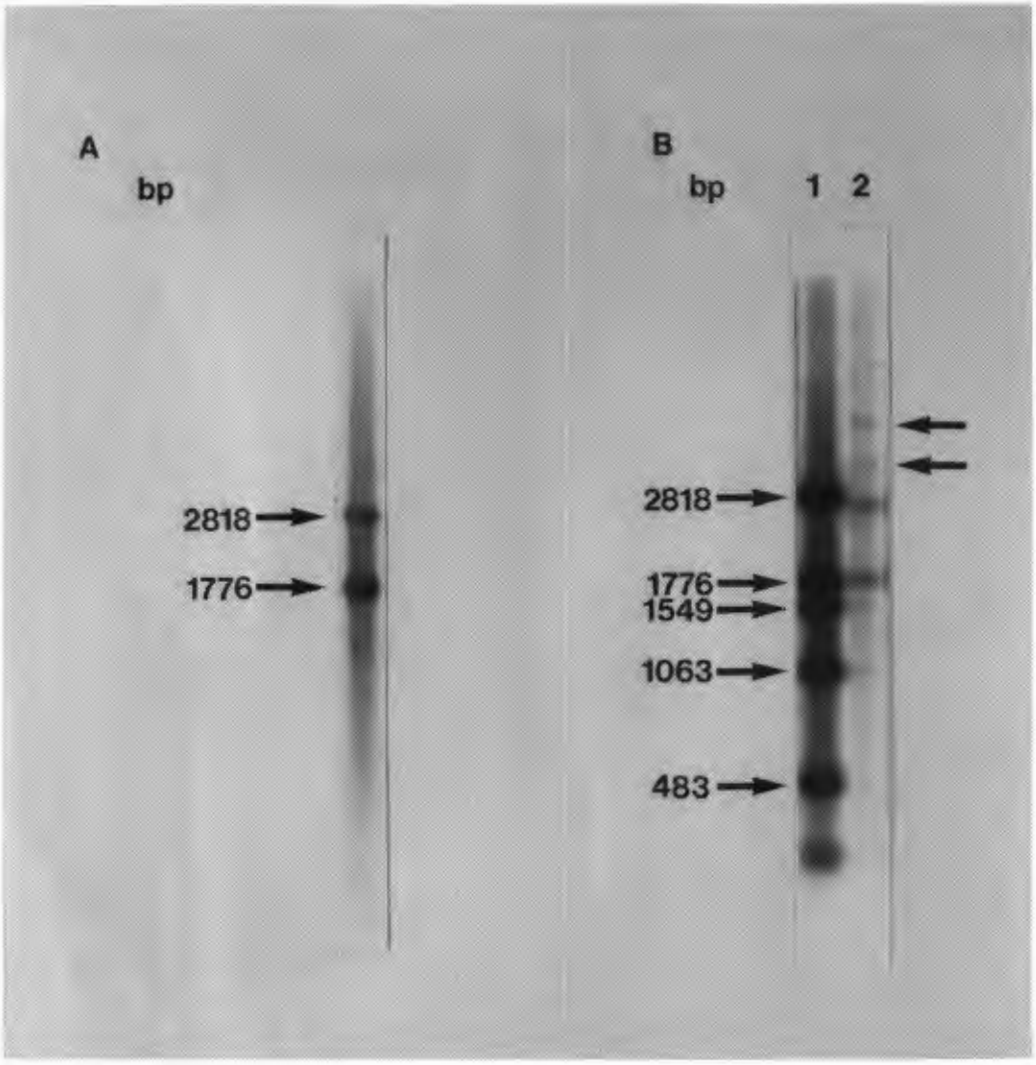

Figure 5: Autoradiograph showing a HPV 16 prototype, a HPV 16 prototype together with additional bands and an unclassified HPV.

Autoradiograph of agarose gel electrophoresis fractionated *Pst*I fragments of total DNA from biopsies obtained from patients with carcinoma of the cervix. The biopsy DNA was hybridized with cloned HPV 16 DNA under conditions of moderate stringency.

Figure 5a: "Unclassified" HPV (possibly an integrated HPV 16)

Figure 5 b: Lane 1: HPV 16 prototype.

