

VIRAL GENE DETECTION IN ORAL NEOPLASMS USING THE POLYMERASE
CHAIN REACTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
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BY

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* * * * *

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To my wife and family for their dedication, love, and tolerance of those late nights in the lab.

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CHAPTER I. INTRODUCTION

Introduction

In this chapter the author discusses the pathology of oral cancer and comments on its etiology. The background of the study, the significance associated with oral carcinogenesis, and the limitations and objectives of this study will also be discussed.

A. Background and Statement of the Problem

Oral cancers are responsible for one to two percent of cancer deaths in the United States. Efforts at prevention and early detection have had little effect on the incidence of oral cancer. The survival statistics for oral cancer have not been dramatically improved by advances in surgical, radiation or chemotherapy. Recent advances in molecular biology have provided considerable insight into the pathogenesis of several types of cancer and have suggested new strategies for prevention or therapy. The researcher proposes to use similar approaches to advance our understanding of the

molecular mechanisms involved in oral carcinogenesis. Specifically, the researcher will examine tissues from hyperplastic lesions of oral epithelium, benign neoplasms, and carcinomas to detect oncogenic papillomaviruses. These molecular defects will be correlated with the histological diagnoses of the oral lesions. These goals will be accomplished by experiments utilizing polymerase chain reactions and dot blot hybridizations to detect specific DNAs.

B. Objectives of the Study

The goal of the proposed research is to correlate molecular evidence of viral infection with the histological appearance of hyperplastic lesions, benign neoplasms and malignant neoplasms of oral squamous epithelium. Archival specimens will be analyzed to detect infection with human papillomavirus. The use of paraffin embedded specimens allows the researcher to analyze viral DNA from biopsies kept on file in the Surgical Pathology and Oral Pathology Laboratories at the Medical University of South Carolina. Using these specimens will permit retrospective investigation and provide the investigator with a large variety of histopathological diagnoses. Only biopsy material gathered and processed from January of 1988 until July of 1991 will be used due to the

possibility of DNA fragmentation in older specimens (Resnick et al., 1990).

Procedures will encompass the use of polymerase chain reactions, dot blot hybridizations, and procedures used for the preparation and analysis of products gathered from these techniques. The investigator will be looking for the presence of the human papillomavirus and further specifying the viral type found in the specimens.

C. Significance of the Problem

Oral cancer constitutes up to 5% of all malignant tumors in most countries of the Western hemisphere (Silverberg and Lubera, 1989). Over 90% of these oral malignancies are squamous cell carcinomas (SCC) (Verbin et al., 1985). It is estimated that in 1989, 17,700 new cases of intraoral SCC will be diagnosed in the United States and approximately 8650 people will die of this disease (Silverberg and Lubera, 1989). Five year survival rates for patients with oral cancers have improved only slightly over the last 50 years, with overall rates in the range of 30 to 40 percent (Mashberg and Samit, 1989). The stage of disease at the time of diagnosis is an important prognostic factor. Among patients who presented with Stage I and II, 49-71% survived for five years. In contrast, only 13-39% of patients who presented with Stage III

and IV had a five year survival rate (Verbin, et al., 1985). Unfortunately, most oral carcinomas are detected only after they have become symptomatic. Although the oral cavity is easily accessible to visual examination, programs promoting early detection have had relatively little impact on this disease. Oral cancer is more common in men, although recent data indicate an increasing incidence among females. The age at the time of first diagnosis is usually after 40, with one study showing 93% of the cases occurring between 40 and 70 years (Verbin, et al., 1985). These findings may suggest that malignant transformation of oral squamous epithelial cells requires prolonged exposure to exogenous or environmental carcinogens. The existence of an environmental component in oral carcinogenesis is also supported by the observation that anatomic sites most at risk for oral malignancy (floor of mouth, lateral borders of tongue and retromolar trigone) are characterized histologically by thin nonkeratinizing epithelium (Pindborg, 1985). Thus, the proliferating basal epithelial cells in these areas are less protected from ingested carcinogenic compounds or agents. The specific etiologic factors responsible for oral cancers are poorly understood and many influences have been considered, including tobacco use, alcohol, papillomavirus, herpes simplex II virus and Candida infections. Of these, epidemiologic studies most convincingly implicate tobacco and alcohol use as important etiological factors (Pindborg, 1985).

Identification of premalignant lesions is essential to effective cancer prevention and is an important step toward understanding the pathogenesis of malignant neoplasms. The term leukoplakia has been defined clinically as a white patch or plaque of the oral mucosa that cannot be characterized clinically or pathologically as any other disease. Although leukoplakia has often been considered a "precancerous" lesion of the oral cavity, only 2-4% of white lesions are in situ or invasive carcinoma and only 4 to 6% of leukoplakias develop into cancer over a prolonged period of clinical observation. In contrast, 90% of erythroplakic lesions in high risk sites are severe dysplasias, carcinoma in situ or invasive carcinomas (Pindborg, 1985). The redness of these lesions is related to a reactive inflammatory infiltrate attracted to the developing squamous cancer. In contrast, the leukoplakic lesions show increased keratinization, representing an adaptive response to adverse environmental factors.

A variety of hyperplastic lesions and benign squamous epithelial neoplasms are encountered in the oral cavity. These include verruca vulgaris, Heckt's disease (focal epithelial hyperplasia) and squamous papillomas. These vary in their clinical and histological appearances, but all behave in a completely benign fashion, with essentially no risk of progression to carcinoma (Guggenheimer et al., 1985). The fundamental molecular differences between these benign

proliferative lesions of oral squamous epithelium and malignant or premalignant lesions are not well understood.

Although the morphologic features of oral carcinomas have been thoroughly characterized and epidemiologic associations with oral cancer have been studied extensively, relatively little information is available concerning the molecular events which occur during oral carcinogenesis. Recent technical advances in molecular biology have provided methods for detecting nucleic acids of DNA and RNA tumor viruses. Three paradigms of modern cancer research (tumor virology, oncogene activation and growth factor expression) have greatly increased our understanding of carcinogenesis. For this reason, the investigator proposes to correlate morphology of oral hyperplastic lesions, dysplasias, benign neoplasms and malignant neoplasms with infection by oncogenic viruses (papillomaviruses). This information should advance our understanding concerning the presence of HPV in oral neoplasms.

CHAPTER II: REVIEW OF LITERATURE

A. Historical Aspects

Genital warts were recognized in antiquity. They were recorded by erotic poets and were described by the ancient physicians as "condylomas" or figs; in addition, in the early second century Soranus wrote a chapter on "Warty Excrescences in the Female Genitalia" quoted by Campion et al., 1987. Papillomavirus like some other viruses appear to have a strong correlation between infection and the subsequent development of malignancy. Other viral examples include hepatitis B virus and the subsequent development of hepatocellular cancer, the Epstein-Barr virus (nasopharyngeal cancer, Burkitt's lymphoma), and Human T cell leukemia virus (HTLV I - Adult T cell leukemia/lymphoma) (Shibata, et al., 1989).

The papillomavirus was the first DNA tumor virus to be isolated and characterized from the cottontail rabbit (the Shope papillomavirus) (Campion et al., 1989).

B. Epidemiology and Pathogenesis

Two percent of routine Papanicolaou smears and ten percent of the sexual disease clinic smears in the United States are dysplastic (Dallas et al, 1989). Ninety percent of cervical cancers and their metastases contain specific papillomavirus DNA types considered to be "high risk" (HPV 16, 18); furthermore, these types are capable of integrating into genomic DNA, leaving intact early genes (E6 and E7 open reading frames) (ORF) that continue to express c-myc and c-ras oncogene mRNA (Broker, 1987). The E6 and E7 ORF have an important role in altering growth properties in the cell (transformation); furthermore, these ORF's are responsible for replication and maintenance of a high number of viral DNA copies (discussed further on pages 11-13, 16).

Tobacco metabolites have been found in cervical mucus of women who smoke and are considered to be among the carcinogenic agents which account for a two to four times increased risk for ensuing cancer development (Gissmann, 1987). In the past, the herpes virus was implemented as playing a causative role in oncogenesis; however, with the new technical advances in molecular biology, this has been disproved (Gissmann, 1987). It should be pointed out that herpes virus may still be a cofactor.

Four hundred and fifty thousand (450,000) cases of cervical cancer occur each year in the world along with 150,000 other cases of squamous cell cancers of the female

genital tract. High risk areas have a forty five to fifty percent incidence (of the 600,000) of death (Syrjanen et al., 1989). The risk in western countries for the development of cancer is around one percent - worldwide around ten to thirty percent (Gissman et al., 1989). Squamous cell carcinoma accounts for seven percent of cancer in the United States and twenty percent in developing countries (Roman and Fife, 1989). Fortunately, the time from primary infection to cancer is several decades.

Latent human papillomavirus (HPV) infection in "normal" epithelium has been identified using molecular biological techniques. These subclinical infections produce minor epithelial changes which histologically and cytologically are difficult to detect; furthermore, the time interval between a latent infection and a clinical manifestation or resolution of the infection has not been determined (Syrjanen et al., 1989).

Recently, evidence has been presented indicating that respiratory papillomatosis may be secondary to HPV infection (Levi et al. 1989). The fetal infection is picked up during passage through the infected genital tract of the mother at birth. It may remain latent for decades (juvenile or adult onset). Another mode of transmission for the viral particles may be oral sex (Levi et al., 1989).

Co-factors may include acquired immune suppression factors that adversely affect immune competence such as renal transplant patients and patients infected with the human

immunodeficiency virus (HIV). Oral contraceptives are thought to increase the prevalence of HPV infection in the body (Koutsky and Wolner-Hanssen, 1989). There is also an increase in prevalence of genital warts in pregnancy (due to altered hormonal status) because temporary immunosuppression during pregnancy may allow the HPV positive cell to escape immune surveillance (Koutsky and Wolner-Hanssen, 1989). Glucocorticoid treatment causes an increased rate of HPV gene expression and an increased growth activity of the HPV 18 cell line. Additionally, vitamin deficiencies may be a co-factor in oncogenesis (Roman and Fife, 1989).

Tumors related to HPV infection are monoclonal; accordingly, only one of the large number of HPV positive cells is able to escape immunological controls or suppressor genes on genomic DNA needed for normal cell development. It is postulated that mutagenic events may induce the cellular DNA repair systems to cause DNA breakage and trigger recombinational events which favor the integration of viral HPV DNA into the host genome (cellular DNA) (Gissmann, 1989). Cell hybrids from Hela cells (known to contain 10-50 copies of HPV 18) and normal keratinocyte fibroblasts reveal that the transformed cells may be suppressed by gene products present in normal cells that down regulate the papillomavirus gene expression (Dallas et al., 1989). Consequently, carcinogenesis from an HPV-infected cell is favored if the cell is consistently exposed to co-factors in the form of

mutagens, inactivating the cellular genes (Koutsky and Wolner-Hanssen 1989).

C. Viral Structure

The human papillomavirus belongs to the family Papovaviridae. The DNA of the virus is surrounded by a polyhedral capsid with 20 faces. This icosahedral structure is composed of 72 capsomeres (basic building block of the virus shell). The virus has covalently closed, circular, double stranded DNA (same for all papovaviruses (SV40, PV, BK and JC). The difference between these papovaviruses and the human papillomavirus is that HPV has a larger capsid - fifty five versus forty five Å in diameter and larger chromosomes - 7.9 Kilobases versus 5.2 Kilobases. HPV has no lipid membrane envelope (Smotkin, 1985). The major capsid is 54,000 daltons (d) while the minor capsid is around 76,000 d. Additionally, the molecular weight is 5,200,000 daltons with an adenine/thymine to guanine/cytosine ratio of 58:42.

There are regions of HPV chromosome with the potential to code for proteins. These regions are fundamentally important to the HPV genome and are termed open reading frames. ORF's are conserved and code for messenger RNA (mRNA). Several ORF's may code for one specific mRNA. The regions of the ORF's are termed E (early) and L (late) with the E ORF's controlling cell transformation and DNA replication and the L ORF's containing two long ORF's that code for viral capsid proteins.

There are more than one E and L region ORF's; consequently, they are given numbers depending upon their relative lengths. Logically, E region ORF's are expressed early in the virus life cycle when new viral particles are made while the L region ORF's are expressed late in the infection. The following are true for ORF's. Broker indicates:

- (a) E₁ - "is directly involved in DNA replication"
[Prevents over replication which might prematurely kill the cell].
- (b) E₂ - role in "efficiency of cellular transformation".
- (c) E₄ - alters cell structure or takes part in viral particle formation.
- (d) E₅ - has transforming activity, codes for smallest viral transforming protein.
- (e) E₆ - "has transforming activity and alters growth properties of cells in culture. Retained in human cervical cancer cell lines". Maintains high numbers of copies of viral DNA in the cell.
- (f) E₇ - "affects the rate of initiation and regulates copy number of episomal viral DNA". Retained in human cervical cancer cell lines.
- (g) L₁ - codes for a major capsid protein.
L₁ is expressed in the outer, highly differentiated koilocytes; however, HPV 16 may

produce little capsid protein and thus may escape immunohistochemical detection.

(h) L₂ - minor capsid protein (Broker, 1987)

The Upstream Regulatory Region (URR) is a 600-900 base pair region which contains no protein coding sequence and is located between the end of L and the beginning of E. The URR plays a role in regulating signals important in DNA replication. It should be noted that the URR is not deleted when viral integration into cellular DNA takes place (Smotkin, 1989).

D. Genetics

Unlike most viruses which are divided into subtypes depending upon their serological differences, comparing HPV genomes for relatedness is accomplished by solution hybridization to analyze DNA:DNA heteroduplexes. The isolates which are largely resistant to DNAase that degrade single strand mismatched regions are considered the same; however, if the heteroduplexes degrade more than fifty percent the viral DNA is considered to be a new type, and if heteroduplexes degrade less than fifty percent but more than twenty five percent, the virus is given a new category such as a or b (Roman and Fife, 1989).

HPV DNA has been cloned into E. coli plasmids related to pBR322 or pU18/19 which carry a DNA replication origin and

selectable drug markers. HPV contains runs of nucleotides recognized by restriction endonucleases, allowing for molecular studies.

E. Life Cycle

HPV type is considered specific for a single species, type of epithelium, and anatomical location; in addition, these viruses infect epidermal squamous cells. The subcolumnar reserve cell, which eventually undergoes metaplasia to a basal cell, most likely is the target for HPV infection. It has been suggested that wounding is essential to permit physical access of virus particles to the basal cells. Wound healing may encourage the establishment of the infection due to accelerated cell division, prompting capillary growth and an increased blood supply at the healing site (Broker, 1987).

During the initial infection, the virus invades the basal cell via specific binding of a viral capsid protein with a cell receptor. Early (E) genes induce the infected basal cell to undergo hyperplasia (acanthosis). "Immediately after the HPV's DNA is transported to the nucleus, the viral DNA replicates to a high copy number due to the lack of viral regulatory proteins present in the nucleus. Subsequently, the E7 and E1 modulator functions eventually stabilize the copy number of the viral DNA to about 20 to 50 copies per cell" indicates Broker (1989).

Secondly, there is an intermediate phase in which the virus stabilizes its DNA replication so that it is synchronous with that of cellular DNA, insuring that the basal cells maintain adequate viral copies to allow the virus to persist in a latent state. This phase is considered to be an episomal or non-integrated viral DNA phase. As Broker indicated in 1987, "At this point, maintenance of the episomal state (infection with a "low risk"HPV) is entirely dependent on the carboxyl terminal 2/3 of the E₁ ORF and E₅ ORF. After this point, most of the subsequent steps are carried out by host deoxyribonucleotide triphosphate pools".

As the squamous cells of the basal strata rise and differentiate into intermediate and superficial squamous cells, there is a renewal of viral gene expression allowing for the viral DNA to replicate to an extremely elevated level (late genes). Thus virus particles are assembled and viral capsids are developed. Lastly, as the cells de-differentiate and become more anaplastic, the normal viral life cycle becomes blocked so that in high grade dysplasias and cancers new viral particles are not assembled. Simply stated, the morphogenesis of a high grade intraepithelial lesion, squamous cell carcinoma, or adenocarcinoma most likely kills the majority of the virions. It should be noted that the progression from mild dysplasia to invasive cancer may be influenced by co-carcinogens. More importantly, host cell replication may be affected by the oncogenic HPV process in

high grade dysplasias due to the viral insertion of the E6, E7 ORF's into the host genomic DNA near host proto-oncogenes. In addition, if viral oncogenes are inserted near these proto-oncogenes, multiple mutations in cellular genes may occur and some of these mutations may activate the oncogenes (examples include c-src, c-ras, c-myc) and result in progression of the oncogenic process from a benign or pre-invasive lesion to one of malignancy (Smotkin, 1989). It has been suggested that integration might interrupt functions of genes coding for cellular interfering factors which would paralyze defense mechanisms that the cell might normally use for down regulating aberrant growth and further viral transcription (Broker, 1987). DNA replication can be followed by cell culture (Woodworth et al., 1989).

F. HPV Types

As of 1991, there are sixty different HPV types based on their nucleic acid sequences. About one fourth of these are found in the female genital tract and include: HPV 6, 11, 16, 18, 31, 33, 39, 41-46, 51-56, and 59 (Koutsky and Wolner-Hanssen, 1989). HPV 6 and 11 are generally thought to be associated with benign, exophytic condylomas and low grade dysplasias or cervical intraepithelial neoplasia, grade one (CIN I), while 16 and 18 are found more often in high grade dysplasias. HPV 16 has been detected in ninety three percent

of uterine cervical cancers and HPV 18 has been detected in eleven percent of cervical cancers (Syrjanen et al, 1987). The types with the highest rate of progression to cancer are HPV 16 (forty five percent) and HPV 18 (twenty three percent) (Syrjanen et al, 1987). The genomes of HPV 16 and HPV 18 have been completely sequenced. It has been noted that three out of ten normal cervical sections adjacent to regions diagnosed as dysplastic on histological section containing HPV 16 or 18 (confirmed using the polymerase chain reaction) are also positive for HPV. These types of infections would be considered latent, subclinical, or early infections (Shibata et al., 1988).

