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Detection of Human Papillomavirus DNA Sequences in Oral Squamous Papillomas

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DETECTION OF HUMAN PAPILLOMAVIRUS DNA SEQUENCES IN ORAL SQUAMOUS PAPILLOMAS

by
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DEDICATION

To God, for the strength in pursuing this degree in spite of the obstacles.

To my children, Patricia, Ignacio, Leda, Omar, and Moudar, for their love and understanding. Especially to my daughter Patricia, for her help at home which made this project possible.

VITA

The author, Martha von Thun Jano, was born on March 5, 1954, in Aragua de Barcelona, Venezuela.

During her middle and high school years, Dr. von Thun Jano lived in Argentina, United States and Mexico. In 1972, she completed her high school education in the specialty of Humanities at Colegio San Jose de Tarbes and in 1974, received a degree in the specialty of Science.

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In August, 1988 Dr. von Thun Jano joined Loyola University of Chicago to obtain a Master Degree in Oral Biology and in 1991, she obtained the License to practice General Dentistry in the state of Illinois.

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INTRODUCTION

Papillomaviruses are a group of highly specialized, transmissible, host and target specific DNA viruses which infect the squamous epithelia and cause benign and occasionally, malignant hyperplastic, papillomatous and/or verrucous proliferations in skin and mucosa of a wide range of animals and humans.[1,2,3]

In vertebrate animals, papillomaviruses produce papillomas in the skin of the rabbits which either regress, persist or undergo malignant transformation.[4] Also, they have the ability to stimulate the dermal fibroblasts inducing formation of fibroblastic tumors of the brain, polyploid tumors of the urinary bladder in cattle, sarcoma-like tumors in the skin of the horse, connective tissue tumors (fibromas, fibrosarcomas, chondromas) in hamsters and fibromas in a type of mice.[5] In the oral cavity of vertebrate animals, the papillomavirus is known to cause oral papillomas in dogs, which might progress to squamous cell carcinomas.[6] The bovine papillomavirus type 4 causes alimentary tract papillomatosis in cattle which might become malignant, if fed with braken fern[7] or immunosuppressed with azathioprine.[8]

In humans, papillomaviruses have been identified in

numerous proliferative disorders including active keratosis, bowenoid papulosis, carcinomas of penis and vulva, cervical intraepithelial dysplasias, condyloma acuminata, epidermodysplasia verruciformis, kerathoacanthomas, low grade epithelial dysplasias, melanomas, skin warts, squamous and verrucous carcinomas (Bushke-Löwenstein tumor). In the oral cavity, human papillomaviruses have been identified in condyloma acuminatum, epithelial dysplasias, papillomas, warts, focal epithelial hyperplasia, laryngeal papillomas, "hairy" leukoplakias, leukoplakias, lichen planus, squamous and verrucous carcinomas.[9,10,11]

There are more than 60 types of human papillomavirus (HPV) identified so far and more than 10 have been identified in oral lesions.[12] The type of human papillomavirus determines the histology of the lesion, the quantity of virus production and its malignant transformation.[13]

The oral squamous papilloma is a generic name that includes papillary and verrucal epithelial growths. The squamous papilloma is the most common benign epithelial tumor of the oral cavity, although some investigators regard it as potentially pre-malignant, that can occur at any age, but mainly in the third through the fifth decades. Any region of the oral mucosa might be affected, but the most common sites are the hard-soft palate-uvula complex, dorsum and lateral tongue borders and lips.

Clinically, the oral squamous papilloma appears as a painless, white or pink small (the majority measures 25 to 30 mm in diameter), usually well circumscribed, pedunculated, exophytic growth with a granular or cauliflower-like surface. Histologically, the oral squamous papilloma consists of many long, thin finger-like projections of stratified squamous epithelium extending above the surface of the mucosa with overlying ortho/parakeratin, supported by a central fibrovascular connective tissue core, containing an inflammatory infiltrate. In 80% of the cases, it may have secondary and tertiary branchings that are covered by orthokeratin or parakeratin or both. The essential feature is a proliferation of the spinous cells in a papillary pattern. Mitotic activity is prevalent and variable levels of cellular atypia are present. Koilocytosis of the epithelial cells in the upper layers may be found which is thought to be indicative of viral infection. Koilocytes are cells where the cytoplasm have suffered vacuolation and as a consequence the nuclei appear as sorrounded by an halo or empty space. The human papillomavirus is an etiologic agent of the oral squamous papilloma, HPV types 2, 6, 11 and 16 have been related to these lesions.

Differential diagnosis of the oral squamous papillomas should be made with oral condyloma acuminata and oral verruca vulgaris, since clinical and histological

similarities exist, making it difficult to differentiate among them.

Clinically, the oral condyloma acuminatum is a painless, soft, sessile, sexually transmitted viral papillomatous lesion. The color is similar as or lighter than the surrounding tissue. Rare in the oral cavity, it is mostly seen in adults but also has been observed in children who have been sexually abused. The sex partner will have similar lesions in such case.

Histologically, the oral condyloma acuminatum exhibits broad papillary folds with bulbous appearing rete ridges and parakeratin crypts. The stratified squamous epithelium is often parakeratinized but it may also be non-keratinized. The upper spinous cell layer shows koilocytotic changes, there is acanthosis and sometimes ballooning type of nuclear degeneration as seen in focal epithelial hyperplasia. Human papillomaviruses are the etiologic agents of condyloma acuminatum. HPV types 6, 11, and 16 have been associated with these lesions.

Clinically, the oral verruca vulgaris is a painless, papillary lesion with white surface projections due to keratin production. It may be undistinguishable from papilloma. While common in skin of children and young adults, it is uncommon intraorally. When present in oral mucosa, the verruca vulgaris is mainly located on the palate followed by the alveolus, gingiva, tongue, commissures and

buccal mucosa. They exhibit varied clinical presentations from focal exophytic lesions to multiple or spreading verrucous lesions.

Histologically, the oral verruca vulgaris may present three different types of patterns:

1. Acropapilloform, which consists of both angulated and rounded papillomatous projections and abrupt cupped margins. It is the most common pattern.

2. Acroform, which consists of acutely angulated verrucous projections with discrete margins. It is the second common pattern.

3. Cryptoform, which consists of bulbous acanthotic invaginations, cornified crypts and discrete margins. It is the least common of the variants.

The majority of the oral verruca vulgaris cases, present both ortho and parakeratin. In some cases, koilocytes might be seen in the granular and upper spinous cell layer, where they can appear as small, vacuolated cells with pyknotic nuclei or as larger, pale cells with small dark nuclei.

The human papillomaviruses are the etiologic agents of the oral verruca vulgaris. HPV types 2 and 4 have been associated with these lesions.[14,15,16,17,18,19,20,21,22]

The methods used until now for the detection of HPV infection of cells are:

1. Hematoxylin-eosin stain, used for the observation of histomorphologic changes present in tissues such as

koilocytes and other cellular/nuclear atypias. Performed on fixed tissues mounted on slides. Method not sensitive for the detection of HPV.

2. Electron microscope, used for the observation of viral particles but viral particles must be assembled. Performed on fixed tissues mounted on slides. Method not as sensitive as melecular genetics.

3. Immunoperoxidase stain of HPV antigen, used for the observation of viral proteins. Performed on fixed tissues mounted on slides. Method not as sensitive as molecular genetics.