Lane 2: HPV 16 prototype, together with additional

bands (marked with arrows)

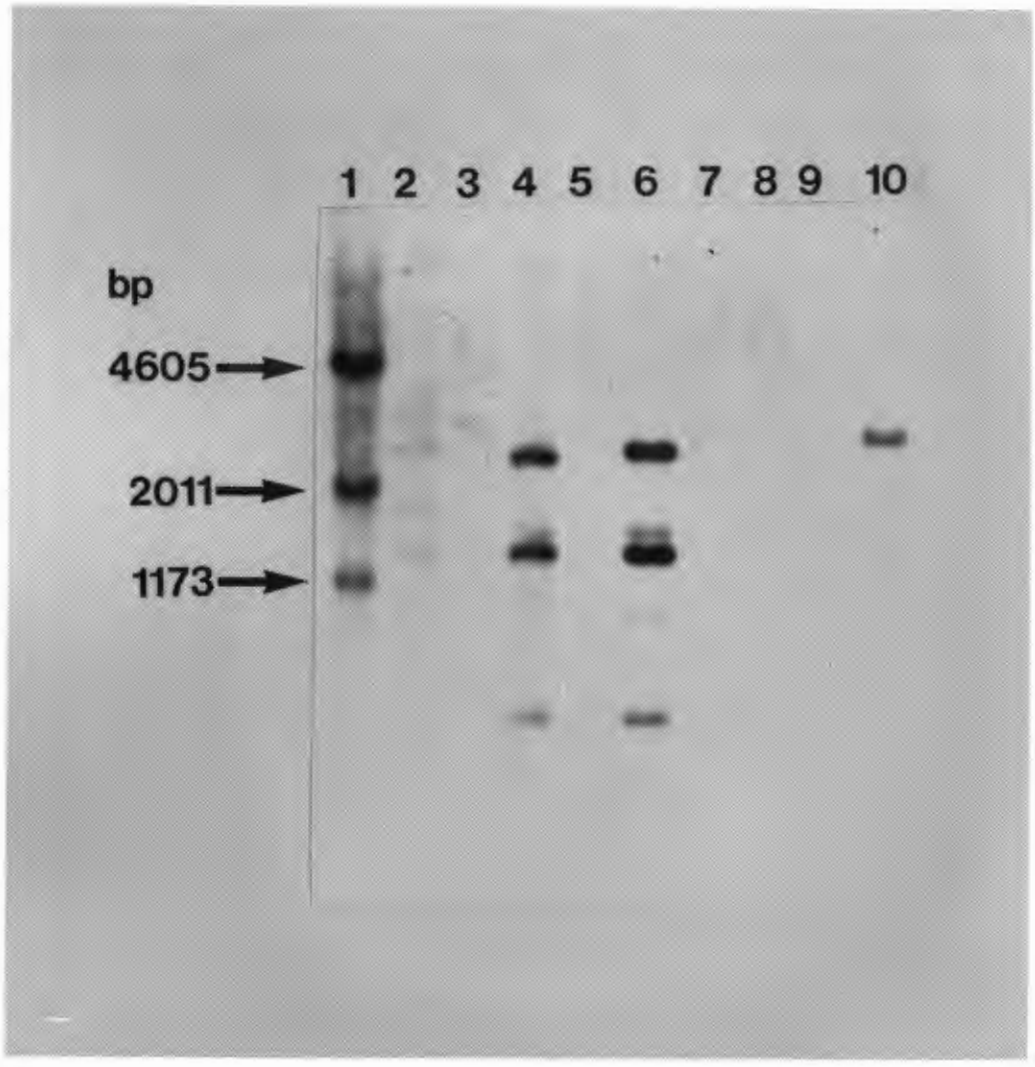

Figure 6: Autoradiograph demonstrating a HPV 33 prototype, together with an unclassified HPV type.

Autoradiograph of agarose gel electrophoresis fractionated DNA fragments of total DNA from biopsies from patients with carcinoma of the cervix. The biopsy DNA was hybridized with cloned HPV 33 DNA under conditions of moderate stringency.

Lane 1: HPV 33 prototype

Lanes 2, 4 and 6: Biopsies containing HPV 16 DNA cross-hybridizing with the cloned HPV 33 probe.