In addition to being detected in low grade cervical lesions, HPV 6 and 11 are also associated with juvenile/adult onset laryngeal papillomas and papillomas of the oral cavity (Levi et al., 1989). Other neoplasms associated with papillomavirus include oral focal epithelial hyperplasia (HPV 13), invasive carcinoma of the oral cavity (HPV 16), and transitional cell carcinoma of the bladder (HPV 16) (Campion, 1987).

G. Immunology

It is generally thought that the persistence of a virus is measured by the virulence of the infecting agent and the host's ability to control the spread of the virus. The immune response involves HPV inactivation by antibody, complement mediated lysis of viral particles or virus infected cells coated with the appropriate antibody, and lysis of infected cells by antibody dependent cytotoxic cells (ADCC) or specific cytotoxic T lymphocytes. T-helper, T-suppressor, B-cells, natural killer (NK) and killer (K) lymphocytes, monocyte/macrophages (MPS cells), including Langerhans cells are all involved in the immune response to invading HPV. However, there is no correlation between proportions of these cell types and the outcome of the disease (Syrjanen et al., 1987) .

After initial HPV infection, macrophages (or histiocytes) form the first line of defense. There is also a decrease of Langerhans and T cells resulting in a localized deficiency in cell-mediated immunity thereby neutralizing the NK cells effect (Campion et al., 1987).

H. Cell Culture

There are three papillomavirus induced carcinoma cell lines. They include: Hela (HPV 18 containing 10-50 copies per cell), Siha (HPV 16 containing 1 to 2 copies per cell), and

Caski (HPV 18) (Kiyabu et al., 1989). Within these lines, further modulation is evident by HPV 16, 18, 31 and 33 in that they will immortalize primary cultures of human foreskin keratinocytes and exocervical epithelial cells (Woodworth et al., 1988). Additionally, there is integration of viral DNA into one or more sites in the host genome near cellular proto-oncogenes (C-src, C-ras, C-myc) (Smotkin, 1989). Moreover, C-myc oncogenes are amplified in cervical cancer because mRNA levels are elevated. Immortalization only occurs with those HPV's associated with cancer (types 16, 18, 31, 33); episomal HPV 6 and 11 infected cells senesce, resemble normal human keratinocytes, and do not immortalize. Those that immortalize resemble squamous cells at 100 population doublings and grow rapidly in serum-free media after subculturing with no terminal differentiation. If transferred back to a medium with fetal bovine serum, most cells cease to proliferate with the exception of a few colonies which continue to divide and differentiate via an acquired resistance to terminal differentiation (Woodworth et al., 1989).

DNA analysis of the immortal cell lines suggest that the viral genome is rearranged due to its integration within the cellular DNA. Immortalized cell lines express viral RNA transcripts that differ from those which have senesced but this has little consequence to the type of HPV DNA (Woodworth et al., 1989). "High risk" HPV DNA has also been shown to

transform human fibroblasts, and NIH3T3 cells (Dallas et al., 1989).

I. Cytologic and Histologic Detection

Koilocytosis, dyskeratosis, hyperkeratosis, parakeratosis, nuclear wrinkling, bi-nucleation, multi - nucleation, and epithelial spikes are all morphologic changes which are found using conventional cytological or histological evaluation for HPV. Dysplasia may be considered the result of HPV infection. Criteria associated with dysplasia include cells with hyperchromatic nuclei, nuclear enlargement, increased nuclear/cytoplasmic ratios, increased chromatin granularity, aberrations in nuclear shape, increase in cellular pleomorphism, and abnormal keratinization. Histopathologically, dysplasia may represent disorderly maturations of the squamous epithelium, an increase in the numbers of immature, primitive cells from the basal lamina toward the surface, variable epithelial thickness, and increased mitotic activity. It should be pointed out that cytology alone is three times more effective than HPV antigen detection demonstrable by immunocytochemistry for the identification of HPV (Sato et al., 1987). As dysplasia increases, koilocytosis decreases; furthermore, the incidence of koilocytosis correlates with the concentration of mature

HPV virions in a cell, which also correlates well with immunocytochemistry. Therefore, dysplasia is a fair indicator of HPV detection/diagnosis but an excellent indicator of "early" HPV detection/diagnosis. (Sato et al., 1987).

J. Antigen Detection

The major capsid proteins of all animal and human papillomaviruses are antigenically cross-reactive; therefore, immunological detection may be of some value with patient samples using commercially available group-specific antisera (Woodruff et al., 1980). The method utilizes an avidin-biotin peroxidase complex directed against the HPV capsid antigen. Initially, the biopsy specimen is fixed in ten percent formaldehyde and embedded in paraffin. Secondly, a 0.5% H₂O₂ solution and methanol is used to quench endogenous peroxide activity, and the specimen is incubated with a twenty percent normal swine serum (to reduce non-specific staining). Third, the biopsy is incubated with anti-bovine papillomavirus serum (anti-BPV) at 1:400 and also incubated with swine anti-rabbit immunoglobulin. Finally, the specimen is incubated with rabbit peroxidase and counterstained with hematoxylin and eosin. If the nuclei stain brownish/red they are considered positive for the viral antigen (Ag) (Woodruff et al., 1980).

Unfortunately, only episomal DNA can be identified by immunological probes due to the decreased amount of detectable

L₁ protein in higher grade dysplasias. Since this percentage declines as CIN increases and HPV antigen expression is directly related to the degree of squamous differentiation, these types of probes are not considered complete diagnostic tools for the detection of HPV. Immunohistochemistry is somewhat effective in detecting HPV Ag in benign condylomas, but is ineffective when probing dysplasias and or neoplastic cells since viral capsid proteins are poorly expressed as cells become undifferentiated. Dysplastic cells fail to mature, therefore the virions fail as well, leaving a reduction of virions in these lesions as well as episomal DNA (Yu-Kai et al., 1989).

K. Transmission Electron Microscopy

Electron microscopy (EM) can be used for the detection of immature virions not detectable by immunocytochemistry (found in basal/parabasal cells) because these cells contain a small number of virions which may not exhibit the classical morphologic signs (koilocytosis, dyskeratosis). Although EM may provide information on the location and number of virions per cell the search for viral particles is extremely time consuming, thus hindering its clinical utility (Sato, 1987).

L. Detection of HPV Using Molecular Biological Techniques

a. Hybridization Theory

In order to understand the current techniques used in the molecular diagnosis of HPV, it is important to briefly discuss the concept of hybridization. Hybridization can be conducted under conditions in which all HPV's can be detected, considered as low stringency, or in which the specific type can be documented (high stringency) (Roman and Fife, 1989).

The technique involves the annealing of two DNA strands due to complementations of their nucleotides (i.e., adenine, thymine, guanine, and cytosine). The optimal rate of duplex matching can be found by raising the temperature of the sample to where the temperature denatures half of the duplexes. If the hybridization occurs under standard conditions (high stringency - $T_m=25\frac{1}{2}^{\circ}\text{C}$) the percent mismatch tolerated between the nucleotide bases on the complementary strands is around thirteen percent (Roman and Fife, 1989). The degree of mismatch tolerated is increased to thirty - three percent when the hybridizations occur under conditions considered as low stringency ($T_m=43\frac{1}{2}^{\circ}\text{C}$). Additionally, stringency of hybridization can be varied by adjusting the formamide concentration. Therefore, the overall stringency of hybridization is determined by the formamide concentration and temperature (Roman and Fife, 1989).

b. Generating probes

The generation of probes as molecular markers for the detection of HPV, has been historically performed via the cloning of the HPV genome into a bacterial plasmid related to pBR322 (Maniatis et al, 1982). After reproducing in vitro millions of copies of the genome, the specific marker is released from the plasmid and cut with an appropriate restriction enzyme. It is then analyzed by gel electrophoresis.

Nick translation enables the researcher to randomly introduce nicks into the nucleotide sequence of the DNA via incubation with deoxyribonuclease I. Characteristically, E. coli DNA polymerase I removes bases processively in the 5' to 3' direction (from the nicked site) and fills in that same region with ³²P labeled nucleotides. The labeled nucleotides can now be used for hybridization analysis (Roman and Fife, 1989).

c. Southern Blot Hybridization

From the inception of molecular biology, the Southern blot analysis has generally been considered the gold standard in hybridization techniques. Briefly, the total cellular DNA must be extracted from fresh tissue and subsequently digested with restriction enzymes (usually Pst I in the case of HPV).

Each of the DNA fragments are separated with agarose gel electrophoresis. Consequently, a smear of DNA is denatured, and transferred to a nitrocellulose filter. After transfer, the DNA on the filter can be hybridized under low stringency conditions with a cocktail containing several HPV types (labeled with ^{32}P) to determine if there is HPV present in the sample. Subsequently, those types that are identical will be retained on the filter at high stringency, while other hybrids (HPV, but not the specific probe types) will be liberated from the filter.

It has been noted that as little as one copy of HPV DNA per cell can be detected if ten milligrams of cellular DNA is extracted as starting material. The Southern blot can also determine if the HPV DNA is episomal or integrated.

There are several disadvantages to this technique. This procedure is quite time consuming, thereby limiting its possible clinical diagnostic uses and it provides no information on the localization of viral DNA within the cell. Moreover, large quantities of DNA (10 mg) are required. This procedure limits its use to fresh biopsy material. Other forms of clinical material such as direct scrapes or smears (easier derived but contain lower amounts of cellular DNA) can not be used. Therefore, the Southern blot eliminates retrospective studies; moreover, there are times when genomes will be detected by in situ hybridization and not by Southern blot (Roman and Fife, 1989).

d. Dot Blot Hybridization

Dot blot hybridizations are similar to the Southern blot (often called modified Southern) with the exception that these hybridizations are used only under high stringency conditions. As with Southern blots, extracting the total cellular DNA is required; however, only 300-500 nanograms are needed. In this procedure, DNA is denatured, neutralized, and applied to a nitrocellulose filter; furthermore, replicate nitrocellulose filters can be probed with HPV DNA. The dot blot method utilizes an oligomer (produced by DNA synthesizer) which is end labeled with gamma ^{32}P . A positive signal is considered at least two times stronger than a negative signal (Roman and Fife, 1989).

Dot blot's are highly sensitive: all that is needed is one viral copy per genome (1 to 2 picograms of HPV DNA) when 500 nanograms of cellular DNA is applied to the filter (Maniatis et al., 1982).

e. Reverse Dot Blot Hybridization

This technique is the reverse of the Southern blot - in this case the researcher places the HPV DNA on the filter and

labels the cellular DNA. The results are similar to dot blot hybridizations for it gives information as to whether the HPV DNAs are related, but not whether they are related over their entire genomic length. In contrast to dot blot hybridizations, the reverse dot blot can be used for low or high stringency conditions. The disadvantage is that it requires a separate labeling reaction for each of the cellular DNAs (Roman and Fife, 1989).

f. in situ Hybridization (Tissue in situ hybridization) (ISH)

With this procedure, tissue sections or smears are probed directly to provide information on the localization of the HPV genome within the cell. In contrast to immunoperoxidase detection, it distinguishes a myriad of HPV positive cells. Accordingly, in benign neoplasms, the viral copy number increases as differentiation increases (from basal to parabasal cell differentiation to koilocytosis), but decreases through the late stages of CIN. Current dogma suggests that the viral life cycle is blocked in high grade dysplasia and invasive carcinoma so that new viral particles are not made. The development of these lesions is a biological dead end for HPV. Therefore, the antigen detection may be falsely negative, but the advantage of in situ hybridization is its sensitivity for locating the rare positive cells. Probes used for in situ

hybridization are labeled with ^3H (tritium), ^{35}S (sulfur), or biotin. Biotinylated probes tend to be more stable, but confer a higher background of non-specific binding. ^3H has a very low background due to long term exposure to emulsion but the lengthy incubation time hinders potential clinical value. ^{35}S tends to have a higher background than tritium, but results are more rapid. As with reverse dot blots, all results exhibit the relatedness of HPV DNA but not the full genomic identity.

Within in situ hybridization, DNA probes can discriminately detect DNA or RNA. In this instance, biotin or radiolabeled sense RNA probes (identical to mRNA) will detect DNA copies. Antisense RNA probes will detect mRNA and is highly sensitive when compared to the sense probes because of the increase number of copies of mRNA as compared to DNA.

Other advantages of in situ hybridization include: the T_m for RNA-RNA hybrids is $10\frac{1}{2}^\circ\text{C}$ higher than for DNA-DNA hybrids thereby enabling the procedure to be carried out under more stringent conditions. The background tends to be lower due to ribonuclease treatment; furthermore, the researcher has the advantage of using dark field illumination when analyzing the tissue sections under the microscope and one can perform retrospective studies involving paraffin embedded tissue documenting the continuity of an HPV type in a histologic section. Using ISH, the researcher can document a carcinoma in situ merging into full blown carcinoma.

In comparison to Southern blots, if HPV DNA is in a subpopulation of cells, ISH will allow for identification of the virus and may identify two different HPV types in the same cell. With the Southern blot technique, one doesn't know if the DNA resides in the same cell or in different cells.

The disadvantage of this technique is its prolonged incubation time (^3H , ^{35}S), which hinders the clinical utility (Roman and Fife, 1989).

g. Filter in situ Hybridization (FISH)

Much like in situ hybridization, FISH allows for the detection of DNA sequences present in smears or scrapes. With this technique, cells are applied to a filter and the filter is treated, disrupting the cells and denaturing the DNA; however, unlike the dot blot hybridization, there is no need for DNA extraction.

Handicaps of FISH lie in the fact that the technique is one half to one third as sensitive as the Southern blot - the signal strength is often difficult to distinguish from negative hybridizations (Roman and Fife, 1989).

h. Nucleic Acid Sandwich Hybridization (NASH)

NASH involves cloning HPV into the bacteriophage M13 and the plasmid pBR322. This technique avoids the use of autoradiography to detect HPV. M13-HPV DNA is bound to a filter and the pBR322 sequences are radiolabeled. A cocktail containing radiolabeled DNA fragments and disrupted cells from the sample smear are added to a filter under hybridization conditions and if HPV is present, it will anneal to the M13-HPV DNA on the filter and concurrently anneal to the radiolabeled HPV DNA. Subsequently, a scintillation counter is used to count the bound radioactivity (indicating the presence of HPV). Unfortunately, NASH tends to have too many variables (multiple cloning vectors) and limits the researcher to only HPV presence (Roman and Fife, 1989).

i. Polymerase Chain Reaction (PCR)

PCR consists of selectively amplifying target DNA sequences through repeated cycles of denaturation, annealing with oligonucleotide primers complementary to specific regions of the target HPV sequence type, and primer product extension with Taq Polymerase (or recombinant Taq) in the presence of excess deoxynucleotide triphosphates. The desired nucleotide sequence will increase exponentially as the number of cycles rises, allowing for the ensuing detection with a radiolabeled complementary base pair oligomer (Figure 1). The

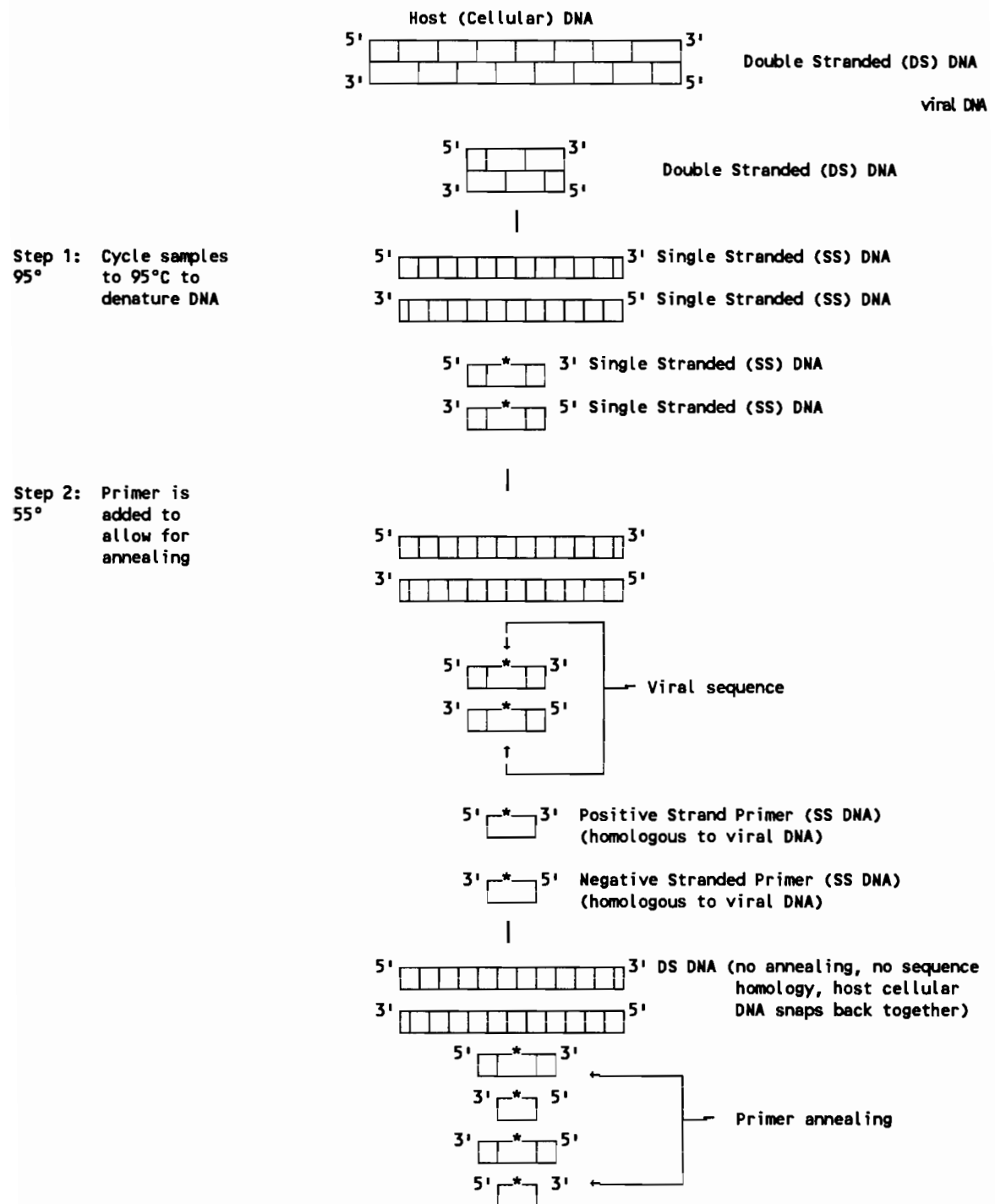


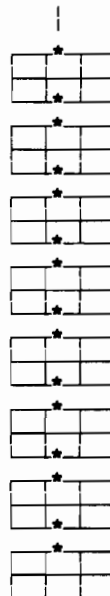
Figure 1. Polymerase Chain Reaction Theory

Figure 1. Polymerase Chain Reaction Theory (continued)

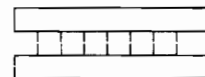
Step 3: Primers extend
via Taq
polymerase
and excess
nucleoside
triphosphates



... After two reactions ...