4. Southern blotting, used for the observation of cleaved viral DNA subjected to electrophoresis, then hybridized. Requires fresh or fresh-frozen specimen; sensitivity of detection about 10^5-10^6 copies of the genome; labor intensive. Research tool, most reliable method for HPV typing.

5. Dot blot hybridization, used to observe uncleaved denatured viral DNA blotted onto membranes, then hybridized. Requires fresh or frozen specimen; sensitivity of detection similar to Southern hybridization; difficult to discriminate weak signal from background. Research tool for screening of HPV types.

6. DNA "in situ" hybridization, used to observe viral DNA localized to cells on sections using probes. Can be

performed on fixed tissues; sensitivity of detection
variable; labor intensive. Research tool,
commercial kits available.

7. Polymerase chain reaction, is used to observe viral DNA
   fragments amplified, but not localized to specific cells.
   Performed on fresh or fresh-frozen specimens as well as
   in fixed specimens. Sensitivity of detection 10 to 100;
   potential for false-positive results because the test can
   pick up even low-level contamination. Research tool, the
   most sensitive technique, contamination problems,
   rapid.[23,24]

   The objectives of this investigation were:

   First, to detect HPV DNA in oral squamous papillomas by
the use of "in situ" hybridization, which localizes specific
nucleic acids, DNA or RNA, in this case DNA sequences,
within the cells of tissue specimens. This was accomplished
by using wide spectrum and type specific probes. The wide
spectrum probe (WSP) targets genomic DNA of HPV types 6, 11,
16, 18, 30, 31, 33, 35, 45, 51 and 52 but does not
distinguish among them. The type specific probes (TSP)
target genomic DNA of HPV types 6, 11, 16, 18, 31 and 33
individually.
The "in situ" hybridization method was selected for this
investigation since it preserves the morphology of the
tissue, thus allowing to associate abnormal morphology with
HPV infection and to visualize the stratum in the epithelium

where HPV DNA is located which is not possible with the
other techniques mentioned above.

Eleven of the HPV types targeted by the wide spectrum and
specific probes have been implicated in different types of
epithelial lesions:

> HPV type 6 have been detected in cervical
> intraepithelial neoplasia, genital warts (condyloma
> acuminatum), laryngeal papillomas, oral
> warts/papillomas, verrucous carcinoma (Buschke-
> Löwenstein tumor), vulvar carcinoma.
>
> HPV type 11 in cervical epithelial neoplasia, genital
> warts, laryngeal papillomas, oral papillomas, penile
> carcinomas.
>
> HPV type 16 in cancers of the anogenital region, high-
> grade intraepithelial lesions, lung carcinoma, oral
> carcinoma, oral hyperkeratotic lesions, oral mucosa.
>
> HPV type 18 in carcinoma of cervix and penis, invasive
> carcinomas of the anogential region, macular lesions of
> epidermodysplasia verruciformis.
>
> HPV type 30 in laryngeal carcinomas.
>
> HPV type 31 in carcinoma of cervix and high-grade
> intraepithelial lesions of the anogenital region.
>
> HPV type 33 in Bowenoid papulosis, carcinoma of cervix,
> cervical intraepithelial neoplasias.
>
> HPV type 35 in carcinoma of cervix, cervical
> intraepithelial neoplasias.
>
> HPV type 45 in genital warts, invasive carcinoma of the
> cervix.
>
> HPV types 51 and 52 in high-grade intraepithelial
> lesions of the cervix.[25]

One hundred papilloma specimens were first assayed by
the Avidin-Biotin-Peroxidase Complex for
immunohistochemistry technique which detects HPV antigen.
The papillomas that yielded positive results with the use of

antibodies to HPV proteins were assayed with the wide
spectrum probe. And in turn, those specimens that yielded
positive for HPV DNA with the Wide Spectrum Probe, were each
in turn assayed with all the type specific probes
individually.

The immunohistochemistry technique was used as a method of
selection of the papillomas to be tested with the Wide
Spectrum Probe, although the absence of HPV antigen does not
rule out the presence of HPV DNA.[26]

Second, to establish "a risk profile for malignancy",
in order to determine which lesions have the potential for
becoming malignant according to the type of HPV present.
This profile would be based on the following criteria:

1. "Low risk" lesions are those induced by HPV types
    6, 11, 42, 43 and 44. These types have very
    low potential for malignant transformation.

2. "Intermediate risk" lesions are those induced by
    HPV types 31, 33, 35, 51, 52 and 58. These types
    might or not induce malignant transformation.

3. "High risk" lesions are those induced by HPV types
    16, 18, 45 and 56. These types have a high
    potential for malignant transformation.[27]

Third, to establish the location of HPV DNA in the
epithelium, since it is usually found in the nuclei of
keratinocytes in the stratum granulosum and stratum
corneum.[28] But, could it also be found in the basal layer?

Fourth, to determine which HPV type is present in each papilloma specimen, due to the fact that each papilloma could be infected with more than one type of HPV DNA.

Fifth, to compare histomorphologically the papillomas which express HPV DNA and those papillomas which do not express HPV DNA, based on the following criteria:

1. Presence or absence of orthokeratin or parakeratin or both. Is the epithelium hyperkeratinized?

2. Presence or absence of koilocytosis.

3. Presence or absence of acanthosis.

4. Presence or absence of cellular atypia.

5. Presence or absence of basal cell layer replication.

As a result, I expected to find features characteristic of viral infection such as: hyperkeratosis, koilocytosis, dyskeratosis, papillomatosis and acanthosis.

And based on the findings of this investigation, a re-definition of "oral squamous papillomas" was suggested.

REVIEW OF RELATED LITERATURE

Genus Papillomaviruses of the Papovaviridae family, are classified as such by virtue of their capsid structure and biochemical composition. They induce tumors in skin and mucosa and their name comes from the Latin, meaning papilla, nipple, pustule and the Greek suffix "oma", to denote tumor.[29] Each virus is named after its natural host in its English or Latin form.

The "type" of papillomavirus is determined, based on the degree of DNA homologies. In case of less than 50% cross-hybridization under stringent conditions, two isolates are considered as independent; if more than 50% cross-hybridization, but incomplete, then they are considered sub-types (ex. HPV type 6b [b=subtype]).[30] When tested under stringent conditions, the members of individual groups cross-hybridize from less than 1% to 40%.[31,32] HPV types 6 and 11 which affect the mucosa, show 25% of cross hybridization and very similar biological properties.[33,34]

In general, the sequences of all papillomaviruses are highly homologous, specially within E1 and L1, which leads to cross-hybridization between their DNAs under conditions of reduced stringency.[35]

The human papillomaviruses (HPV) are small, non-enveloped, composed of icosahedral particles which contain DNA and protein. The DNA is composed of 8000 base pairs, circular, double-stranded, encapsulated in an outer protein shell of 72 capsomeres, in a skew structure either left-handed (cotton tail rabbit papillomavirus), or right-handed (human papillomavirus).[36] After negative staining, the capsomeres appear as hollow cylinders of equal height and width, which are connected at their base by fibrous bridge-like structures.[37] All the genetic information is localized on one strand. The viral genome is divided into:

a. An early region (about 4.5 kilobases [KB]), which contains eight reading frames E1-E8, which are necessary for viral replication and transformation.