Lane 10: Unclassified HPV type.

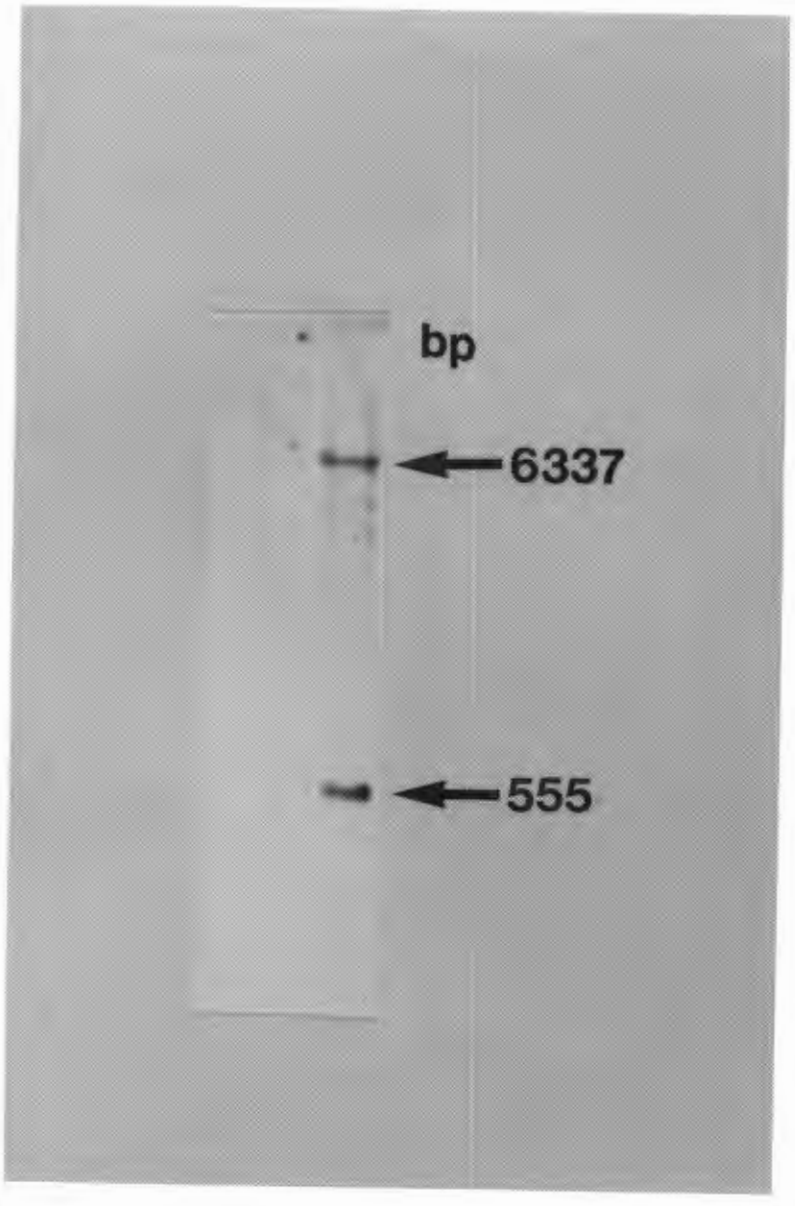

Figure 7: Autoradiograph demonstrating a HPV 18 prototype.

Autoradiograph of agarose gel electrophoresis fractionated *PstI* fragments of total DNA from biopsies from patients with carcinoma of the cervix. The biopsy DNA was hybridized with cloned HPV 18 DNA under conditions of moderate stringency. The left hand lane contains no detectable HPV 18 DNA. The right hand lane shows a biopsy containing HPV 18 DNA from a patient with an adenocarcinoma of the cervix.