Viral DNA (DS DNA)



Host DNA (remains the same)

x 40 reactions

oligonucleotide primers and probes which correspond to flanking sequences of the HPV genome are synthesized by a DNA synthesizer (Applied Biosystems, California) and purified on a polyacrylamide-urea gel.

PCR is either amplified to levels that can be detected by gel electrophoresis, or dot blot hybridization. If the target oligo sequence is absent, HPV DNA amplification will not occur and the test is considered negative.

The region amplified depends upon the desired sequences needed for HPV detection. Most are specific for each HPV type and the primers involve amplifying areas of the E₆ or L₁ intron (Shibata et al., 1989). Unfortunately, this involves using a different primer for each HPV that is possible for detection. Since there are over sixty species, it would be a very time consuming experiment to use PCR on each HPV type. Moreover, only five types have been completely sequenced (Dallas et al., 1989). Other types are lost to possible detection using type specific primers. Most researchers are nevertheless only concerned with the HPV types considered as "high risk", or those which have transforming capabilities (HPV 16 and 18, 31, 33) (Shibata, Fu, et al., 1988). This method is suitable for those types which are known to be involved in cervical cancer but leaves possible pitfalls if analyzing unknown HPV types in other mucosal lesions.

A positive HPV PCR signal does not recognize the entire HPV genome, only those sequences from the HPV genome which were selected for amplification (Dallas et al., 1989).

Recently, universal primers (or primers that recognize several of HPV types) for identification of the presence of HPV DNA have been developed. Positive specimens must be subsequently typed with specific primers as mentioned earlier (Resnick et al., 1990 and Dallas et al., 1989).

There are several advantages to using the polymerase chain reaction. The procedure can be performed in less time than it takes to perform conventional molecular analysis if one excludes DNA extraction and molecular detection. The technique requires only 0.5 μ m tissue sections or comparable amounts of material on smear or scrapes (Shibata et al., 1989). PCR is rapid, highly sensitive (detects 0.00001 copies per cell) and effective in all types of specimens (Roman and Fife, 1989).

Although the advantages make this procedure a useful tool for the evaluation of clinical material, it does not allow visualization of the target DNA. However, in most cases, the clinical management of HPV may only require identifying its presence, or the possibility of a "high risk" infection and not cellular location (Kiyabu et al., 1989). Due to the extreme sensitivity of this procedure, careful attention must be given to avoid contamination from extraneous DNA (or DNA from other specimens). Cross-contamination of samples can be avoided by

meticulous cleaning of all utensils with xylene between cutting specimen blocks and proper handling of the extracted DNA.

M. Relationship of Oral Neoplasms to Human Papillomaviruses

A. Pathogenesis

Perhaps the most significant recent advance in understanding the pathogenesis of benign and malignant squamous proliferative lesions of several organs (genital, cutaneous, laryngeal, oral) has been the detection of human papillomavirus DNA in these lesions (Phelps and Howley, 1989; zur Hausen, 1987). Papillomaviruses are associated with papillomas, fibropapillomas, dysplasias and carcinomas in various animals including cows, horses, sheep, deer, elk and rabbits. As mentioned earlier, several types of human papillomaviruses (HPV) and papillomaviruses of nonhuman species have been cloned and sequenced, and found to display considerable homology. Like many other DNA tumor viruses, the papillomavirus genome includes early and late genes. The former are responsible for transformation, replication and control of gene expression while the latter encode structural capsid proteins (Phelps and Howley, 1989).

B. Etiology

Female and male genital carcinomas have been most convincingly linked to papillomaviruses and this evidence has been previously discussed (see pages 7-22).

Among oral lesions, HPV DNA has been detected in squamous papillomas, condylomata, verrucae, focal epithelial hyperplasias and carcinomas (Eversole, et al., 1987a, 1987b, 1988; Syrjanen, et al., 1987). Focal epithelial hyperplasia (Heck's disease) usually contains HPV13 or HPV32 DNA (Scully, et al., 1988). The remaining lesions may show infection with HPV types 2, 6, 11, 16 or 30 (Scully, et al., 1988). Greer, et al. in 1987 indicated that five of twenty seven smokeless tobacco associated keratoses were infected with HPV types 2 or 6. Sixteen of 77 smokeless tobacco keratoses contained HPV capsid antigen (Greer, et al., 1988).

The pathogenetic significance of oral HPV infection is clouded somewhat by two facts. First, even highly sensitive techniques such as Southern blot hybridization detect HPV DNA in only a small percentage of invasive carcinomas (Kiyabu, et al., 1989; Maitland, et al., 1987). Similar studies using in situ hybridization have found HPV infection in 11.8% of oral cancers (Syrjanen, et al., 1988). The second troublesome fact is that some studies have detected HPV 16 related sequences in up to forty two percent of patients with no clinically

apparent lesions (Maitland, et al., 1987; Scully, et al., 1988).

C. Current Information

The presence or absence of HPV sequences in various oral lesions has been studied extensively using Southern blot or dot blot hybridizations and by DNA-DNA in situ hybridization (Eversole, et al., 1987a, 1987b, 1988; Ostrow, et al., 1987; Syrjanen, et al., 1986, 1987, 1988). However, the most sensitive method for papillomavirus detection, the polymerase chain reaction (PCR) amplification of DNA, has not been applied to studies of large numbers of malignant and premalignant oral lesions. The researcher is aware of only one study which has utilized PCR for HPV identification in oral lesions, and this study used primers for only HPV types 16 and 18, and examined only carcinomas but not other leukoplakic or erythroplakic lesions (Kibayu, et al., 1989).

CHAPTER III : PROCEDURES

Experimental Organization

A. Principle

The goal of the proposed research is to correlate molecular evidence of viral infection with the histological appearance of hyperplastic lesions, benign neoplasms and malignant neoplasms of oral squamous epithelium. Archival specimens from the Oral Pathology and Surgical Pathology Laboratories at the Medical University of South Carolina will be analyzed to detect infection with human papillomavirus.

B. Working Hypotheses

1. Many proliferative lesions of oral squamous epithelium are composed of HPV infected cells. This hypothesis will be tested by utilizing PCR amplification.
2. Different HPV types are present in different lesions, with high grade dysplasias and carcinomas containing those types with E6, E7, and L1 genes capable of in vitro

transformation. HPV presence will be determined by agarose gel electrophoresis and HPV type will be detected by dot blot hybridization with type specific probes corresponding to oligomer products from PCR amplification (Figure 2).

3. Productive HPV infection is present more often in early or low grade proliferative lesions than in carcinomas. Results of analyses for viral nucleic acids will be correlated with histology.

Description and Selection of Specimens

Patient specimens for analysis will be selected to include representative examples of hyperplastic lesions, dysplasias, benign neoplasms, non-invasive and invasive carcinomas. The carcinomas will include various grades (well, moderately and poorly differentiated) as well as an example of verrucous carcinoma. Whenever possible, sections will be chosen which also include portions of histologically normal epithelium to serve as internal controls. Cases will be selected from the files of the Surgical Pathology and Oral Pathology laboratories at the Medical University of South Carolina.

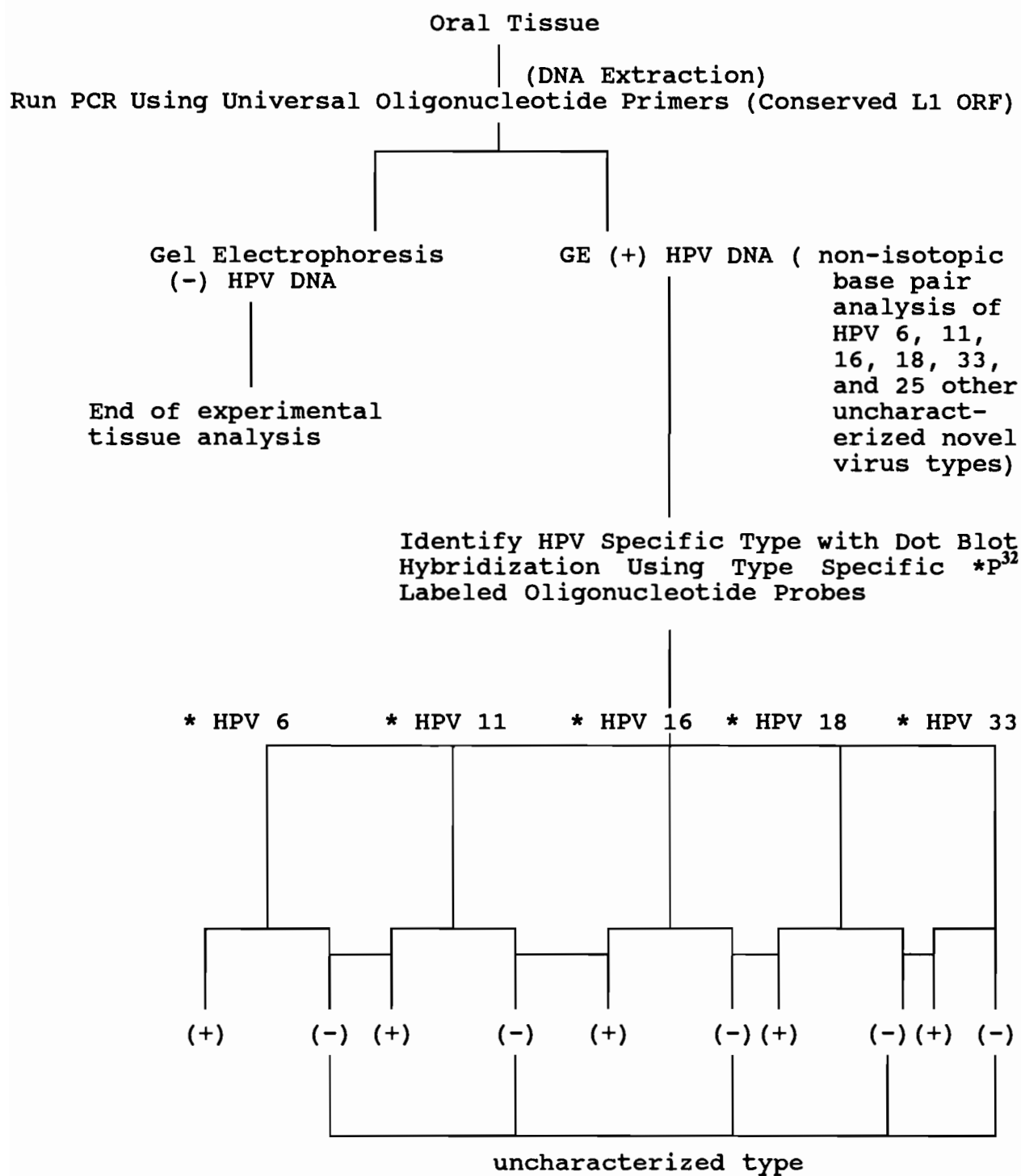


FIGURE 2. DETECTING HPV DNA IN ORAL NEOPLASMS USING THE POLYMERASE CHAIN REACTION

Extracting DNA from Paraffin-embedded specimens

(Protocol performed after modification of a procedure described by Wright and Manos, PCR Protocols, Innis et al., 1990)

A. Materials (Perkin Elmer Cetus Corp., Norwalk, CT)

1. Oral Tissue sections
2. xylene
3. 100% ethanol
4. Proteinase K (20 mg/ml stock solution)
5. Digestion buffer [50 mM Tris (pH 8.5), 1 mM EDTA, plus 0.5% Tween 20]

B. Methods

Prepare sections (10-20 μ m thick) from blocks of fixed embedded tissue (see page 73). Trim excess paraffin from the block before slicing. Cut the sections and remove them from the microtome dry, handling the sections with clean tweezers. Place tissue section into a 0.75-ml microfuge tube. Avoid cross-contamination of samples by meticulous cleaning of all utensils with xylene between cutting each block and changing

microtome blades between specimens. Label the microfuge tubes on the caps with a Sharpie permanent pen.

Deparaffinizing Sections

Per specimen section

1. Add 0.75 ml of xylene to each tube. Close tubes, mix at room temperature for about 30 minutes.
2. Pellet the tissue and any residual paraffin by centrifugation (5 minutes in microfuge at 14,000 xg).
3. Remove the xylene from each sample with a clean Pasteur pipette.
4. Repeat steps 1, 2, and 3 to eliminate residual paraffin.
5. Add 0.5 ml of 100% ethanol, close and mix gently.
6. Pellet tissue for 5 minutes at 14,000 xg.
7. Remove almost all of the ethanol, leaving a small portion (~ 20 μ l) in each tube.
8. Repeat steps 5, 6, and 7 to remove the solvent.
9. Cover the tubes by stretching Parafilm across the top of the tube, poke several holes in it, and dry the samples under vacuum to evaporate ethanol.

Proteinase Digestion

1. Add 100 μ l of digestion buffer containing 1 μ l of Proteinase K to the extracted, dried samples. Samples containing large amounts of tissue should be re-suspended in 200 μ l of digestion buffer with 2 μ l of Proteinase K.
2. Incubate for 3 hours at 55 $\frac{1}{2}$ °C.
3. Incubate at 95 $\frac{1}{2}$ °C for 9 minutes in a thermal cycler to inactivate the protease. Do not assume the temperature of the block is the same as that within the sample (monitor the sample temperature with a thermal probe).
4. Pellet any residual paraffin or tissue by centrifuging in a microcentrifuge for about 30 seconds. Use 10 μ l of the supernatant for the amplification.
5. Store the sample at -20 $\frac{1}{2}$ °C.