b. A late region (about 2.5 KB), composed of two L1 and L2 open reading frames, which are the potential coding regions.

c. A regulatory or long control region (LRC), (about 1 KB), which contains the origin of replication and the control elements for the transcription and replication.[38]

The E6 and E7 are the transforming proteins. The E7 proteins bind to the retinoblastoma tumor suppressor gene product (pRB), with a preference for the underphosphorylated "active" form of pRB. The E7 proteins derived from the "high risk" HPVs bind to pRB with a higher affinity than the E7 proteins from the "low risk" HPVs. The "high risk " HPV E6

proteins can associate with the tumor suppressor p53 in
vitro and enhance their degradation, which presumably
accounts for the very low levels of p53 in cervical
carcinoma cell lines. The expression of E6 protein
substitutes for mutations within the p53 gene and seems to
lead to loss of functional p53. While, HPV negative
anogenital cancers show evidence of clonal somatic mutation
within p53. Suggesting that expression of a mutant p53
protein contributes to the development of HPV negative
tumors, which results in the expression of an altered p53
protein that may gain positive transforming activity, in
addition to losing wild type function. Therefore, HPV
negative cancers, are more aggressive in nature than HPV
positive cancers since they express a mutant p53.[39,40]

The virus enters the mucosa through a loss of
continuity in the epithelium, where it infects the basal
layer. Here, the virus causes transformation of basal cells,
but do not undergo lytic replication, since basal cells are
non-permissive to viral replication. As keratinization takes
place, cells become permissive to viral replication.
Keratinocyte differentiation then, results in viral DNA
replication, synthesis of structural proteins and induction
of virus-specific cytopathic effects. In humans the
incubation period varies between 3 and 18 months. As
epithelium differentiates, there is early (non-structural)
protein synthesis and episomal DNA replication, which will

either produce a condyloma or low grade epithelial lesion or latent infection or integration of viral DNA into host cellular genome (HPV types 16, 18, 31). Integration causes inactivation of tumor suppressor genes of host cells which accompanied by co-factors, could result in malignant transformation. The above takes place with comcomitant productive DNA synthesis and late (capsid) protein synthesis with assembly of viral particles. As desquamation of epithelium occur, cells are shed off into the environment, which results in re-infection and transmission of the virus.[41,42] But, progression of a benign papilloma to a malignant carcinoma, is accompanied by cessation of productive viral replication and alteration of the pattern of keratinization.[43]

HPV is transmitted through transplacental transmission, contamination during passage of fetus through birth canal, contact with an infected person, autoinoculation as in the case of thumb-sucking habits,[44] sex or fomites.[45] Reports of congenital condyloma acuminatum,[46] juvenile laryngeal papillomatosis,[47] presence of HPV DNA in the oral cavity, foreskin[48] and amniotic fluid of newborn babies,[49] and the presence of HPV DNA in both cord and venous blood of newborns babies born to mothers with HPV DNA in peripheral blood mononuclear cells, suggest transplacental transmission of the virus.[50] Newborns acquired laryngeal papillomas from genital HPV infection by one of three ways: By direct

contact, between the fetal larynx and the infected genital tract of the mother, by transplacental transmission, or by post-natal contact.[51]

Sexual transmission results from oro-genital sex or contact with infected partner through an abrasion in the mucosa.[52,53] HPV type 16 DNA, has been detected in the semen from male partners of women with HPV infection by the polymerase chain reaction. The male harbored same HPV type as the women.[54]

There exists an immune response in organisms infected by HPV, which is most evident in cases of immunosupression which predisposes the individual to HPV infection,[55] and in regressing warts.[56] Antibodies to E7 protein are found in sera of about half the cases of invasive cervical carcinomas associated with HPV-16 but less frequently in control sera or in sera of carcinoma cases not associated with HPV-16.[57]

In the treatment of HPV-induced lesions, surgical removal is the most frequent method used. Interferons which have general antiviral, antiproliferative and immunomodulating effects, are also being used. In one study, 1/3 of refractory genital condylomas disappeared and an additional 1/3 of the condylomas decreased in size but interferons have been less effective in the treatment of respiratory papillomas.[58,59] Autogenous vaccines have been effective with variable success. It is thought that immunization with capsid proteins (L1 and L2), may prevent

infection and that immunization with transforming proteins (E6 and E7) may prevent progression to malignancy or reduce tumor load in established cancers. The best prevention of HPV infection, is the avoidance of high risk behavior.[60]

According to the literature review between the years of 1965 to 1992, the following studies of different lesions of the oral cavity have been conducted in order to detect the presence of HPV in different oral lesions:

1. Oral hairy leukoplakia was studied in thirty two patients infected with human immunodeficiency virus (HIV), with light microscope. Ultrastructural studies were conducted in 20 of the cases, where viral particles of the human herpes virus were detected in 17 of the cases and no HPV viral particles were found. Immunohistochemical studies were made in 26 cases using a rabbit antiserum to papillomavirus where nuclear positivity was observed in 25 of cases.[61]

2. Eighty-eight formalin-fixed, paraffin-embedded invasive squamous carcinomas were studied for HPV types 16 and 18-related DNA sequences following in vitro gene amplication using the polymerase chain reaction (PCR). Nine of the 25 (36%), oropharyngeal squamous carcinomas contained HPV DNA sequences, including 4 of 10 (40%) laryngeal, 3 of 8 (38%) buccal and 2 of 7 (29%) glossal tumors. Of the 44 cases that contained viral DNA, HPV type 16 was detected in 41 cases (93%) and HPV type 18 in 5 cases (11%), while both types were found in 2 cases, (one anal and one vulvar).[62]

3. One hundred and fifty six formalin-fixed, paraffin-embedded biopsies from 40 patients with surgically treated oral squamous carcinoma were analyzed for the presence of HPV infection by histopathologic evaluation, DNA "in situ" hybridization and PCR. Morphologic changes suggesting infection by HPV were found in 16 out of the 40 patients (40%), HPV DNA was demonstrated in one of the lesions by "in situ" hybridization, with a biotin-labelled probe, for types 6, 11, 16 and 18, and with the PCR technique, samples from 11 of the 40 patients proved to contained HPV DNA. Of these, HPV- 6 was demonstrated in one case, HPV-16 in 9 cases, and HPV-18 in one case. HPV DNA was exclusively detected in the biopsies showing carcinoma tissue or its adjacent precancer lesions.[63]

4. A total of 206 benign oral mucosal biopsy specimens were studied by the indirect immunoperoxidase (IP-PAP) technique to detect HPV antigens. Forty five of the 92 oral squamous papillomas/condylomas studied were positive for the HPV antigen. Also, 144 surgically treated benign oral lesions (which were included in the 206 lesions listed above), were analyzed using an DNA "in situ" hybridization technique with a 35S-labeled HPV (radioactive), to demonstrate HPV types 6, 11, 13, and 16, in routinely processed, paraffin-embedded biopsy specimens. HPV DNA was present in 45 of the 144 lesions, being found in 33.8% of the papilloma/condyloma group. Fifteen of these 45 lesions also expressed HPV

antigens. The most frequent HPV type was HPV-11, representing 37.8% of the DNA positive lesions. HPV-16 DNA was found in 4 of the papilloma/condyloma lesions, (from a total of 92).[64]