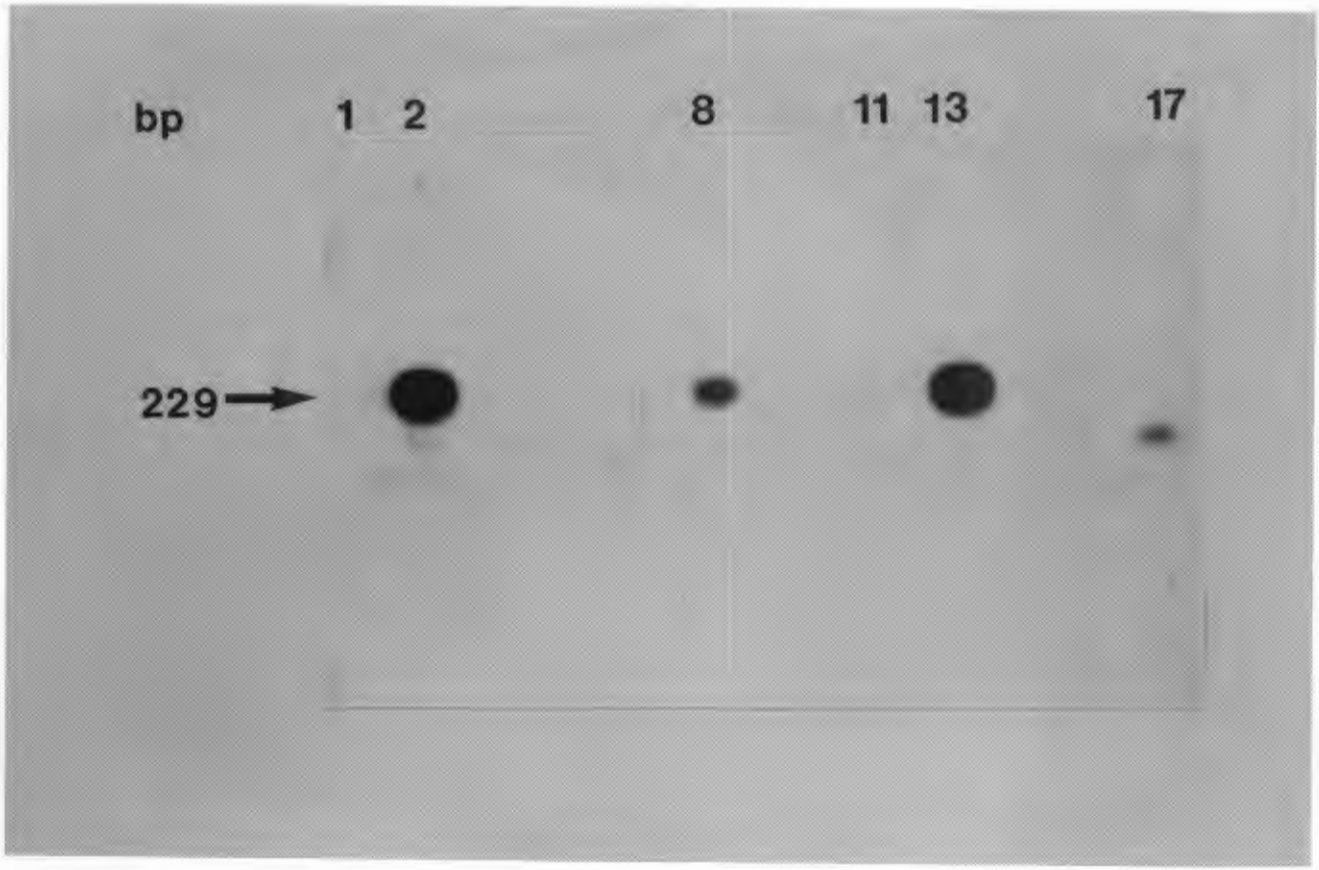

Figure 8: Autoradiograph showing selective DNA amplification of HPV 16.

Autoradiograph of an agarose gel electrophoresis of amplified HPV 16 DNA sequences using the polymerase chain reaction.

Lane 1: Negative control (No DNA)

Lane 2: Positive control from a patient with detectable HPV 16 DNA on Southern blot hybridization.

Lanes 8, 11 and 13: Amplified HPV 16 sequences in biopsies with no detectable HPV 16 DNA on Southern blot hybridization.