Polymerase Chain Reaction

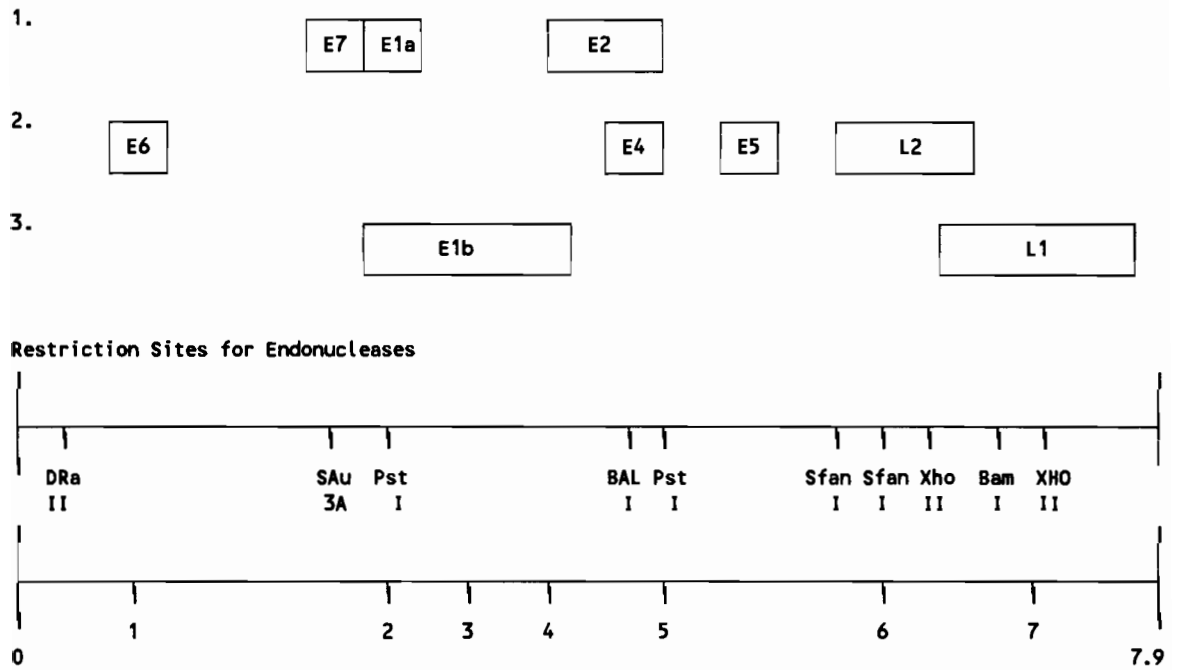
The use of paraffin-embedded tissues provides the molecular biologist with the means of analyzing archival specimens and conducting retrospective studies; consequently, the researcher is not limited to having to obtain fresh tissue from the patient each time a potential patient receives a biopsy. Gathering additional fresh tissue is not always feasible with the clinician, patient, or in the interest of time. Southern blot analysis has recently been performed on preserved tissues; however, this procedure lacks versatility and may result in false negative diagnosis due to the fact that DNA from older specimens is often found not to be completely intact. Accordingly, many cases of interest which require analysis may be found only using archival specimens. PCR is an extremely sensitive technique which allows the researcher to examine the DNA in fixed, paraffin embedded archival tissues. In addition, the efficiency of this methodology permits DNA detection in a relatively short period of time, while permitting the investigator to analyze a greater number of patient samples. PCR analysis can be cultivated using existing patient specimens from the archival files of surgical pathology. Specimens 40 years old have been successfully amplified using PCR (Shibata et al. 1989)

There are only 5 HPV genomic types which have been completely sequenced: these are HPV genomes 6, 11, 16, 18, and 33 (Schwarz et al 1983; Dartmann et al 1985; Cole and Danos 1987; Cole and Streeck 1986). It would be possible to amplify each individual HPV genomic type with each individual specific primer to isolate the presence of the virus in oral tissue. This process, although used most frequently in the past, is time consuming and laborious. One primer which would amplify all 5 types of HPV could be used. For these reasons, the investigator decided to use a universal or "consensus"(degenerate) primer pair which shares interspersed regions of DNA sequence homology. These regions are common uniquely within the open reading frames E1 and L1. By comparing the DNA sequences of genital HPV types 6, 11, 16, 18, and 33, regions were identified with homology 20 to 25 bp in length from the L1 open reading frame (ORF). Unlike oligomers derived from the E6 or E7 ORF, these primers also successfully amplify types 1, 5, 8, 26, 27, 30, 31, 35, 39, 40, 41, 42, 43, 45, 47, 48, 51-55, 57-59, and at least 25 other yet unidentified types of HPV (Innis et al., 1989)). Universal primers were originally used to amplify genital papillomaviruses but common HPV types have been found to infect other organ systems (oral cavity, larynx, lung, bladder). As recently as February 1991, "consensus"primers were developed from the E6, E7 ORF which is involved in viral transformation. However, in a comparative study, the L1

primers and the E6, E7 primers were found to be identical in amplification using a variety of pre-invasive and invasive lesions with the exception of one specimen. Both primers were equally successful,; therefore, using the E6, E7 primers instead of the L1 to detect integrated viral DNA was inconsequential (personal communication, Michele Manos). Even in instances where episomal L1 is not detected using Southern blot, most tumors have small episomal viral DNA, although in small amounts. The amount is of little consequence because of the capability of PCR to amplify as little as 1 copy of nucleic acid. The added advantage of the L1 over the E6, E7 primers is its ability to amplify other novel HPV types, yet uncharacterized which may infect other areas such as the oral cavity. For these reasons, the investigator chose to use the L1 primers (Figure 3).

The sequences of oligonucleotide consensus primers (primer for the positive strand and for the negative strand) are degenerate in several positions (at which one or more possible nucleotides are inserted during synthesis of the oligonucleotide) to render them complimentary to HPV 6, 11, 16, 18, 33 (Figure 4). The products of amplification are around 450 bp when visualized on agarose gel electrophoresis (Ting and Manos, 1990).

Open Reading Frames (ORF) HPV 16



Universal Primers

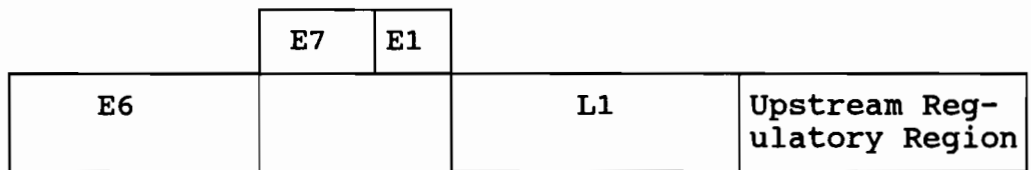


Figure 3. HPV Genomic Map

Positive Strand Oligonucleotide Sequence (20-mer)

5'GCM***CAGGGW**♥CATAAY***AATGG**

Negative Strand Oligonucleotide Sequence (20 mer)

5'CGTCCM*AR***R**#GGAW♥ACTGATC

Predicted size of HPV Amplification Products are @450 base pairs

*M = A + C	#R = A + G	♥W = A + T	♣Y = C + T
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FIGURE 4. HPV UNIVERSAL PRIMERS (Ting and Manos, 1990)

A. Materials

(Ting and Manos, 1990 with modifications)

1. sample DNA
2. 500 nM positive primer (50 μ M stock)
3. 500 nM negative primer (50 μ M stock)
4. 200 nM dNTPs (ATP, CTP, GTP, TTP)
5. 2.5 units of Taq polymerase
6. 10x PCR buffer (500mM KCL, 40m MgCl_2 , 100mM Tris (pH 8.5)
7. glass-distilled, sterile H_2O to bring the volume to 100 μ l
8. Mineral oil (ensures heat conductivity)
9. HPV-positive control (HPV 16) and HPV-negative control (water)
10. (b-globin) primer pair to check amplification

A cocktail would be made to include (per reaction):

1. 10 μ l of 10x PCR buffer
2. 1.0 μ l each of positive and negative primer
3. 2 μ l of each dNTP
4. 0.5 μ l of Taq polymerase (2.5 units)
5. 69.5 μ l of H_2O

90 μ l total

B. Methods

Pre-Thermal Cycling

1. Determine the total number of reactions (plus 1) to be run.
2. Prepare a cocktail that contains all reagents for PCR (except sample DNA).
3. Add 100 μ l of mineral oil to each tube.
4. Dispense 90 μ l of reaction cocktail into each PCR tube.
5. Add 10 μ l of extracted DNA to each tube.
6. Spin the tubes for 30 seconds in a microcentrifuge to collect reaction mixture under oil.

Thermal Cycling Conditions

* It is imperative that the conditions for thermal cycling be measured as the temperature of the sample and not the block.

1. Initial Step: Denature 95°C, 1 minute
(Taq Pol may be added here)
Annealing 55°C, 1 minute
Extension 72°C, 2 minutes
2. Subsequent Steps:
Denature 95°C, 1 minute
Annealing 55°C, 1 minute
Extension 72°C, 2 minutes
Number of cycles 39
3. Final Step: Extend for 5 minutes at 72°C.

Analysis

A. Agarose Gel Electrophoresis

Gel electrophoresis is used to analyze the products of the PCR reaction. DNA found around the 450 bp area is considered to be HPV in nature. The specific HPV product sizes are: HPV#6=448bp, HPV#11=448bp, HPV#16=451bp, HPV#18=454bp, HPV#33=448bp; however, the investigator is simply looking for the presence of HPV at this point - if found, subsequent detection of the specific type of HPV will be analyzed using dot blot hybridization procedures (discussed in the next section).

1. Materials and Methods

AGAROSE GELS

0.8% gels (Sigma Corp., > 99% pure)

* Tris acetate EDTA

MINI (25 mL)	MEDIUM (150 mL)	HALF-LARGE (250 mL)
0.2 g agarose	1.2 g	2 g
24.5 mL dH ² O	147 mL	245 mL
0.5 mL 50 x *TEA	3 mL	5 mL

Ethidium Bromide

(10 mg/mL stock)

0.5 μ l3 μ l5 μ l

Heat until almost boiling (don't boil over), swirl until in solution, repeat until completely dissolved. Allow to cool to 60°C. Add Ethidium Bromide, swirl. Pour into prepared form, no bubbles. Allow to harden ~30 minutes, remove comb.

Buffer

MINI (200 mL)

1000 mL

2000 mL

196 mL dH₂O

980 mL

1960 mL

4 mL 50 x TEA

20 mL

40 mL

4 μ L Eth. Bro.20 μ L40 μ L

(10 mg/mL)

50 x TEA Buffer, pH 7.6 (1 L)

Maniotis sec 6.7

2 M Tris-base

242 g

Dissolve in ~ 400 mL dH₂O

Add:

0.5 M *EDTA (pH 8.0) 100 mL

glacial acetic acid 57.1 mL

* Ethylenediaminetetraacetic acid

Bring to final volume of 1 L.

B. Dot Blot Hybridizations

Dot blot hybridizations allow for the specific detection of each HPV type through complementary base pair annealing with PCR products. The samples/specimens considered positive for HPV via PCR amplification and gel electrophoresis analysis can be used to perform this procedure. Hybridizations are performed using type specific probes for HPVs 6, 11, 16, 18, and 33 (Figure 5).

Prolonged annealing and amplification required in the polymerase chain reaction often leads to nonspecific products being produced in addition to the specific DNA choosing to be amplified. Performing dot blot hybridizations will eliminate these undesired products.

Initially, the non phosphorylated oligomer probe (18 - 20 mer) is labeled with gamma ^{32}P adenosine triphosphate (ATP) to a specific activity of $.3 \times 10^6$ to 1×10^7 c /mM. This is accomplished using a 5' DNA terminus labeling system. DNA phosphorylation will be accomplished and the labeled probe can

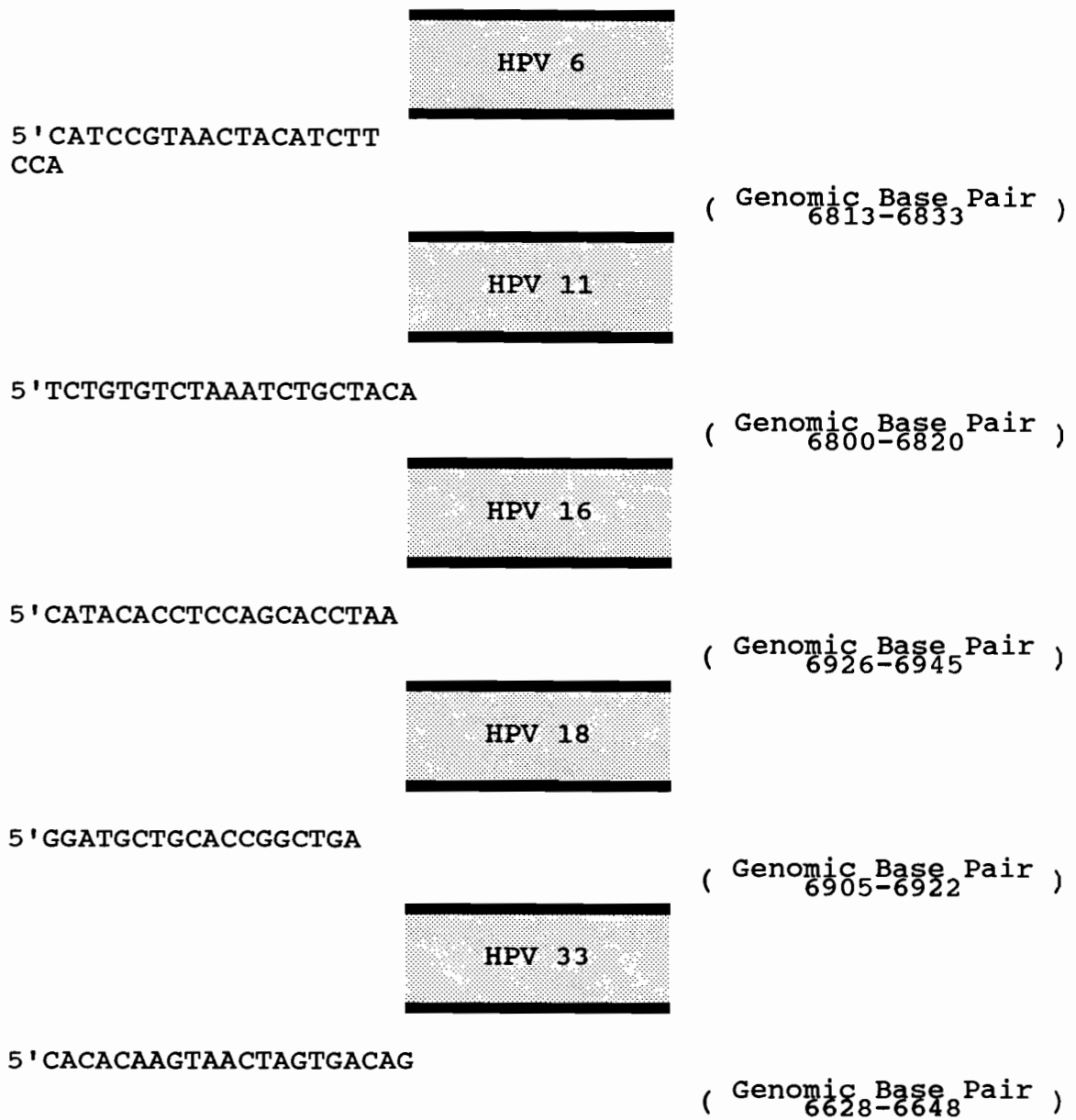


FIGURE 5. HPV TYPE SPECIFIC PROBES (Ting and Manos, 1990)

Zeta-Probe membranes are used for dot blot analysis. The DNA is heated in alkali, then filtered directly onto the Zeta-Probe membrane.

1. 5' DNA terminus labeling

a. Materials

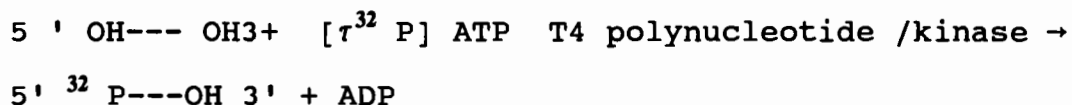
1. T4 Polynucleotide Kinase; 5U/ μ L; (50 mM Tris-HCl, pH 7.5; 25 mM KCl; 5 mM DTT; 0.1 μ M ATP; 50% (v/v) glycerol; 0.2 mg/mL BSA)
2. Forward Reaction Buffer (5X); (300 mM Tris-HCl, pH 7.8; 75 mM 2-mercaptoethanol; 50 mM $MgCl_2$; 1.65 μ M ATP)
3. Control DNA (Lambda DNA, Hae III-cleaved; 0.3 mg/mL) (20 mM Tris-HCl, pH 8.0; 0.1 mM Na_2 EDTA)

Methods

(Modified from protocol of the Bethesda Research Laboratories)

This system end labels the 5' end of the DNA oligonucleotide which is 18 - 20 base pairs in length, depending upon the HPV specific type (see Figure 4). A forward

reaction is used to allow for the phosphorylation at a specific activity of 1×10^7 cpm/pmol. The reaction is seen as follows:



1. Phosphorylation of DNA - add the following components, in order:

- a. HPV DNA - 100 nanograms
- b. Forward reaction buffer (5X) - $1 \mu\text{l}$
- c. $[\gamma^{32} \text{ P}]\text{-ATP}$ - $200 \mu\text{C}$ (if ethanol is present in the $[\gamma^{32} \text{ P}]\text{-ATP}$ (4500 Ci/mmol), the solution should be lyophilized in the reaction tube, and sterile distilled H_2O added to a final volume of $5 \mu\text{l}$ or $1 \mu\text{l}$).
- d. T4 polynucleotide kinase (5 units) - $2 \mu\text{l}$.

Total volume = $10 \mu\text{l}$

Incubate for 37°C for 30 minutes, determine cpm incorporated, and EtOH precipitate the labeled DNA by adding 0.5 volume $7.5 \text{ M NH}_4 \text{ OAc}$, 2 volumes EtOH (-20°C). Store at

-70°C for 30 minutes. Collect the DNA by centrifugation at 15,000 rpm for 5 minutes. Collect and resuspend the pellet (usually invisible) in 50 μ L 10 μ M Tris-HCl (pH 7.5), 0.1 μ M EDTA. Repeat precipitation. Alternatively, a column can be utilized for rapid separation of labeled DNA from free isotope (see below).

Nucleic Acid Cleanup

(Bethesda Research Laboratories, Gaithersburg, MD.)

1. "Invert column several times to resuspend settled gel. Remove top cap followed by the bottom stopper and allow the excess buffer to drain by gravity. Discard buffer.
2. Place the column in a collection tube and centrifuge for five minutes in a swinging bucket or horizontal rotor centrifuge at an rpm which will generate a centrifugal force of $1100 \times g$.
3. If any liquid remains in the top of the column or if the liquid in the collection tube is in contact with the tip of the column, discard the collected buffer and re-spin for an additional 30 seconds. Discard buffer and collection tube.
4. Very carefully apply the sample (50-100 microliters) directly to the center of the column, allowing the liquid to drain into the gel bed between successive drops of the sample. (Careless sample application may result in lower nucleotide retention due to channeling of nucleotides down the slides of the column.)

5. Place the column containing the applied sample in the second collection tube provided and centrifuge for four minutes at a minimum centrifugal force of 1100 x g.
6. The purified nucleic acid solution will be in the collection tube.
7. Discard the column."