5. A 33-year-old immunosuppressed man, developed bowenoid papulosis on his genitalia, velvety papules and plaques in his mouth and invasive squamous cell carcinoma of his tongue. All three lesions tested positive for HPV type 16.[65]

6. DNA biotinylated probes for HPV types 6, 11, 16, and 18 were tested in cytostin preparations of fine-needle aspirates obtained from 5 squamous cell carcinomas of the head and neck (four metastatic in lymph nodes and one primary). All five carcinomas showed reactivity with probe 11, four were strongly reactive, and one also reacted weakly with probe 16. HPV types 6 and 18 showed no reactivity.[66]

7. Ten specimens from 5 patients diagnosed as fibropapilloma were analyzed with the electron microscope. Specimens were taken from the proliferative lesion and from the normal sorrounding mucosa. HPV viral particles were seen in the non-keratinized epithelial cells as spherical particles 40-55 nm in diameter, mostly in the cytoplasmic matrix and nuclei (especially on their chromatin masses), of the intermediate and superficial layers, which did not form a crystal array. Some viral particles were distributed in the extracellular spaces of the intermediate layer, and they

were hardly observed in cells of the basal/suprabasal and prickle layers. All the membranous organelles of the epithelial cells were devoid of viral particles. There were no significant differences in the HPV distribution between the cells derived from the proliferative lesions and those derived from the surrounding normal mucosa.[67]

8. Two hundred and seventeen tissue samples from various human malignancies were examined for the presence of HPV DNA with a low-stringency filter hybrydization technique, these techniques were sensitive enough to crosshybridized with all known papillomavirus DNAs, both animal and human. Approximately, 2% of the cancers contained HPV DNA, these included carcinomas of the lung, coecum, tongue and neck. Thirteen of 4 cancers, contained HPV-16-related nucleotide sequences.[68]

9. Eighteen salivary gland pleomorphic adenomas, including 9 with a t(3;8) (p21;q12) as the sole chromosome abnormality, were tested for the presence of HPV DNA and for rearrangements of the cellular flanking regions of a HPV-16 integration site maping to 3p21. None of the tumors expressed HPV DNA or related sequences or rearranged allele of the integration site.[69]

10. Thirty-three cases of hairy leukoplakia in HIV-positive patients were examined. Twelve out of 14 cases studied under the electron microscope showed only herpes viral particles but 95% of the 33 cases were positive for HPV antigen when

studied by immunohistochemistry.[70]

11. In sections of 373 formalin-fixed, paraffin-embedded samples from benign and precancerous leukoplakias and squamous cell carcinomas, HPV DNA was identified in 56 of the specimens by the "in situ" hybridization technique.[71]

12. Seven biopsies from carcinomas of the tongue were analyzed for the presence of HPV sequences by Southern blot analysis. One tumor was positive with HPV type 2 under conditions of high stringency and two were positive for HPV type 16. All tumors contained high copy numbers of their respective viral DNA. The cleavage pattern of the HPV-2 positive cancer differed from the established HPV-2 prototype and one of the HPV-16 positive cancer also differed from its prototype.[72]

13. Biopsies from 40 cases of oral squamous cell carcinomas were examined with light microscope to look for features of HPV infection and by immunoperoxidase to detect the presence of HPV antigen. Morphological signs of the flat-type HPV lesion were found in 4 cases (10%), of the inverted type in 3 cases (7.5%), and of the papillomatous type in 9 cases (22.5%). Koilocytosis was seen in most of the antigen positive lesions, 5 of which were of the papillomatous type, 2 of the inverted type and 1 of the flat type.[73]

14. Forty-five oral papillary lesions classified clinically and histologically as condylomatous papilloma, hyperkeratotic papilloma and oral wart were subjected to

histopathological evaluation and tested by immunoperoxidase for HPV group-specific antigens. HPV antigen was found in 10% of 31 condylomatous papillomas, 22% of the 9 hyperkeratotic papillomas, and 60% of 5 oral warts. Koilocytosis of varying extend was regularly observed and quantified.[74]

15. HPV types 4, 16 and 18 were detected in biopsies of normal and malignant human mucosa by Southern blot hybridization and polymerase chain reaction (PCR). HPV DNA from types 4, 16, 18 were detected in three different carcinomas but viral DNA was only found in adjacent dysplastic and normal tissue with PCR. The HPV-18 DNA detected by Southern blot hybridization showed an altered restriction pattern in region E1 of the viral genome; however direct nucleotide sequencing of PCR products from the E6 open reading frame showed no sequence alterations in either normal or malignant samples.[75]

16. Five papillomas, five leukoplakias and six carcinomas were investigated for the presence of papilloma group-specific antigens, with genus-specific papillomavirus antibodies and viral DNA, with cloned HPV types 11 and 16 DNA, used as probes in Southern blot hybridization at conditions of different stringency. Four of five papillomas, four of five leukoplakias and three of six carcinomas reacted with HPV DNA probes and revealed some stained cells after exposure to HPV antibodies. HPV-16 was found in one

carcinoma and HPV-11 in another case of carcinoma.[76]

17. Lesions of the oral mucosa, from 10 patients suffering from focal epithelial hyperplasia (FEH), were studied for the detection of HPV DNA by blot hybridization technique. Four of the patients showed HPV type 13 DNA while the other six patients showed a new HPV type, tentatively named HPV-32. Also, oral papillomas from 14 additional patients were analyzed, 5 of which expressed HPV DNA: HPV-6 was detected in a condyloma and a papilloma, 2 showed uncharacterized HPV DNAs and one showed HPV-32, the latter showing histologic characteristics of FEH. Thus, FEH was conceived as a disease associated with HPV types 13 and 32.[77]

18. Twenty formalin-fixed, paraffin-embedded specimens of human immunodeficiency virus (HIV) positive patients, were evaluated for the presence of HPV genus-specific antigen, HPV- 2/4, 6/11 and 16/18 DNA and Epstein-Barr virus (EBV), by "in situ" hybridization. Three cases exhibited HPV antigen in the cytopathologically altered spinous layer keratinocytes and only one exhibited HPV types 16, 18 or related sequences. While, 19 cases were found to harbor EBV DNA, in the upper spinous layer koilocytes.[78]

19. Seventeen oral epidermoid carcinomas, 3 oral papillomas and 17 normal gingival tissues were tested for the presence of HPV types 6, 11, 16 and 18 sequences, by Southern blot hybridization. In 76.4% of the oral carcinomas and in all the papillomas, HPV type 16 episomal sequences were

detected. None of the samples contained HPV types 6, 11, or 18 sequences. Examination of the habits, showed that 59% of the patients were quid chewers and 82% were smokers.[79]

20. Seventy-seven tissue samples from patients with either degree I, II, or III smokeless tobacco keratoses were examined for the presence of HPV antigen by immunocytochemistry. The antigen was found in sixteen (20.78%) cases.[80]

21. "In situ" hybridization, was used to detect HPV DNA types. Of 100 cases of benign leukoplakias, 4% of non-tobacco-related and 10% of smokeless tobacco-related lesions harbored viral sequences. Of the dysplastic lesions, 3%, alternatively, 17% and 20% of the verrucous hyplerplasia and verrucous carcinomas harbored HPV.[81]