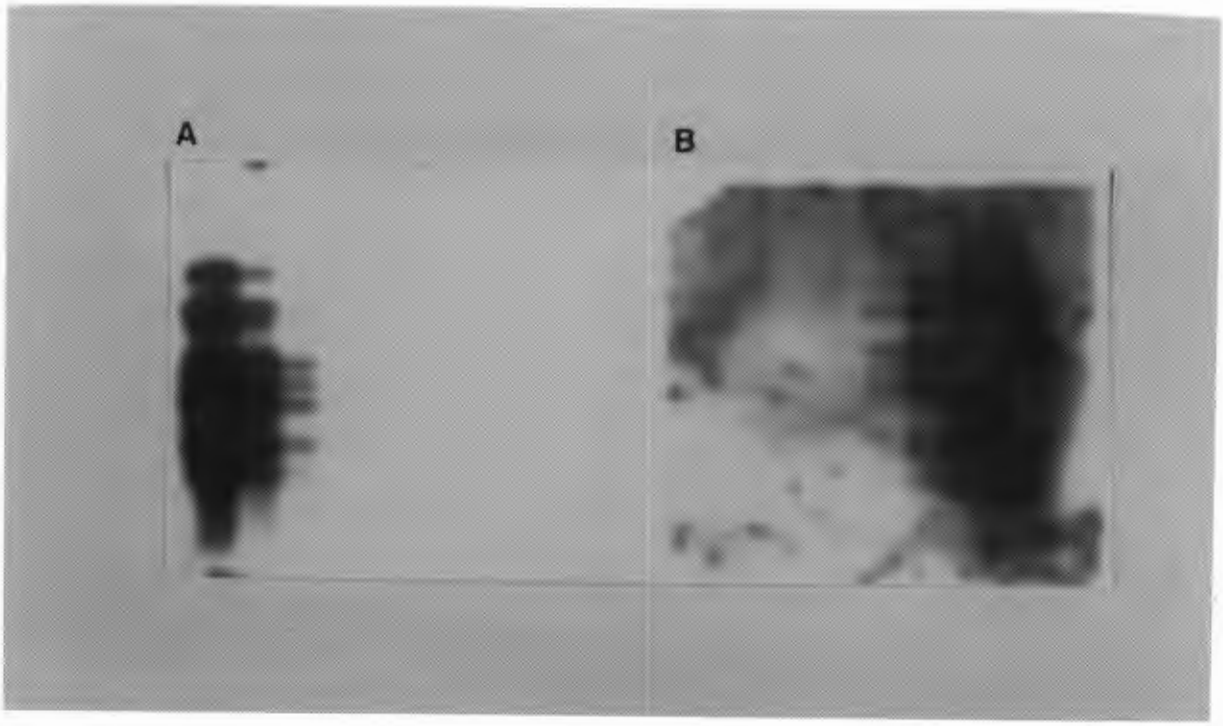

Figure 9: Two autoradiographs comparing conditions to suppress background radioactivity.

Figure 9a: The nylon membrane was treated with a 5% solution of a commercial blocking agent (Boehringer Mannheim, FRG) dissolved in 6xSSC with 30% formamide and 10% Dextran sulphate.

Figure 9b: This shows a nylon membrane treated with a pre- hybridization solution containing 6xSSC, 30% formamide, 0,02% salmon sperm and 0,5% fat-free milk powder.

8. Appendix

Pst*1 digestion of Human Papillomaviruses

MW (bp)	HPV 16	HPV 18	HPV 31	HPV33	HPV35
	2818	6337	2830	4605	4560
	1776	555	1650	2011	1770
	1549	457	1570	1173	530
	1063	441	1100	120	440
	483	67	450		400
	216		200		
			100		

*Data correlated from the Papillomavirus reference chart compiled by E-M De Villiers.

Pst*1 analysis and nucleotide sequence of HPV 16

***Pst*I fragment**	**Nucleotide sequence**
2818	875 - 3692
1776	7003 - 875
1549	5238 - 6787
1063	3692 - 4755
483	4755 - 5238
216	6787 - 7003

*From Baker *et al*, 1987

Open Reading Frames of HPV 16*

Open Reading Frame	First nucleotide	Nucleotide preceding Stop Codon
E1a	859	1167
E1b	1104	2810
E2	2725	3849
E4	3332	3616
E5	3863	4096
E6	65	556
E7	554	855
L1	5526	7151
L2	4133	5653

*From Baker *et al*, 1987

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