Pre-hybridization : Materials and Methods

1. Heat one twentieth of the PCR product in a total volume of 0.5 mL with a final concentration equal to 0.4 M NaOH, 10 mM EDTA at 100°C for 10 minutes.
2. Wet a sheet of Zeta-Probe membrane by immersing it in distilled water.
3. Assemble the microfiltration apparatus with the pre-wetted Zeta-Probe membrane. Make sure that all the screws or clamps have been tightened under vacuum to prevent cross-well contamination. Rinse wells with 0.5 mL TE or H₂O. Apply vacuum until wells are empty but **not** dry.
4. Apply a 0.5 mL DNA sample into each appropriate well without vacuum.
5. Start vacuum until the wells are just dry.
6. Rinse all wells by placing 0.5 mL of 0.4M NaOH in each, then apply vacuum until all wells are quite dry.
7. Disconnect the vacuum, disassemble the apparatus, and rinse the membrane briefly in 2 x SSC. Air dry the membrane. The dried membranes are stable at room temperature. If hybridization is not to be undertaking within two days, vacuum dry the blotted Zeta-Probe membrane at 80°C for 30 minutes. The membranes can be

stored dry between two pieces of filter paper in plastic bags at 23-25°C.

2. Hybridization Protocol for Oligonucleotide DNA Probes : Materials and Methods

Seal the blotted Zeta-Probe membrane inside a heat sealable bag. Prepare the following solution for pre - hybridization:

³²P-kinased oligonucleotides HPV 6, HPV 11, HPV 16, HPV 18, HPV 33

5X SSC (Standard Saline Citrate)

20mM NaH₂PO₄, pH 7.0

7% SDS (Sodium Dodecyl Sulfate)

10X Denhardtts

Denatured herring sperm DNA @ 100mg/ml

1. The carrier DNA must be denatured before adding it to the hybridization solution by heating at 100°C for 5 minutes, followed by rapid cooling in ice.
2. Cut one corner of the plastic bag and pipet pre - hybridization solution in, then reseal the bag.
3. Incubate at 50°C for 0.5-24 hours.

Hybridization:

1. Immediately before use, fragment and denature the probe and carrier DNA as follows. Dissolve the radio-labeled

probe in 0.1ml of 0.2M NaOH, add carrier DNA, mix, and centrifuge briefly to consolidate the solution. Pierce a fine hole in the tube cap and place the tube in a heating block at 100°C for 5 minutes, followed by rapid cooling on ice.

2. Clip corner of bag, remove pre - hybridization solution, and replace it with same buffer.
3. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 50°C for 4-24 hours. **NEVER LET THE MEMBRANES DRY.**

Washes:

1. Open side of bag with scissors and remove fluid with low suction into hot vacuum flask.
2. Remove filters and place in pre-warmed wash solution. **DO NOT LET FILTERS DRY.** Note - All filters hybridized with the same probe can be washed together.
3. Wash twice 30-45 minutes at 50°C with about 300-500 mL each wash, discard down radioactive sink.

3X SSC

10X Denhardt's

5% SDS

25 mM NaH₂PO₄, pH 7.5

bring to final volume of 1000 mL

4. Wash once for 30 minutes at 50°C with about 300-500 mL each wash, discard down regular sink.

1X SSC

1% SDS

bring to final volume of 1000 mL

5. Wrap in Saran Wrap after last wash (avoid wrinkles) and expose to film, using intensifying screens to sandwich filter and placing side transferred against gel next to film. Develop in 1-2 days.

6. To strip wash two times in 500-1000 mL of:

0.1 x SSC	5 mL/L of 20 x SSC
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0.5% SDS	5 g/L
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at 95°C for 20 minutes. Keep wet. Rewrap and store at -20°C. Blot Saran with toweling before checking with overnight exposure.

EXPECTED RESULTS

The use of universal primers for the detection of HPV within oral neoplasms should allow for a more complete picture concerning the presence of this virus because of the broad spectrum of viral types defined by these primers. The consensus primers should elucidate the presence of HPV in lesions which to this point have not been associated as carrying the viral genome due to the fact that probes have not been developed to detect any sequences other than HPV 6, 11, 16, 18, 33. These viral types are known to be associated with cervical cancer of the uterus; however, the possibility that these may also be the only types implicated in viral infection of oral tumors or lesions may be misleading. The research should show not only the presence of virus in these lesions which to this date has gone undetected but also demonstrate an increased association of infection due to the sensitivity of the technique (PCR) over the conventional molecular techniques used to diagnose this disease. However, if viral presence is found on the gel electrophoresis but can not hybridize with the type specific ^{32}P labeled complementary probes (HPV 6, 11, 16, 18, 33) to the types specified above, the researcher must assume the viral type is one which is included in the litany

of complementary types specified in the universal primer set.

METHOD OF DATA ANALYSIS

Data will be compared and analyzed using 35mm photographic analysis of the PCR electrophoretic products and the dot blot hybridizations. Graphic and tabular forms will be used to indicate HPV presence and type within oral neoplasms.

CHAPTER IV : RESULTS

SUMMARY OF THE DATA

A. Agarose Gel Electrophoresis

Ninety samples (one hundred and three diagnoses) were analyzed for the presence of HPV after polymerase chain reaction amplification with the universal primers. Samples considered as positive for HPV were seen as possessing a 450 base pair fragment on a 0.8% agarose gel. Standard molecular weight markers were used to determine the size of the electrophoretic products. Control (b Globin) primer pairs were used to insure that the samples were sufficiently intact or amplifiable (the extraction was successful and DNA intact). The b Globin primers simultaneously amplified a 260 base pair fragment which served as an internal control (for examples see Plate XIV). Control (HPV 16) DNA was used to insure the validity of the reaction cocktail (for examples see Plate XV, XVI). Negative controls (water) were used in each set of amplifications to monitor cross over or possible contamination. To reduce the possibility of contamination, all tissues were cut, extracted, and amplified on three different counters in three different rooms; furthermore, each tissue

was cut with its own microtome blade and all utensils were thoroughly cleaned with xylene.

After intercalation of the ethidium bromide with the PCR products and exposure to ultraviolet light, the researcher found the presence of HPV in twenty one paraffin-embedded tissue specimens. Of these specimens, the breakdown of HPV per clinical specimen was as follows (see Table 1 and 2) : Four well differentiated squamous cell carcinomas (for examples see Plates I, II, VII, XXII, and XXIV) (total number analyzed = nineteen), three moderately differentiated squamous cell carcinomas (for examples see Plates III, IV, X, XI, and XXVI) (total number analyzed = fifteen), no poorly differentiated squamous cell carcinomas (total number analyzed = three), three carcinoma in situs (for examples see Plates XII, XIII, XXIII) (total number analyzed = nine), two mild dysplasias (for examples see Plates V, VI, XXI) (total number analyzed = fourteen), two moderate dysplasias (for examples see Plates V, VI) (total number analyzed = five), two severe dysplasias (for examples see Plates V, VI) (total number analyzed = eight), two hyperplasias (for example see Plate VIII) (total number analyzed = four), two inflammations (for examples see Plates III, IV, VIII, IX) (total number analyzed = five), no verrucous carcinoma (total number analyzed = two), and one specimen which was benign or normal (total number analyzed = six).

TABLE 1. Types of Oral Specimens

<u>Diagnosis</u>	<u># of Specimens Analyzed</u>
Benign Normal	6
Inflammation	5
Hyperplasia	4
Dysplasia	<u>27</u>
a. mild	14
b. moderate	5
c. severe	8
Carcinoma in Situ	9
Squamous Cell Carcinoma	<u>37</u>
a. Well Differentiated	19
b. Moderately Differentiated	15
c. Poorly Differentiated	3
Verrucous Carcinoma	2

TABLE 2. HPV Presence in Paraffin Embedded Oral Tissue Lesions

Oral Lesion	HPV Types						/ %
	6	11	16	18	33	uncharacterized	
1. Negative/Normal Benign (N=6)	-	-	1	-	-	-	17
2. Hyperplasia (N=4)	-	1*	2	-	-	-	50
3. Inflammation (N=5)	-	-	2	-	-	-	40
4. Mild Dysplasia (N=14)	-	1*	2	-	-	-	14
5. Moderate Dysplasia (N=5)	-	-	2	-	-	-	40
6. Severe Dysplasia (N=8)	-	-	2	-	-	-	25
7. Carcinoma in Situ (N=9)	-	-	3	-	-	-	33
8. Well Differentiated Squamous Cell Carcinoma (N=19)	-	-	4	1*	-	-	21
9. Moderately Differentiated Squamous Cell Carcinoma (N=15)	-	-	3	-	-	-	20
10. Poorly Differentiated Squamous Cell Carcinoma (N=3)	-	-	-	-	-	-	0
11. Verrucous Carcinoma (N=2)	-	-	-	-	-	-	0
Total	0	2	21	1	0	0	

* Co-infection

- viral type not present in tissue

B. Dot Blot Hybridization

Dot blot hybridizations were performed on each oral neoplasm which was found to be positive for HPV by gel electrophoresis for typing the virus. Identification of the virus was performed by selective hybridization of each positive sample with five different HPV probes hybridized and labeled with ^{32}P . These probes included: HPV 6, 11, 16, 18, and 33.

Hybridization with any of these probes was considered as specific for a unique viral type (for examples see Plates XVII-XXI); furthermore, intensity of hybridization was analyzed against a known control. The following compares the clinical diagnosis with HPV type (see Table II) : HPV 6 was not found in any of the oral neoplasms. HPV 11 was found in one mild dysplasia and one hyperplasia. HPV 16 was found in four well differentiated squamous cell carcinomas, three moderately differentiated squamous cell carcinomas, three carcinoma in situs, two mild dysplasias, two moderate dysplasias, two severe dysplasias, two hyperplasias, and one negative specimen. HPV 18 was found in one well differentiated squamous cell carcinoma, while HPV 33 was not found in any oral neoplasms. All specimens considered to harbor HPV via PCR/ gel electrophoresis analysis were typed with the dot blot hybridizations. There were no uncharacterized types found. All positive controls hybridized (Plate XVII) (HPV 6 was also positive, data not shown).

CHAPTER V : DISCUSSION OF THE RESULTS

INTERPRETATION OF THE DATA

The study proves that human papillomavirus genomic DNA is found in oral lesions, including: leukoplakia, hyperplasia, mild, moderate, and severe dysplasias, carcinoma in situ, and well and moderately well differentiated squamous cell carcinomas. It is interesting to point out that the virus can be found throughout the spectrum of the diseases. Benign lesions such as leukoplakia or hyperplasia were positive for HPV 11 which is associated with episomal or non-integrated viral life cycles. Pre-invasive dysplastic lesions were positive for HPV 16 which is associated with integrated or transforming viral life cycles. Lastly, squamous cell carcinomas were positive for HPV 16 and HPV 18 which are associated with integrated or transforming viral life cycles. There were two cases which had a co-infection or two viral types associated: one well differentiated squamous cell carcinoma had HPV 16 and 11 (both the episomal and transforming viral types) and one well differentiated squamous cell carcinoma harbored types 16 and 18. Overall, papillomavirus infection was found in twenty four percent of

The results of these experiments are consistent with the hypothesis that the transforming viral type may be found in oral neoplasms. In addition, these findings are consistent with the theory that HPV 16 is responsible for the majority of HPV infections associated with mucosal areas. The presence of HPV in oral specimens using the universal primers directed against the L1 ORF indicates that the virus may conserve the E6, E7, L1 genomic sequence in episomal cases and during transformation. Sequences associated with HPV 16 and 18 are known to transform or break off of the viral genome and insert into host genetic material in vitro and are thought to be associated with carcinoma of the cervix in vivo ; moreover, the research may give credence to the same phenomena in oral carcinogenesis due to the findings of HPV 16 and 18.

The universal primers proved to be successful in amplifying the viral DNA. This research on oral tumors allowed for the detection of a 450 base pair fragment which is exclusive to the viral genome. This could not have been accomplished using the Southern blot technique because entire genomic identity is required. This technique avoids the problem where older specimens embedded in paraffin tend to fragment as age increases. This fragmented DNA is a poor template for detection (Resnick et al., 1990). Only small fragments were produced by PCR in this research (L1 is around 450 base pairs) allowing for detection in a much higher

450 base pairs) allowing for detection in a much higher percentage of cases. PCR detects more specifically and can detect as little as one copy of the viral DNA.

In sixty nine cases, there was no PCR product detectable by gel electrophoresis which indicates that there was no HPV present within the tissue. Another reason for the lack of amplification may be due to the fact that the tissue may have been initially poorly fixed and if there was viral DNA present it was too fragmented for PCR. Another consideration could be that another primer designed to amplify another area might be needed to detect the HPV. The only other primer that might be useful would be one exclusively directed against the transforming genotypes such as an E6, E7 primer. This is most likely not the case because these primers have been compared to L1 primers and have yielded (with the exception of one case) identical results (Resnick et al., 1990). Therefore, the L1 region must also be retained in viral transformation or there is episomal DNA present even after transformation. Episomal viral DNA has been reported to be in cases where "high risk" virus types (HPV 16, 18) were found to be integrated into the host DNA (Gregoire et al., 1989). These deductions were discovered because of the sensitivity of PCR to detect as little as 1 copy of the virus where other molecular biological techniques required more DNA for detection and therefore rendered false negative diagnoses. Therefore, there is a drawback to using exclusive E6,E7

primers that fail to detect other novel types. These primers were designed to amplify only HPV sequences associated with uterine cancer; however, these types may not be the same for extra-genital sites if other novel types are involved. This cripples the utility of such a primer.

Those gels which showed positivity for HPV and hybridized with a type specific probe proved that specific virus was present. This proves the usefulness of the universal primers selected to amplify viral sequences in tumors of extra-uterine origin.

APPLICATIONS AND RECOMMENDATIONS

The utility of universal primers coupled with the sensitivity of the polymerase chain reaction could be used on any paraffin-embedded specimen thought to harbor HPV. Mucosal lesions such as those from the esophagus, larynx, or any area composed of squamous epithelium would be candidates for investigation. The urinary bladder might also be considered for possible viral gene involvement.

Clinically, this technique might be used to follow suspicious patients which might be at risk for developing carcinoma. Patients infected by a transforming genotype would be at much greater risk to develop cancer.

Due to the recent development of PCR, very few parameters have been worked out with respect to optimization of amplification. Experimenting with the concentration of the buffers, the concentration of the primers and nucleoside triphosphates are key in experimental investigation.

Lastly, it is important to investigate the difference between the type of temperature controllers available on the market. Each thermal cycler works slightly different and depending upon whether the temperature of cycling is measured off the block of the machine or in the sample with an in sample probe, the temperature used for amplification must be modified to reach optimization. Well to well uniformity differs with some of the products available and caution should

be taken to insure validity of readings between wells. This researcher compared thermal cyclers which used Peltier circuitry and water cooled systems. Peltier circuitry tended to be more efficient because it reached its target temperature more rapidly which optimizes the amount of Taq polymerase in the reaction. The overall amplification time was shorter for Peltier thermal controllers. PCR electrophoretic products tended to be brighter and crisper, indicating a more efficient amplification. However, even between Peltier controllers there were differences in amplification. In my hands, the system designed by MJ Research (Watertown, Mass.) proved to be the most reliable tool (see page 67 for more information on thermal cyclers).

SUMMARY OF THE STUDY

The presence of human papillomavirus was searched for in a variety of lesions from the oral cavity using universal primers and type specific probes coupled with the polymerase chain reaction and dot blot hybridization. Demonstration of HPV in these lesions and its specific DNA's in hyperplastic lesions of oral epithelium, pre-invasive lesions, and carcinomas was successful. These results were compared with the histological diagnoses of these lesions.

Previous research by others indicates that genital papillomaviruses appear to have a strong correlation between infection and the subsequent development of malignancy. In this study, HPV types which appear associated with carcinogenesis of the uterine cervix (HPV 16 and 18) were also found to infect dysplastic and malignant lesions of the oral cavity. This is a significant finding because many HPV types found in other organ systems (larynx, respiratory, skin,) are different from those which are associated with the genital tract. Extra-genital infections harboring viral types other than those found in the cervix increase the complexity already associated with this virus. Investigators may be comforted with the evidence that the HPV types found associated with cervical lesions were the only types found in the oral cavity.

Therefore, new viral types may not have to be discerned. This information may allow future investigators to consider focusing more strongly on these specific viral DNA's and their pathogenesis rather than trying to work on a litany of other viral types (over 60 types at this date) when considering lesions of the uterine cervix and oral cavity. This investigator found HPV 16, the viral type responsible for transformation in cell culture, in a high percentage of oral lesions which harbored the virus. These findings may stimulate future investigations focusing exclusively on the pharmacological interference of this virus.

CONCLUSIONS

The polymerase chain reaction amplification of oral neoplasms was successful in establishing the nexus between papillomavirus infection and subsequent detection. This is the first time a universal primer which allows for the detection of over 25 different types of HPV DNA has been used. Moreover, these polymerase chain reactions have been applied to benign lesions as well as malignant ones to help elucidate the possible correlation between the papillomavirus and oral cancer. The presence of HPV in oral lesions may predispose the patient to subsequent carcinogenesis either as a vehicle for initiation or promotion of squamous cell carcinoma, or simply as a co-carcinogen in the genesis of this disease. The research proves that viral DNA is found in benign, pre - invasive and invasive lesions to include leukoplakia, hyperplasia, inflammatory disorders, dysplasia and squamous cell carcinoma. The detection of the transforming viral types (HPV 16, 18) in these lesions may initiate future studies concerning the role this virus in the ontogeny of oral cancer. The clinical utility of HPV typing may be aided using the PCR method which ultimately may help in reducing this disease.

If the virus plays a key role in the advancement or evolution of oral neoplasia, it would be necessary to investigate the possible pharmacologic interference of the virus with respect to its maturation. The presence of HPV

within lesions of the oral mucosa may facilitate this future investigation.

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Plate I reveals the presence of HPV, using PCR, in two squamous cell carcinomas and one cervical intraepithelial neoplasia (CIN) used as a control. The last lane shows a 1 kilobase pair molecular weight marker used as an indicator for the 450 base pair HPV amplification products.