22. Twenty two cases of oral precancerous lesions (OPL) and 51 cases of squamous cell carcinomas (SCC), routinely processed, paraffin-embedded biopsies were screened for the presence of HPV DNA, with a 35S-labelled probe containing types 6, 11, 13, 16, 18 and 30, by "in situ" hybridization. The lesions which expressed HPV DNA were further analyzed with the six HPV DNA probes, separetely. HPV-6 was found in one case of mild dysplasia, HPV-11 in 2 cases of mild dysplasia, HPV-16 in 2 dysplasias and in 3 carcinomas and HPV-18 in 1 moderate dysplasia and in 3 carcinomas. HPV types 16 and 18 were simultaneously present in one case of carcinoma. HPV-13 and 30 was not detected in any of the

lesions.[82]

23. Oral warts from 17 HIV-seropositive individuals were analyzed by Southern blot. HPV-7 was found in 7 warts, HPV-13 in 1, HPV-32 in 1 and HPV-18 in 1 from a total of 17 oral warts. There was no correlation between HPV type, histopathology and clinical apperance of the lesions studied, except that the flat warts (FEH types), contained HPV types 13, 18 and 32 (one of each).[83]

24. One hundred and forty oral squamous cell papillomas (SCPs) were subjected to immunoperoxidase-PAP method to detect HPV antigen. Positive intranuclear staining was seen in 41% of the cases, 9% showed both intranuclear and cytoplasmic staining and 50% were negative for the antigen.[84]

25. Four oral papillomas and 7 carcinomas were studied by "in situ" hybridization, with a HPV 16 biotinylated probe under different stringent conditions. Subsequently, a modified biotin-avidin-alkaline phosphatase procedure was used to visualize virus-infected cells. Four papillomas and 4 carcinomas were found to contain HPV DNA. Positive cells were located in superficial and intermediate cell layers in papillomas and in keratinized zones in carcinomas.[85]

26. Seventeen tissue specimens from 9 patients with oral verrucous cancer were obtained, pre-malignant lesions and primary and recurrent tumors were included. Samples were tested for the presence of HPV DNA, by "in situ"

hybridization with biotin-labelled probes. Under conditions of low stringency (Tm-35 degrees), one pre-malignant lesion and lesions from 2 other patients, hybridized with 3 different probes. The pre-malignant lesion also was positive for HPV structural antigen. Under conditions of high stringency, the lesions were each positive for HPV type 2.[86]

27. Sixty-two oral condyloma acuminatum were tested. HPV antigen was detected in 25% of the cases in the upper spinous layer by a immunocytochemistry method, while 85% of the cases expressed HPV types 6, 11 or related genomes, by "in situ" hybridization under conditions of high stringency. One case was found to harbor HPV type 2.[87]

28. Eleven oral verruca vulgaris were examined for the presence of HPV antigen by reaction with antibody to type common antigens and detection by the Avidin-Biotin-Peroxidase Complex method. The specimens were also examined by "in situ" hybridization with biotin-labelled specific HPV type probes. Six of the 11 specimens were positive for HPV antigen and of these 6, five hybridized to the HPV-2 probe and one to the HPV-4 probe.[88]

29. Erosive oral lichen planus (OLPe) lesions from 20 patients were examined with 32P-labeled HPV DNA probes by Southern blot hybridization. HPV type 11 was detected in 6 of the lesions while HPV types 6, 16 and 18 were not detected in any of them. Also, a type-specific polymerase

chain (PCR), reaction assay was used. With the PCR method, HPV type 11 was detected in 8 of the samples, HPV-6 in 5 samples and HPV-16 in 3 samples.[89]

30. DNA extracted from a odontogenic keratocyst was assayed for the presence of HPV type 16 using Southern blot hybridization. The HPV type 16 DNA sequences detected were different from the prototype HPV-16.[90]

31. Eighteen cases of focal epithelial hyperplasia (FEH), were investigated for the presence of HPV antigen by immunohistochemistry and to detect the presence of HPV types 1, 6, 11, 13, 16, 18 and 32 DNA, by "in situ" hybridization employing biotinylated probes. Seven of the cases (39%), expresed HPV group specific antigen while 15 cases (83%), expressed HPV DNA. Nine cases (60 %), expressed HPV-32, five cases (33%), expressed HPV-13 and one case (7%) expressed HPV-11. Two specimens on different sites from the same patient expressed the same type of virus and one patient had, in addition to FEH, a squamous papilloma demonstrating the same type of virus.[91]

32. Two oral lesions, one from the lower lip, the other from the buccal mucosa, from a 30-year-old HIV-positive and immunosuppressed man, were examined by Southern blot hybridization. Under low stringency, both lesions hybridized with a probe coktail comprising HPV types 6, 11, 16, 18, 31 and 33 DNA. Under high stringency, the lip lesion, proved to contain HPV-7, which also was confirmed by "in situ"

hybridization. The buccal lesion was weakly positive with types 11 and 13, but the restriction patterns with Pst I and Bam HI did not fit with the HPVs known so far. Cloning of this new type of HPV is under way.[92]

33. Seventeen cases of focal epithelial hyperpasia (FEH) were investigated by the use of "in situ" hybridization using biotinylated probes of HPV types 1, 6, 11, 13, 16, 18 and 32 DNA. Ten cases were positive for HPV type 13 and 6 cases were positive for type 32 DNA. One case showed doubled infection with types 13 and 32 DNA.[93]

34. Five focal epithelial hyperplasia (FEH) specimens from 4 patients were evaluated for the presence of HPV DNA by Southern blot hybridization. FEH lesions from 3 patients that shared a familial relationship demonstrated HPV-13 DNA sequences or a closely related sub-type and similar clinical features. The other patient harbored sequences very similar to HPV-32.[94]

35. Biopsies from 9 oral papillomatous lesions were analyzed by Southern blot analysis with 32p-labelled cellular DNA, to detect HPV types 1 to 19 and 21 to 26 DNA sequences. Of the three cases diagnosed as focal epithelial hyperplasia, 2 contained HPV-6 related sequences and one HPV-11. One tongue base papilloma and one papilloma of the palate, contained HPV-11. Of the remainding lesions, 2 contained HPV-6.[95]

36. Twenty-one papillomas, 23 ordinary benign keratoses. 13 smokeless tobacco keratoses, 10 verrucous hyperplasias, 10

verrucous carcinomas, 17 squamous cell carcinomas, 3
epithelial dysplasias and 6 lichen planus, were evaluated
for HPV types 6/11, 16/18, 31/33/35, with biotinylated
doubled-stranded DNA probes. Sixty-two per cent (13/21), of
the papillomas were positive for HPV DNA, being the
strongest reactivity for types 6/11. Of the 13 cases, 10
also showed some reactivity with types 16/18 and 31/33/35.
None of the other lesions showed HPV DNA.[96]

37. Twenty representative papillomas were evaluated for the
presence of papilloma genus-specific antigen by
immunoperoxidase technique and for HPV types 2, 4, 6 and 11
by "in situ" hybridization, with biotinylated full-length,
doubled-stranded DNA probes. Only one case exhibited antigen
reactivity. Seven cases (35%), yielded positive for HPV-6 or
11 and one exhibited a dual infection with HPV types 2 and
6.[97]