AGAROSE GEL ELETROPHORESIS

PLATE I

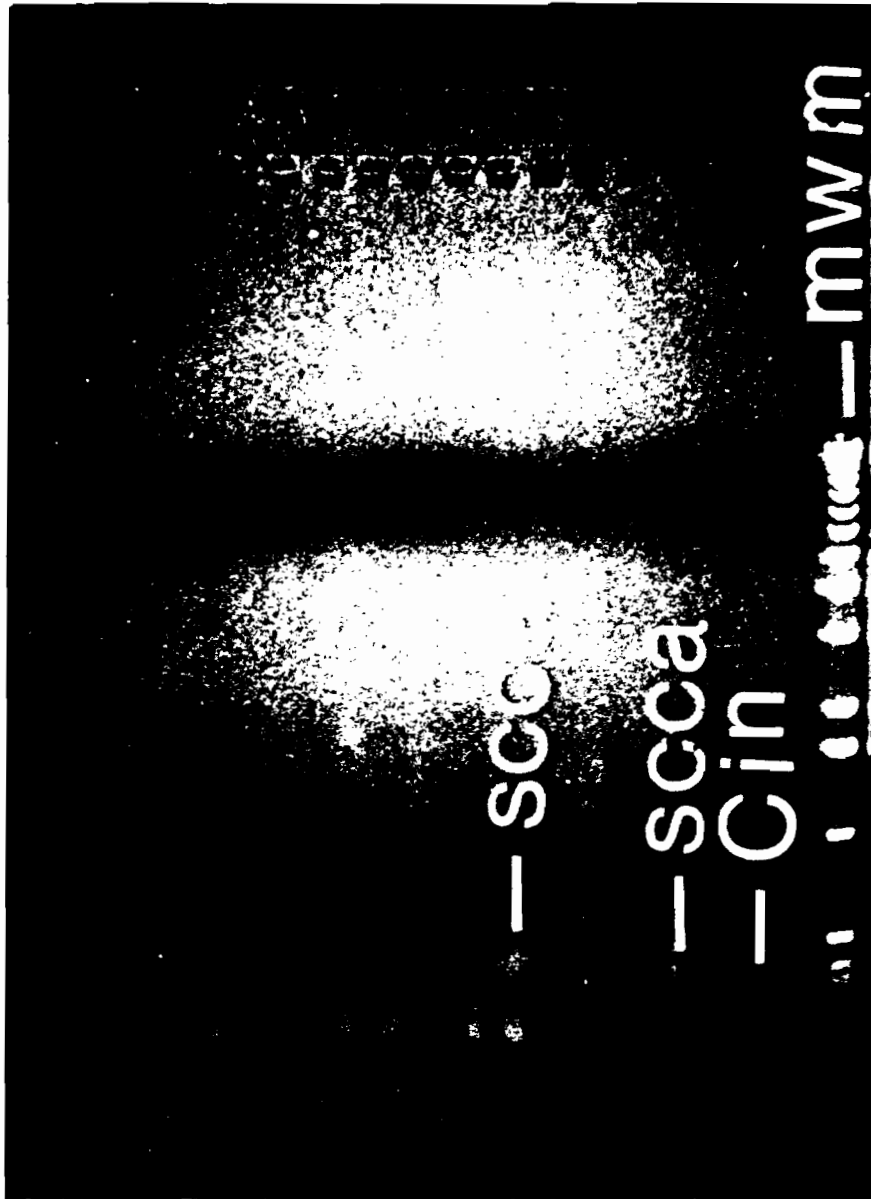


Plate II is a reverse image of Plate I to allow for better visualization of the PCR amplification products.

AGAROSE GEL ELECTROPHORESIS

PLATE II

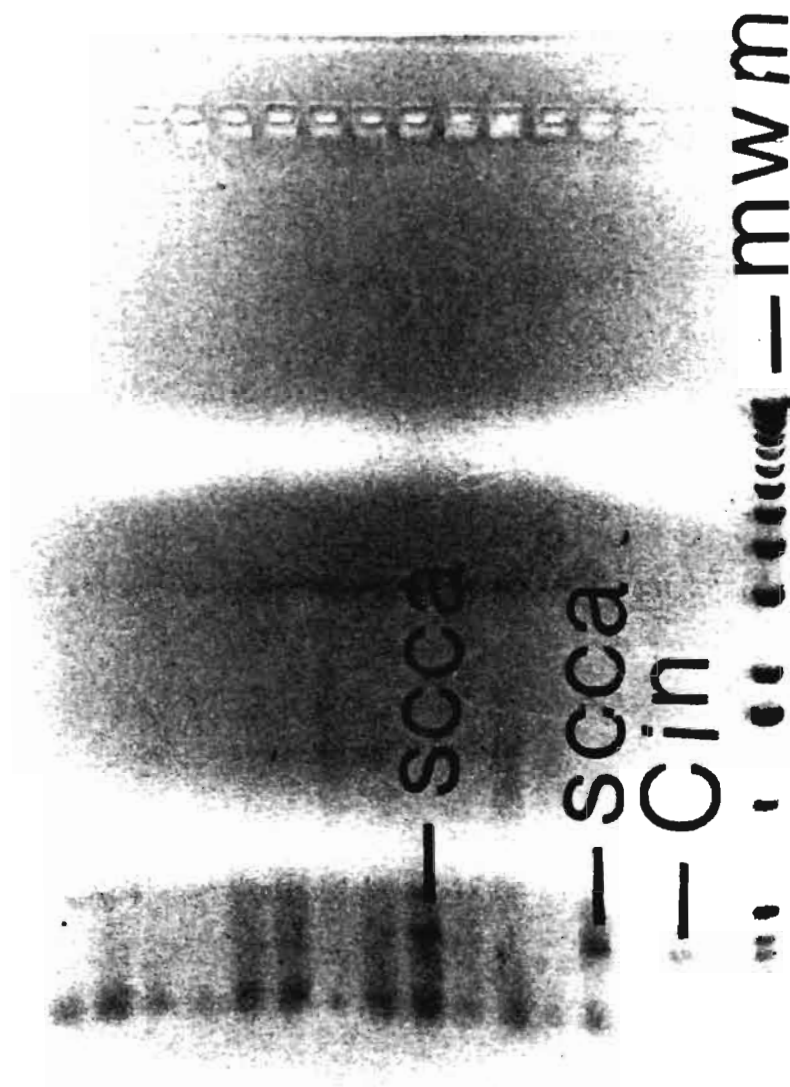


Plate III reveals the presence of HPV in two squamous cell carcinomas, one cervical intraepithelial neoplasia (CIN) used as a control, inflammation, and HPV positive control (HPV 16). The last lane shows a 1 kilobase pair molecular weight marker used as an indicator for the 450 base pair HPV amplification products.

AGAROSE GEL ELETROPHORESIS

PLATE III

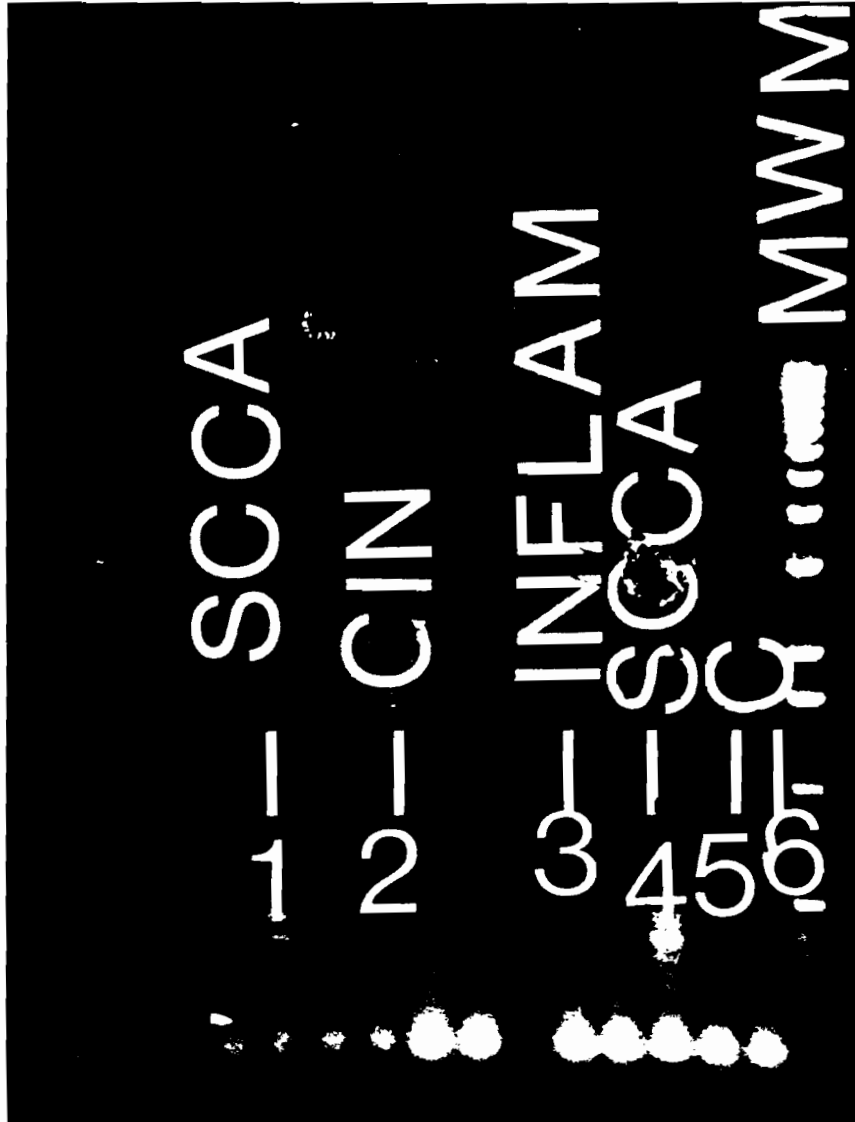


Plate IV is a reverse image of Plate III to allow for better visualization of the PCR amplification products.

PLATE IV **AGAROSE GEL ELETROPHORESIS**

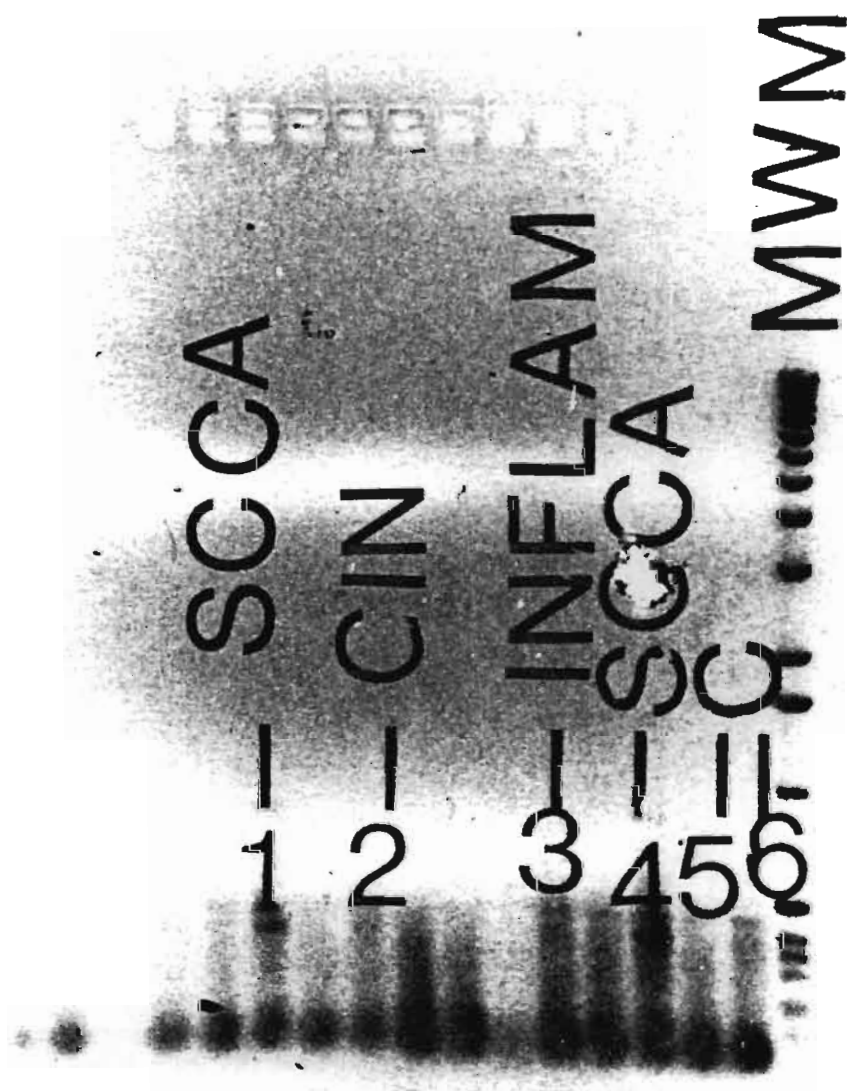


Plate V reveals HPV presence in specimens with a histological diagnosis of mild dysplasia, moderate dysplasia, and two severe dysplasias. b globin products serve as internal controls (MW= 260bp).

PLATE V

AGAROSE GEL ELETROPHORESIS



Plate VI is a reverse image of Plate V to allow for better visualization of the PCR amplification products.

AGAROSE GEL ELECTROPHORESIS**PLATE VI**

Plate VII is a reverse image to allow for better visualization of the squamous cell carcinoma. The last lane reveals a molecular weight marker of 490 base pairs.

AGAROSE GEL ELETROPHORESIS
PLATE VII



Plate VIII reveals the presence of HPV in one hyperplasia and one inflammatory specimen from the oral cavity.

AGAROSE GEL ELETROPHORESIS**PLATE VIII**

Plate IX is a reverse image of Plate VIII to allow for better visualization of the PCR amplification products.

AGAROSE GEL ELETROPHORESIS**PLATE IX**

Plate X reveals the presence of HPV in a squamous cell carcinoma. The last lane shows a 1 kilobase pair molecular weight marker used as an indicator for the 450 base pair HPV amplification products.

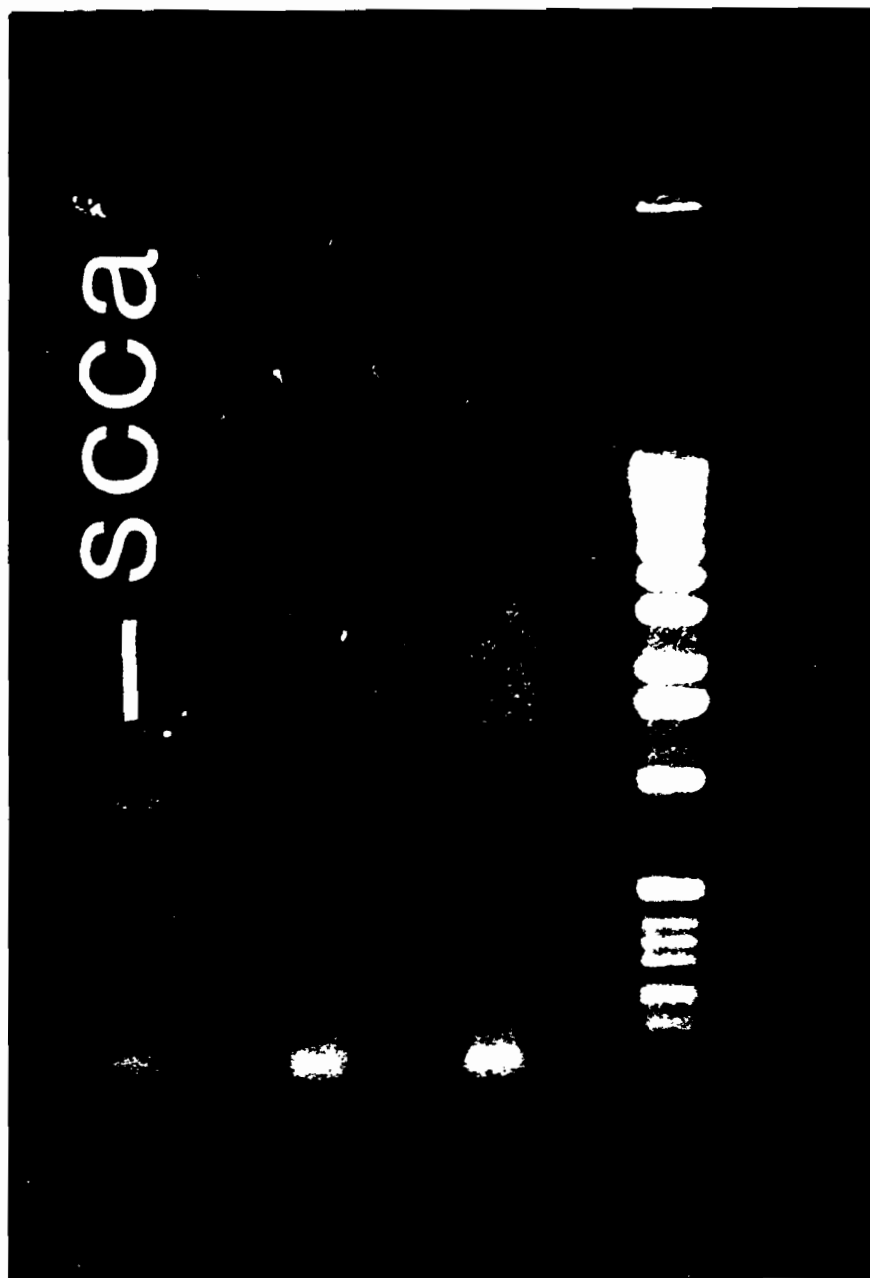
AGAROSE GEL ELETROPHORESIS**PLATE X**

Plate XI is a reverse image of Plate X to allow for better visualization of the PCR amplification products.

PLATE XI **AGAROSE GEL ELETROPHORESIS**

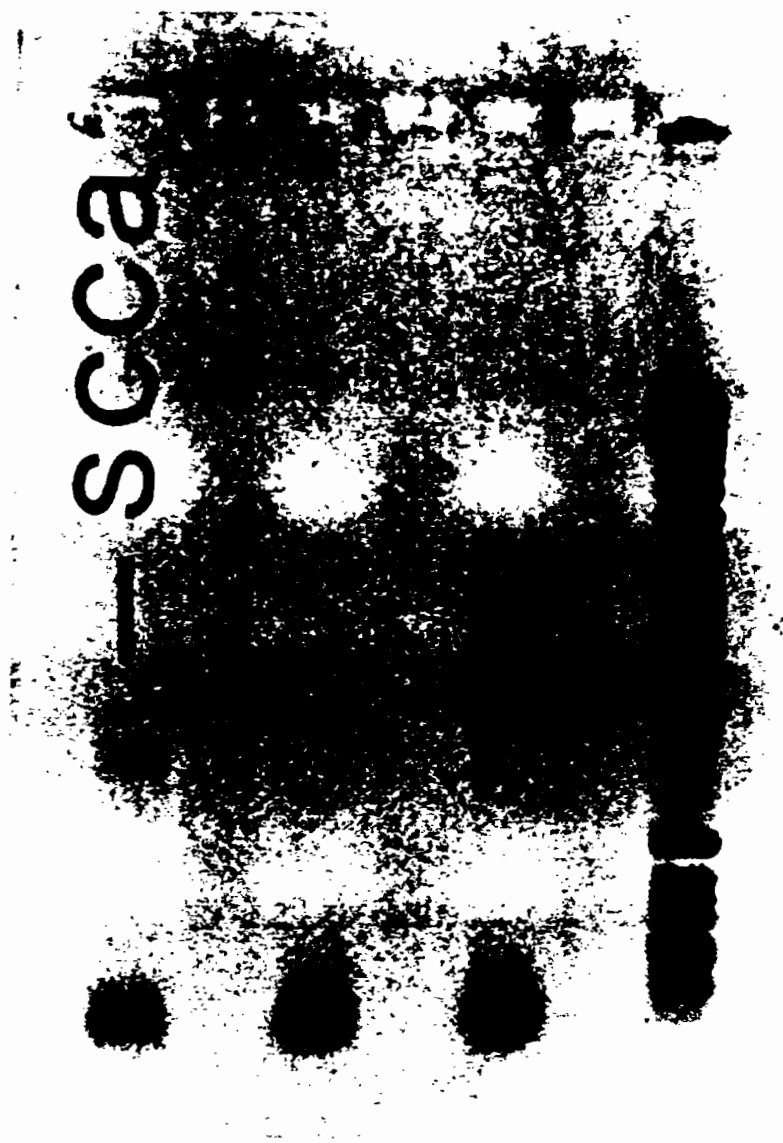


Plate XII reveals the presence of HPV in two carcinoma in situ's . The smaller 260 base pair β globin product in lane one is used as an internal control. The last lane shows a 1 kilobase pair molecular weight marker used as an indicator for the 450 base pair HPV amplification products.

AGAROSE GEL ELETROPHORESIS
PLATE XII

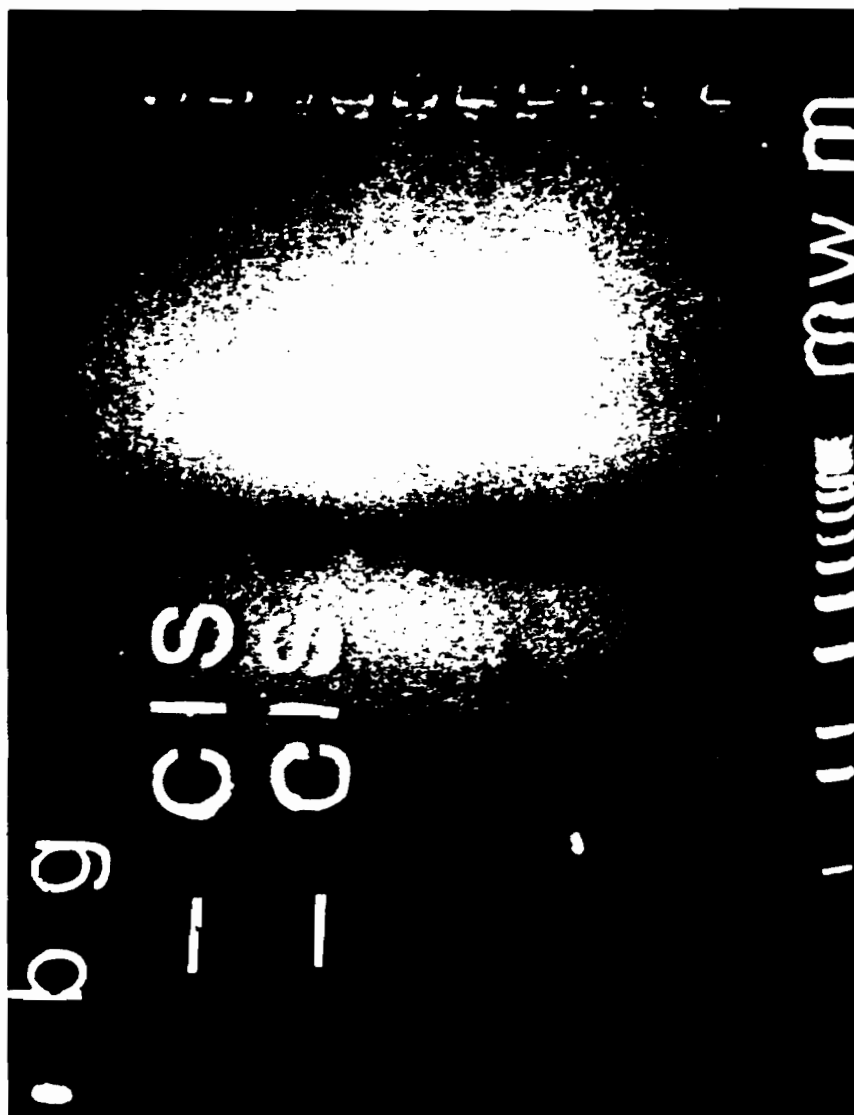


Plate XIII is a reverse image of Plate XII to allow for better visualization of the PCR amplification products.

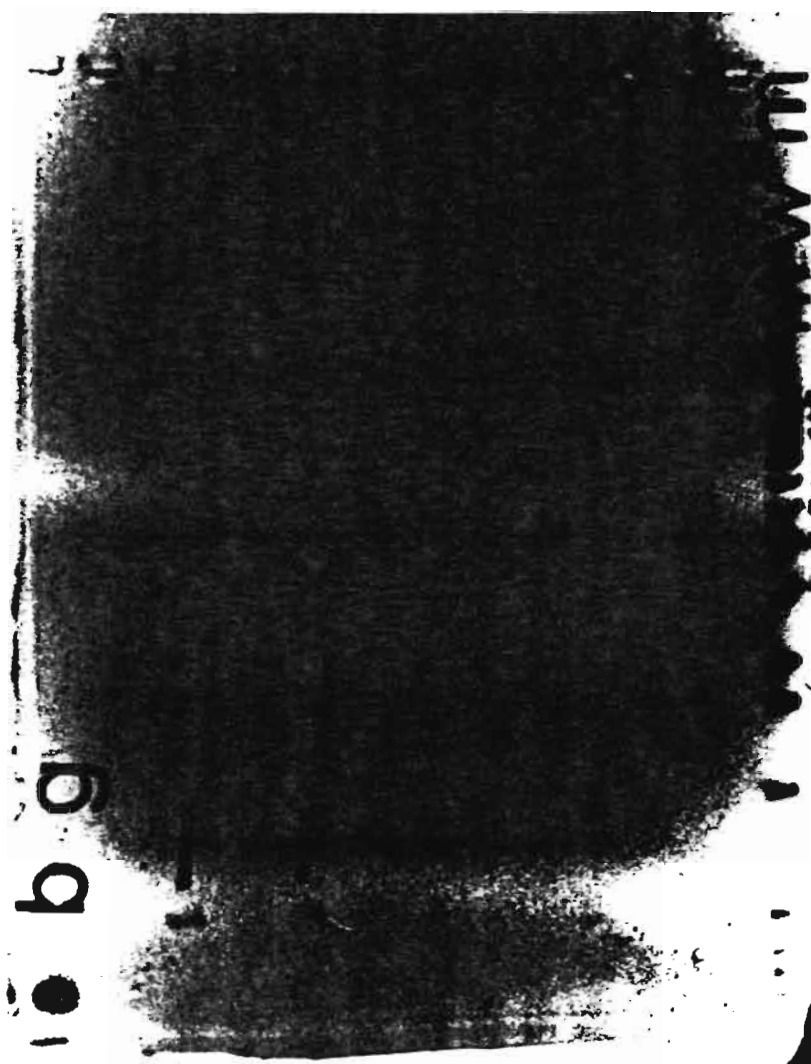
AGAROSE GEL ELETROPHORESIS**PLATE XIII**

Plate XIV reveals β globin products used to ensure that if there were HPV present, amplification would take place. β globin products prove the successful extraction of DNA from the oral tissue.

PLATE XIV **AGAROSE GEL ELETROPHORESIS**



Plate XV is a positive control (HPV 16 recieved from zur Hausen) used to ensure PCR amplification.

PLATE XV **AGAROSE GEL ELETROPHORESIS**

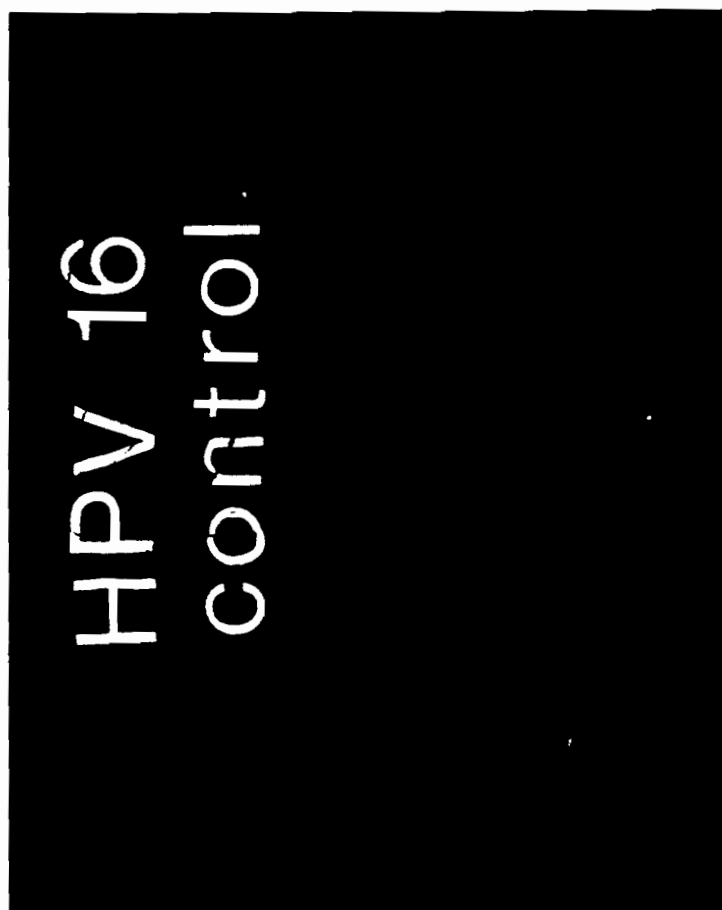


Plate XVI is a reverse image of Plate XV to allow for better visualization of the PCR amplification products.

AGAROSE GEL ELETROPHORESIS
PLATE XVI



Plate XVII reveals HPV specific types found in individual specimens from the oral cavity. Positive hybridization products were considered at least 2X stronger than the negative hybridizations (lanes above POS CON 18 and below carcinoma in situ + HPV 18). A 1+ negative signal is seen two lanes above the POS CON 18. The degree of band intensity coorelates with the number of copies of viral DNA associated with infection.

PLATE XVII

DOT BLOT HYBRIDIZATION

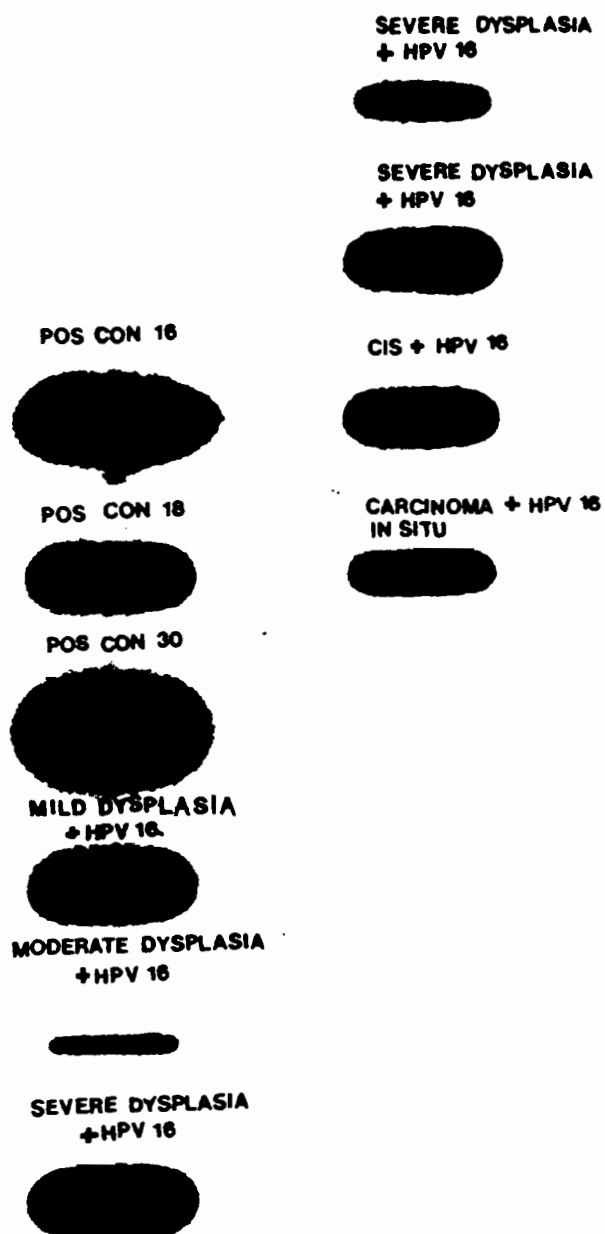


Plate XVIII reveals HPV specific types found in individual specimens from the oral cavity. Positive hybridization products were considered at least 2X stronger than the negative hybridizations. The degree of band intensity coorelates with the number of copies of viral DNA associated with infection.

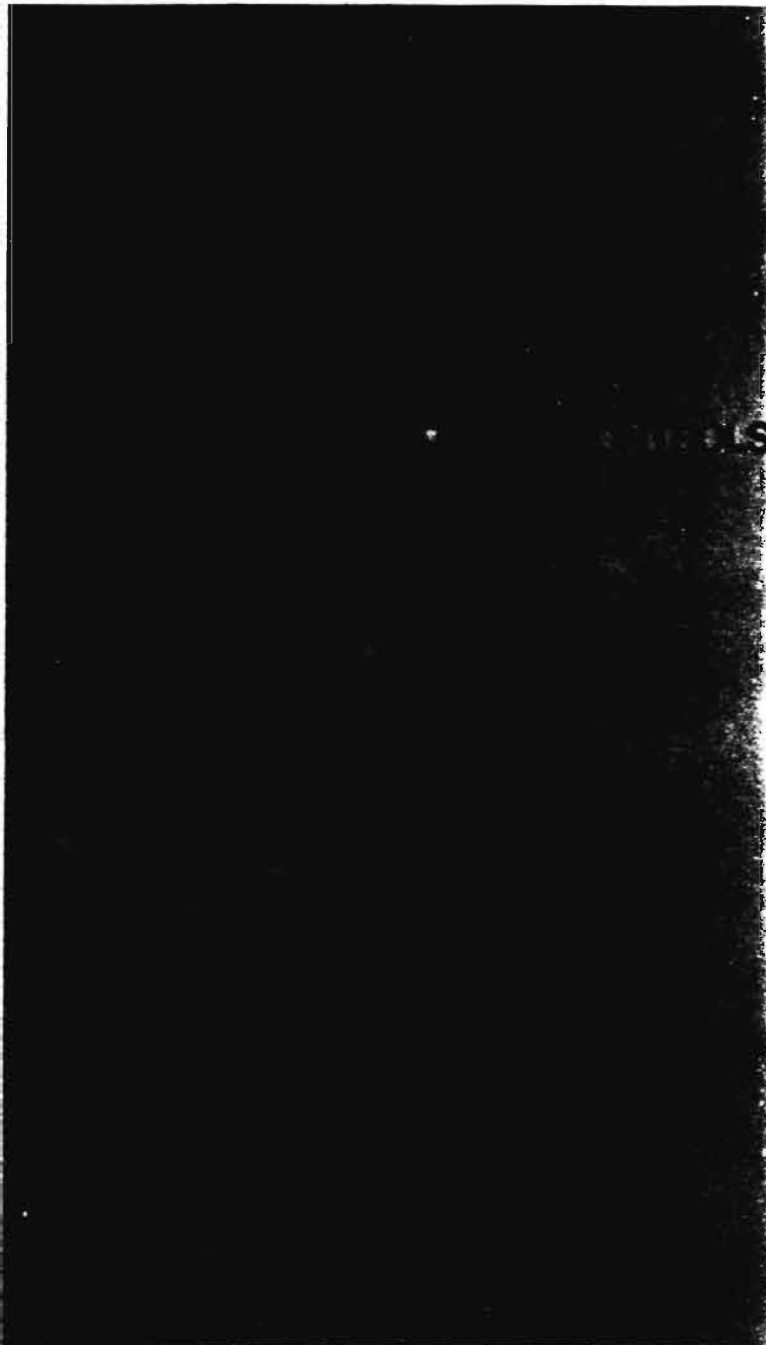
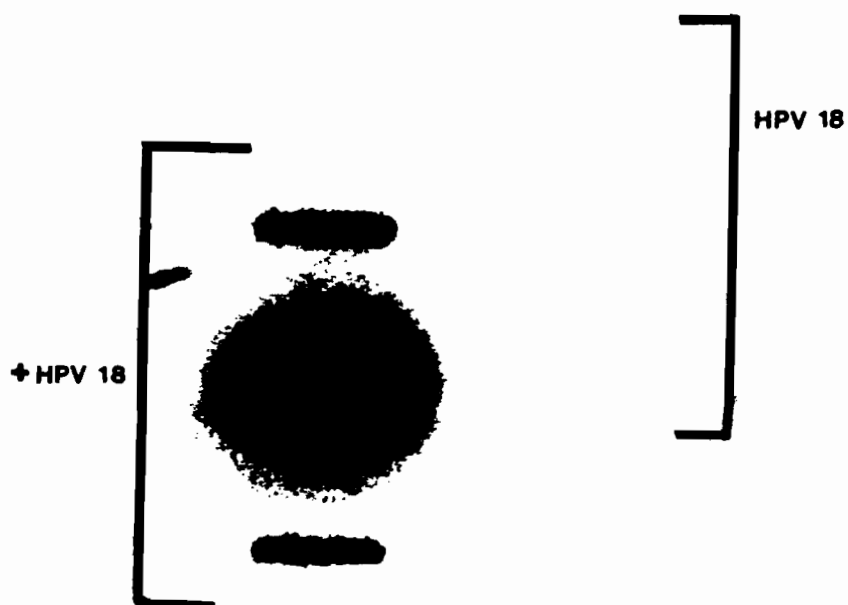
DOT BLOT HYBRIDIZATION**PLATE XVIII**

Plate XIX reveals HPV specific types found in individual specimens from the oral cavity. Positive hybridization products were considered at least 2X stronger than the negative hybridizations. The degree of band intensity coorelates with the number of copies of viral DNA associated with infection.