38. Forty nine cases of primary verrucous or squamous cell
carcinoma were examined by Southern blot hybridization to
detect HPV types 2, 6, 11, 13, 16, 18, and 32.
Approximately, 60% of carcinomas particularly of the mouth,
tongue, pharynx, piriform sinus and larynx, were positive
for episomal viral DNA of HPV types 6, 11, 16 or 18. The
remainding 30 carcinomas, were examined by polymerase chain
reaction amplification assay, for DNA of HPV types 6, 11, 16
and 18, twenty-seven of which were positive for types 16 or
18 DNAs.[98]

39. The sensitivity of detection of HPV DNA in pre-malignant and malignant oral lesions between "in situ" hybridization (ISH) and polymerase chain reaction (PCR), was compared. With both methods, HPV DNA was found in 4 of 24 cases of epithelial dysplasia, 4 of 14 cases of verrucous hyperplasia and 1 of 10 cases of squamous cell carcinoma. The 10 cases of smokeless tobacco keratoses and 3 cases of verrucous carcinoma studied were all negative for HPV DNA. Only a single case that was positive by PCR was negative by ISH. Also, the PCR demonstrated the presence of HPV-16 infection in one case, which hybridized most intensively with the probes for types 31/33/35 in the ISH.[99]

40. Thirty-two surgically treated, paraffin oral lesions were tested by "in situ" hybridization to detect HPV types 6, 11 and 16. A total of 10 lesions express HPV antigen, which were found in 4 of 7 squamous cell papillomas, in 2 of 2 classic condylomas, in 2 of 10 papillary hyperplasias and in 2 of 3 leukoplakia lesions. Two of the squamous cell papillomas contained HPV 6 DNA and 4 contained HPV 11 DNA. One of the condyloma contained both, type 6 and 11 DNA while the other contained only type 11 DNA alone. Both the antigen-positive papillary hyperplasias and leukoplakias contained type 6 DNA. HPV type 16 was found in one lichen planus and one of the 2 squamous cell carcinomas.[100]

41. Fifty radomly selected oral squamous papillomas were tested with the peroxidase-antiperoxidase technique to

detect HPV antigens. HPV-type 1 antigen was found in 2 of the papillomas.[101]

42. One hundred and five oral lesions were analyzed by "in situ" hybridization with biotinylated DNA probes for HPV types 6 and 11; 16 and 18; 31, 33 and 35. Positive hybridization signals were found in 26 specimens and only HPV types 6/11 was detected (24.8%). HPV DNA occurred in 100% of the condyloma acuminatum and verruca vulgaris, in 13.3% of the squamous papillomas, 10% of hyperkeratotic/acanthotic lesions and 0% of malignant and pre-malignant lesions. The tongue (19.1%) and the labial epithelium (17.1%) were infected most frequently. Nuclear staining was seen mostly in koilocytes.[102]

43. Samples from oral, sinus, pharynx and larynx lesions were tested under less stringent (25% formamide, 42 degrees C) and stringent (50% formamide, 42 degrees C) conditions. Three samples (laryngeal papillomas) from 10 benign tumors and 3 of 30 malignant tumors, contained HPV-related sequences. The 3 laryngeal papillomas contained HPV types 11, 6 and 11 or 6, respectively. In the malignant tumors, neither the typical restriction pattern of HPV DNA or viral antigen was detected, suggesting that subgenomic fragments remained integrated in the host cell.[103]

44. A series of 191 oral mucosal tumors were analyzed by indirect immunoperoxidase IP-PAP technique for the presence of HPV structural proteins, of epithelial dysplasia and for

cellular composition (B and T lymphocytes, mononuclear phagocytes,[MPS cell]), of their local inflamatory cell infiltrates using ANAE (acid alpha-naphthyl acetate esterase). HPV structural proteins were disclosed in 85% of focal epithelial hyperplasia (FEH), 75% of condyloma acuminatum (CA) and 41% of squamous cell papillomas (SQP). Mild dysplasia was found in 20% of both CA and SQP and moderate dysplasia was found in 12% of the CA. B cell infiltrate was also more predominant in cases of CA and SQP.[104]

45. One hundred one patients, 66 female and 35 male, with genital condylomata underwent an oral cavity examination. Ninety-nine (99%) practice oral sex and 91 out of these underwent oral biopsies with a diagnosis of condyloma in 48% of 101 specimens collected. Of these 8 of 91 (9%) oral lesions were suspected by naked-eye and on colposcopic examination, 83 of 91 patients were suspected of having oral condyloma. Of these 83 patients, 38 (46%) were confirmed histologically, thus a total of 46 patients with genital condyloma also exhibited oral condyloma and 38 of these 46 patients had condylomas not visible clinically. Twenty cytological oral samples were also collected for DNA filter "in situ" hybridization analysis. HPV DNA genital types were observed in 45% (9/20) of all oral scrapings studied and all were confirmed histologically.[105]

46. Twenty-five oral hairy leukoplakia (OHL) lesions from 35

HIV-infected patients stained with antibody to
papillomavirus common structural antigens. HPV DNA was
detected in 10 of 18 OHL specimens and in 6 of 10 normal
buccal mucosa specimens. Epstein-Barr virus (EBV) was also
being tested in this study. The results indicate that HPV
can be found regularly in histologically normal mucosa.[106]

47. A white spongeous nevus was assayed by Southern blot
hybridization for the presence of DNA sequences homologous
of HPV types 1, 2, 4, 6, 11, 13, 16 and 18. Only HPV-16
homologous DNA sequences were detected at a copy number of
approximately 200 to 250 genome copies per diploid cell. The
viral DNA sequences did not appear to be integrated into the
host cell chromosome.[107]

48. Lesions from twelve patients with recurrent respiratory
papillomatosis were analyzed for the presence of HPV DNA, by
the ViraPap/ViraType hybridization procedure. Biopsies were
obtained from the respiratory papillomas and nondiseased
sites (NDS) of the respiratory tract. Fifty percent of the
patients with papilloma specimens typed positive (6 of 12)
and 40% of the patients typed positive in one or more
biopsies from the NDS (4 of 10). No oncogenic HPV types
16/18 or 31/33/35 were detected and only patients with
multiple, not isolated, papilloma involvement were found to
harbor HPV DNA in NDS. Eighty percent of patients with HPV
infected NDS, required a shorter (no more than 3 months)
treatment interval, compared to 20% of those without HPV

infected NDS. These results showed that HPV infection frequently persists in adjacent, clinically normal sites, and suggested that the extend of NDS involment may predict both the extent of disease and the likelihood of recurrence.[108]

49. Sixteen paraffin-embeded biopsy specimens of nasopharyngeal carcinoma (eight anaplastic and eight well differentiated squamous types), were examined for the presence of HPV types 16 and 18 by polymerase chain reaction. HPV DNA was not detected in any of the specimens.[109]

50. A peripheral ameloblastoma was studied by "in situ" hybridization. Results were positive for HPV types 16/18 but negative for types 6/11 DNA.[110]

51. Twenty-four cases of tongue squamous-cell carcinoma (SCC) were analyzed by dot-blot hybridization technique and polymerase chain reaction method. HPV DNA were detected in 8 cases. One poorly differentiated grade III specimens contained both HPV types 16 and 18; seven other cases contained HPV-16 DNA.[111]