PLATE XIX**DOT BLOT HYBRIDIZATION**

MILD DYSPLASIA
+ HPV 18

SEVERE DYSPLASIA
+ HPV 18

Plate XX reveals HPV specific types found in individual specimens from the oral cavity. Positive hybridization products were considered at least 2X stronger than the negative hybridizations. The degree of band intensity coorelates with the number of copies of viral DNA associated with infection.

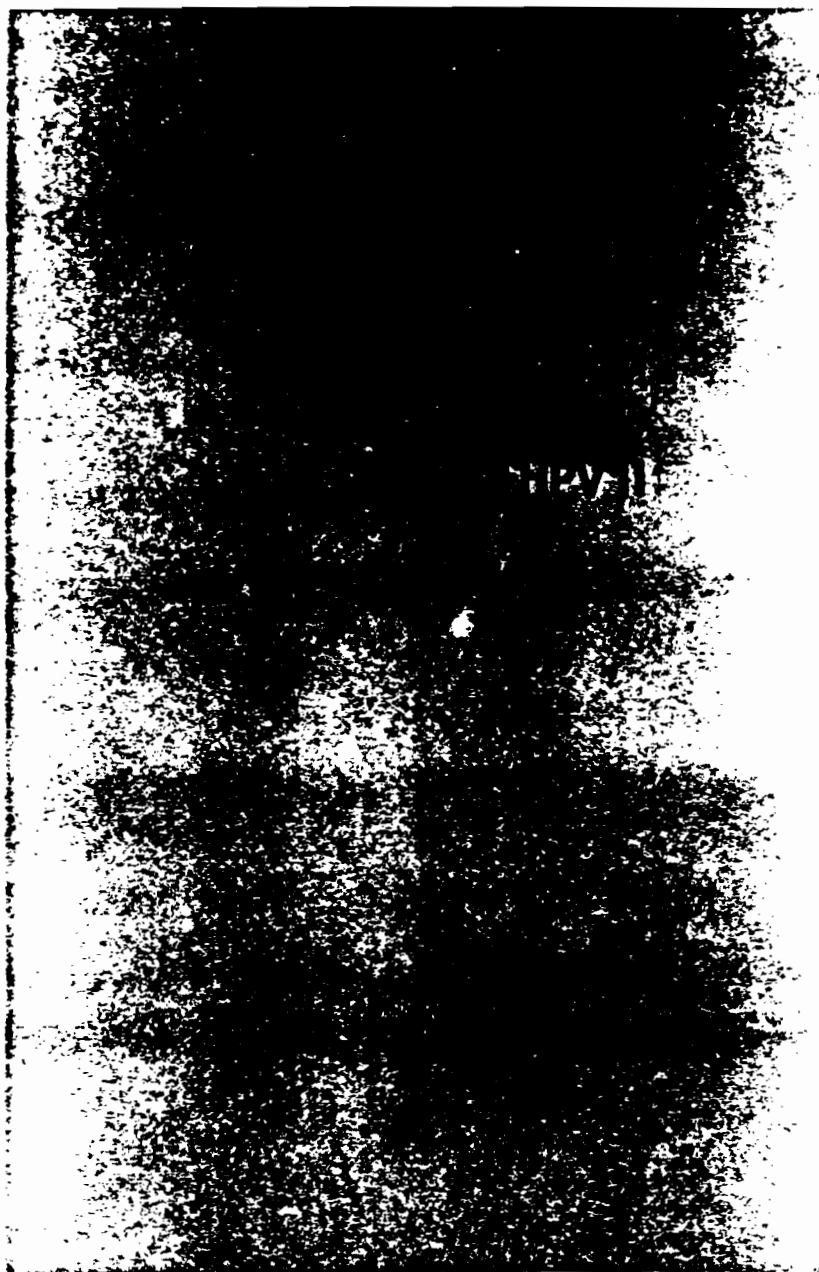
DOT BLOT HYBRIDIZATION**PLATE XX**

Plate XXI is a microscopic view (100 X) of a histological section diagnosed as mild dysplasia. The photo shows the depth of penetration the anaplastic cells invade the epithelium of the oral neoplasm towards the basal lamina. This pre - invasive condition was positive for HPV 16, a subtype responsible for viral integration into the host genome.

HISTOLOGY OF ORAL NEOPLASMS

PLATE XXI



Plate XXII is a case involving squamous cell carcinoma within the submucosa of an oral cavity neoplasm. Note the spiral concentric rings of keratinization indicating the well differentiated state of this tumor. This tumor was positive for HPV 16.

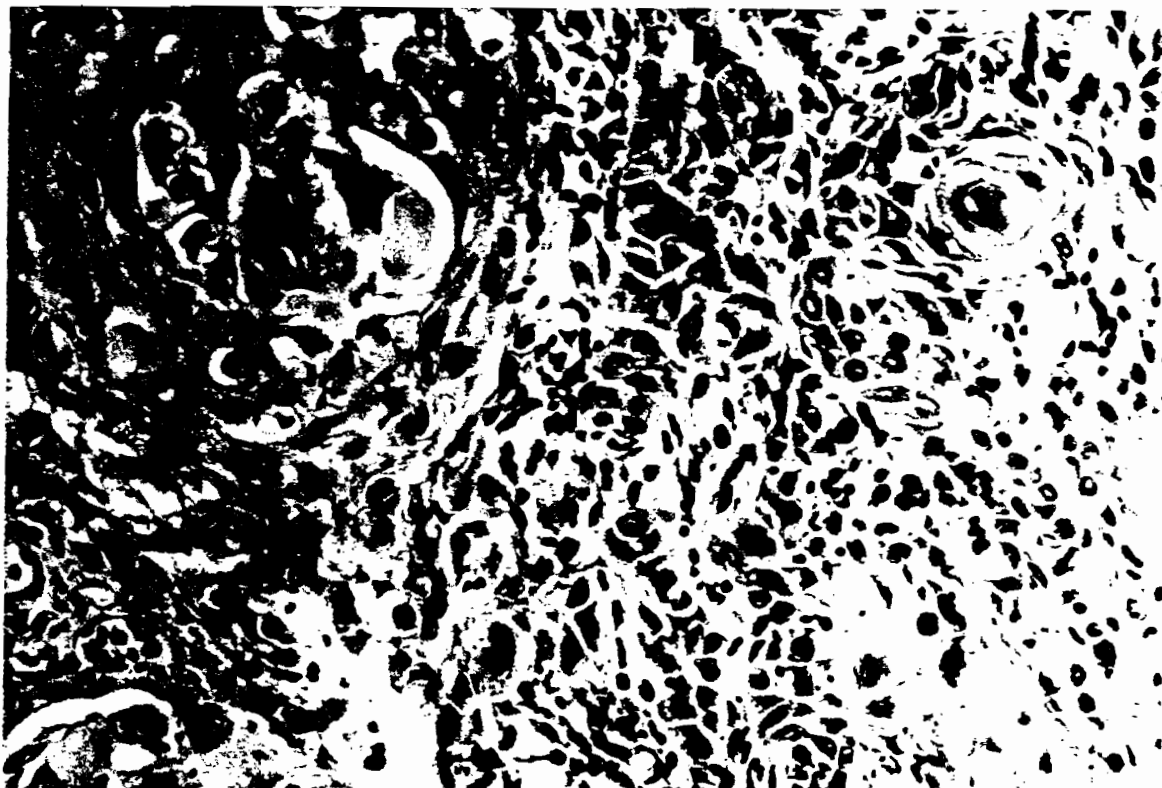
HISTOLOGY OF ORAL NEOPLASMS***PLATE XXII***

Plate XXIII is a microscopic view (100 X) of a histological section diagnosed as carcinoma in situ. The photo reveals the 100 % penetration of the dysplastic cells through the epithelium but without invasion of the basement membrane. This lesion was positive for HPV 16, a subtype responsible for viral integration into the host genome.

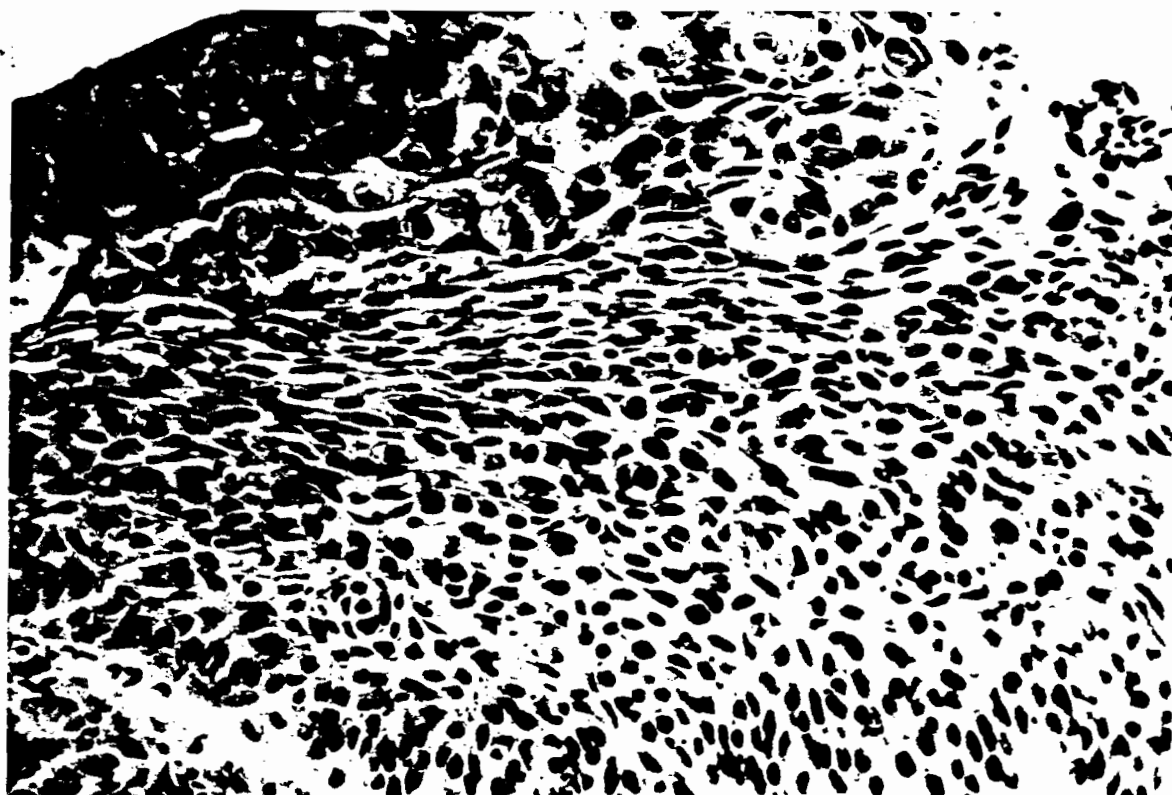
HISTOLOGY OF ORAL NEOPLASMS**PLATE XXIII**

Plate XXIV reveals a papillary tumor of the lower lip which was histologically confirmed as squamous cell carcinoma. This tumor later proved to be positive for HPV 16.

*CLINICAL PATHOLOGY**PLATE XXIV*

Plate XXV : note the white leukoplakic plaque oriented on the buccal mucosa. This specimen later proved to be positive for HPV 11.

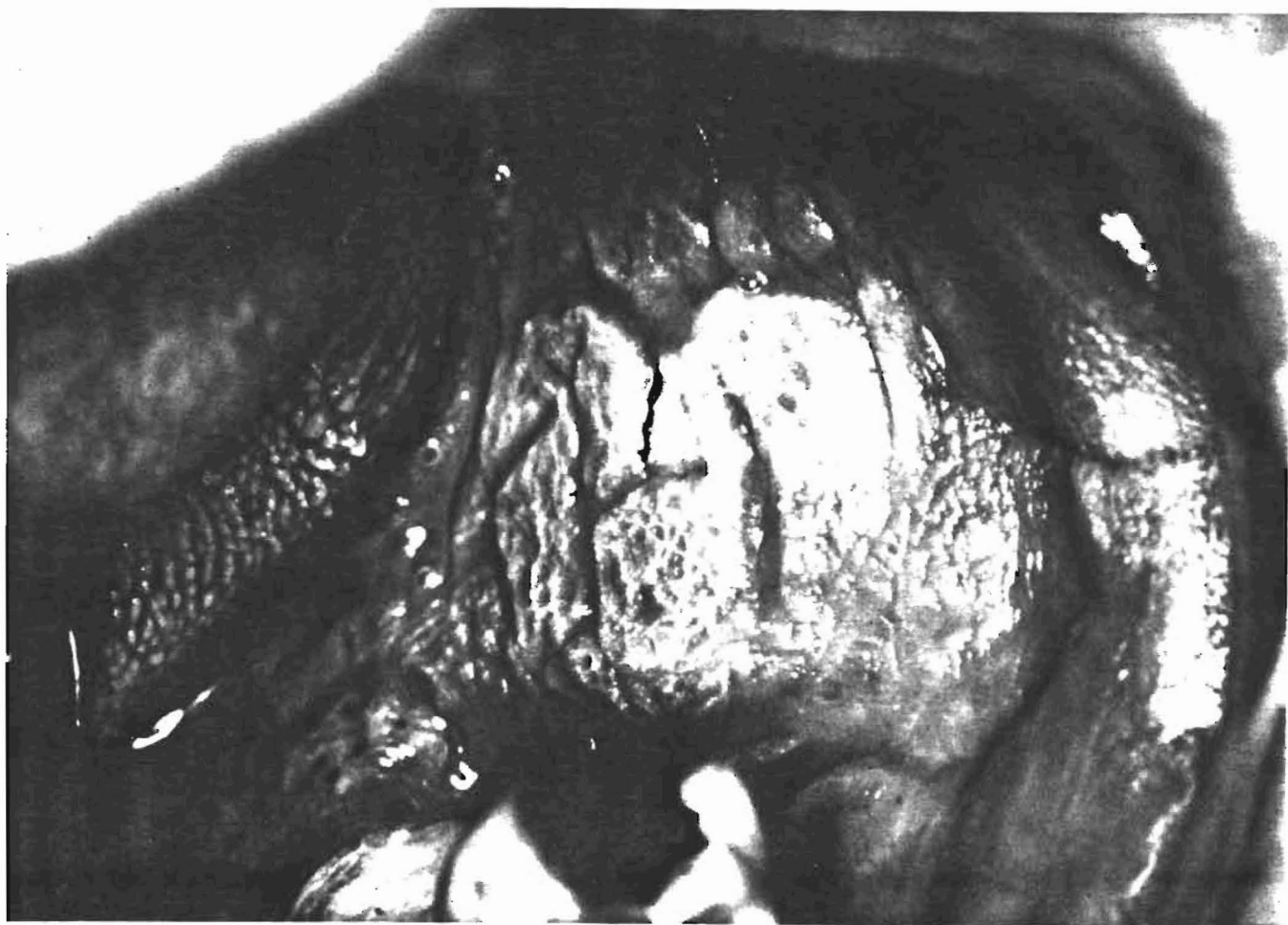
*CLINICAL PATHOLOGY**PLATE XXV*

Plate XXVI : note the leukoplakia on the dorsal surface of the tongue. Histological diagnosis revealed a squamous cell carcinoma and PCR/ Dot Blot hybridization indicated the presence of HPV 16.

CLINICAL PATHOLOGY***PLATE XXVI***