52. Sixty formalin-fixed, paraffin-embedded carcinomas arising from the nasal cavities (NC) and paranasal sinuses were analyzed by using polymerase chain reaction. In cases of squamous cell carcinoma (SCC), (n=49), the authors also compared the clinical features of patients with HPV-positive and HPV-negative results, to determine the clinical

significance of HPV in SCC. HPV types 16 and 18 DNA were
detected in 7 of the 49 cases (14%) of SCC. In the other
histologic types of carcinomas (n=11), neither type was
detected. No significant differences in the clinical
features were observed between the HPV-positive and HPV-
negative SCC.[112]

53. Eight sinonasal carcinomas (one adeno carcinoma, two
undifferentiated nasopharyngeal carcinomas and five squamous
cell carcinomas) were investigated by "in situ"
hybridization and polymerase chain reaction (PCR), for HPV
types 6, 11, 16, 18 and 33. All eight cases were negative by
"in situ" hybridization, while one case was positive for HPV
type 6 by PCR. The HPV DNA positive case was an invasive
papillary squamous cell carcinoma of the maxillary
sinus.[113]

54. Forty benign histologically suspicious of HPV infection
were analyzed with ViraType (Life Technologies, Inc. [LT])
and Pathogene (Enzo Diagnostics, Inc. [ED]), "in situ"
hybridization kits for HPV DNA detection in oral tissue. The
purpose of this study was to compare the sensitivity of both
kits. Specimens were hybridized with DNA probes specific for
HPV types 6/11, 16/18 and 31/33/35 [LT], and HPV types 6/11,
16/18 and 31/33/51 [ED]. Positive hybridization reactions
were seen for HPV DNA type 6/11 only and more often in the
LT (20/40; ED=12/40), probed specimens. HPV DNA sequences
were seen in 100% condyloma acuminata (13/13), 100% verruca

vulgaris (4/4) and 13% of the squamous papilloma, with the LT system. The ED system, yielded positive signals in 77% of condyloma acuminata (10/13), 25% verruca vulgaris (1/4) and 4.4% squamous papillomas (1/23).[114]

55. A case of verruca vulgaris in the larynx of a 37-year-old woman was analyzed using the immnunohistochemistry method, "in situ" hybridization and electron microscope. Papillomavirus capsid antigen, HPV types 6 and 11 DNA and viral particles were detected.[115]

HISTOMORPHOLOGICAL DESCRIPTION OF HPV
WIDESPECTRUM POSITIVE AND NEGATIVE PAPILLOMAS

CASE NO. 1277-92: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and supported by a fibrous stroma. Koilocytes are present in the stratum granulosum. BPV-1 positive, WS negative.

CASE NO. 1227-92: Parakeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections with replication of the basal cell layer which exhibits hyperchromatic nuclei and mitotic figures and supported by a fibrovascular stroma. Few koilocytes are present in the stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 1093-92: Orthokeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections with replication of the basal cell layer and supported by a perivascular stroma. BPV-1 positive, WS negative.

CASE NO. 1090-92: Parakeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary folds with replication of the basal cell layer and supported by a fibrovascular stroma. Koilocytes are present in the stratum granulosum and upper stratum spinosum. BPV-1 negative, WS negative.

CASE NO. 717-92: Hyperparakeratotic, acanthotic stratified squamous epithelial ridges which curve centrally, exhibits a granular layer and is supported by a fibrous stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS negative.

CASE NO. 567-92: Orthokeratotic, stratified squamous epithelium disposed in papillary projections with basal and suprabasal cell layer mitotic activity and supported by a fibrovascular stroma. Koilocytes are present in stratum granulosum and upper stratum spinosum. BPV-1 positive, WS negative.

CASE NO. 564-92: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer, supported by a fibrous stroma. Koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 492-92: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections, basal cell layer replication with mitotic activity in suprabasal and basal cell layers, supported by a fibrovascular stroma. BPV-1 positive, WS positive.

CASE NO: 461-92: Orthokeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections with replication of the basal cell layer and supported by a fibrous stroma. Koilocytes are present in the upper stratum granulosum. BPV-1 positive, WS negative.

CASE NO. 236-92: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and supported by a fibrovascular stroma. Koilocytes are present in stratum granulosum and stratum spinosum. BPV-1 positive, WS positive.

CASE NO. 214-92: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. BPV-1 positive, WS positive.

CASE NO. 1065-90: Hyperparakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and supported by hyalinized fibrous vascular cores of stroma. Koilocytes are present in stratum granulosum. BPV-1 positive, WS negative.

CASE NO. 799-90: Hyperparakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 670-90: Hyperparakeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections with mitotic activity and replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and upper stratum spinosum. BPV-1 positive, WS positive.

CASE NO. 464-90: Hyperortho-parakeratotic, acanthotic stratified epithelial ridges disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS negative.

CASE NO. 242-90: Hyperparakeratotic, acanthotic stratified squamous epithelial fused ridges, obliterating the stromal rete pegs with replication of the basal cell layer. Acanthosis is remarkable. Koilocytes are present in stratum spinosum. BPV-positive, WS positive.

CASE NO. 1067-89: Hyperorthokeratotic, acanthotic stratified squamous epithelium disposed in sharp papillary projections with replication of the basal cell layer and a fibrous supporting stroma. Many large koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 869-89: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrous supporting stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS positive.

CASE NO. 780-89: Parakeratotic, acanthotic stratified squamous epithelium showing papillary hyperplasia, with replication of the basal cell layer and a dense fibrous supporting stroma. Few koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 734-89: Orthokeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections with basal cell replication and a fibrovascular supporting stroma. Many large koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 713-89: Hyperparakeratotic, acanthotic stratified squamous epithelial attenuated ridges disposed in short papillary projections with replication of a hyperchromatic basal cell layer, mitotic activity in the basal and suprabasal cell layers and a fibrous supporting stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS negative

CASE NO. 674-89: Orthokeratotic, acanthotic stratified squamous epithelial sharp, fused ridges disposed in papillary folds and supported by a fibrous stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS positive.

CASE NO. 657-89: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum spinosum. BPV-1 positive, WS positive.

CASE NO: 645-89: Orthoparakeratotic, acanthotic stratified squamous epithelium disposed in sharp papillary projections with replication of the basal cell layer and a fibrous supporting stroma. BPV-1 positive, WS positive.

CASE NO. 637-89: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with basal cell replication and a fibrous supporting stroma. Many koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO: 285-89: Hyperorthokeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with basal cell layer replication and a fibrous supporting stroma. Many large koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 174-89: Parakeratotic, acanthotic, stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. BPV-1 positive, WS negative.

CASE NO. 131-89: Parakeratotic, acanthotic stratified squamous epithelium with a slight corrugated surface, replication of the basal cell layer and supported by a fibrovascular lamina propia with projecting connective tissue pegs, deep to which are neurovascular bundles and interstitial fibrous tissue. Large koilocytes are present in stratum spinosum. BPV-1 positive, WS positive.

CASE NO. 71-89: Parakeratotic, acanthotic stratified squamous epithelium disposed in papillary projectios with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 970-88: Hyperorthokeratotic, acanthotic stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 934-88: Parakeratotic, acanthotic stratified squamous epithelial broad ridges disposed in papillary projections with frequent mitotic activity and replication of the basal cell layer, supported by a delicate fibrovascular stroma. Few koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO: 1076-87: Parakeratotic, acanthotic hyperplastic stratified squamous epithelial broad fuse ridges with mitotically active basal and suprabasal cells and a fibrous supporting stroma. Koilocytes are present in stratum granulosum. BPV-1 positive , WS negative.

CASE NO. 735-87: Para-orthokeratic, acanthotic elongated ridges of stratified squamous epithelium with replication of the basal cell layer and a delicate fibrovascular stroma supporting the capillary projections of epithelia. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 189-87: Hyperparakeratotic, acanthotic, stratified squamous epithelium disposed in papillary projections with basal cell replication and a mature fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and

spinosum, which show chromatin material in the periphery or poikilocytosis. BPV-1 positive, WS negative.

CASE NO. 1106-86: Parakeratotic, acanthotic, folds of stratified squamous epithelium disposed in sharp papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 798-86: Parakeratotic, attenuated, flattened ridges of stratified squamous epithelium with a transition to sharp papillary folds and hyperparakeratosis with basal cell layer replication and a supporting fibrovascular core. Huge koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 765-86: Parakeratotic, acanthotic, stratified squamous epithelium disposed in papillary projections with basal cell layer replication and supported by a fibrovascular stroma showing neurovascular bundles. Slight reactive hyperplasia is seen with a whorling pattern of a benign dyskeratosis near the superficial surface of the differentiating epithelium. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 578-86: Hyperparakeratotic, acanthotic, stratified squamous epithelium disposed in papillary folds, with replication of the basal cell layer, which shows compressed

keratinocytes indicating a reactive hyperplasia and supported by a fibrovascular stroma. Koilocytes are present in stratum spinosum and granulosum and melanocytes in the stratum basale. BPV-1 positive, WS negative.

CASE NO. 485-86: Ortho-parakeratotic, acanthotic stratified squamous epithelium disposed in numerous papillary ridges with piling up of parakeratin in some areas and replication of the basal cell layer, which shows numerous attenuated ridge like projections into the supporting fibrovascular stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 1065-85: Parakeratotic, acanthotic, stratified squamous epithelial elongated ridges disposed in sharp papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. BPV-1 positive, WS negative.

CASE NO. 962-85: Ortho-parakeratotic, acanthotic, stratified squamous broad, fused epithelial ridges, disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. Large koilocytes present in the stratum granulosum, some with pyknotic nuclei. BPV-1 positive, WS positive.

CASE NO. 902-85: Hyperparakeratotic, stratified squamous epithelium disposed in sharp papillary folds and supported by a fibrovascular stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS

positive.

CASE NO. 892-85: Orthokeratotic, acanthotic, stratified squamous epithelium disposed in papillary folds with replication of the basal cell layer and a mature fibrovascular supporting stroma. BPV-1 positive, WS negative.

CASE NO. 816A-85: Ortho-parakeratotic, acanthotic, stratified squamous epithelium disposed in papillary projections with replication of the basal cell layer which appears columnar or cuboidal and palisading as the suprabasal cell layer shows slight variation of the orientation, supported by a loosely arranged mucoid connective tissue containing numerous vascular channels. Basal and suprabasal cells show frequent morphologically normal mitotic figures. Few localized koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 816B-85: Parakeratotic, acanthotic, stratified squamous reactive hyperplastic epithelial ridges with basal and suprabasal cell replication and a fibrovascular supporting stroma. As the papillary projections arise in a branching manner, the surface of the epithelium shows ortho-parakeratinization. One area of the mucosa shows mucoid-like loosely arranged fibrous connective tissue. Many koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 782-85: Parakeratotic, acanthotic, stratified squamous broad and narrow, sharp, elongated epithelial ridges disposed in papillary projections with basal cell layer replication and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum, with a centrally placed nucleus. BPV-1 positive, WS positive.

CASE NO. 481-85: Parakeratotic, acanthotic, stratified squamous broad epithelial ridges disposed in narrow papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. The basal and suprabasal cell show many hyperchromatic nuclei, infrequent, but morphologically normal mitotic figures. BPV-1 positive, WS negative.

CASE NO. 627-85: Parakeratotic, acanthotic, stratified squamous epithelium disposed in raised papillary projections with replication of the basal cell layer, showing occasional morphologically normal mitotic figures and a fibrovascular supporting stroma. BPV-1 positive, WS negative.

CASE NO. 479-85: Ortho-parakeratotic, stratified squamous epithelium disposed in sharp papillary ridges, with replication of the basal cell layer and a fibrous supporting stroma. The basal and suprabasal cells have multiple nucleoli, but few and morphologically normal mitotic figures. Some areas show hyperkeratosis with intercellular filaments characteristic of Candida Albicans. BPV-1 positive, WS positive.

CASE NO. 268-85: Orthokeratotic, acanthotic, stratified squamous fused, broad, epithelial ridges, supported by a fibrovascular stroma. At one point, there rises papillary projections of the epithelium showing hyperorthokeratosis with long, sharp, extending epithelial ridges, supported by a branching fibrovascular connective tissue. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS negative.

CASE NO. 160-85: Parakeratotic, stratified squamous epithelium disposed in sharp papillary folds and supported by a fibrovascular stroma. Few koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 655-83: Ortho-parakeratotic, acanthotic, stratified squamous epithelium disposed in papillary ridges, showing converging borders and supported by a fibrovascular stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 623-82: Hyperparakeratotic, acanthotic, stratified squamous epithelium, disposed in sharp papillary ridges with basal cell layer replication and a fibrovascular supporting stroma. Koilocytes are present in stratum granulosum and spinosum. BPV-1 positive, WS positive.

CASE NO. 244-82: Hyperparakeratotic, stratified squamous epithelium disposed in sharp, papillary folds that converge inward and supported by a fibrovascular stroma with some striated muscle bundles. The stratum granulosum is thick,

showing heavy keratin/hyaline granules. Koilocytes are present in stratum corneum. BPV-1 positive, WS positive.

CASE NO. 153-82: Hyperparakeratotic, acanthotic, stratified squamous broad, fused epithelial ridges disposed in papillary projections with replication of the basal cell layer and a fibrovascular supporting stroma. BPV-1 positive, WS positive.

CASE NO. 143-82: Hyperparakeratotic, acanthotic, broad, fused ridges, disposed in sharp, papillary folds with basal cell replication and a fibrous supporting stroma. The stratum spinosum shows multiple nucleolar like inclusions within the nucleus of the cells. BPV-1 positive, WS negative.

CASE NO. 728-81: Hyperparakeratotic, stratified squamous long, sharp epithelial ridges with plexiform pattern with basal cell layer replication and a mature fibrovascular supporting stroma. Many of the epithelial cells show characteristic nuclear vesiculation with residual single or multiple nucleoli-like structures resembling inclusions. The stratum spinosum shows areas of cytolysis with loss of the entire epithelial cell at sites containing infiltrating monocytes and neutrophils. Koilocytes are present in the stratum granulosum. BPV-1 positive, WS negative.

CASE NO. 200-80: Hyperparakeratotic, acanthotic stratified squamous epithelial ridges disposed in papillary projections and supported by a mature fibrovascular stroma. The basal

cell layer shows some crowding of the epithelium, nuclear edema and prominent nucleoli. The stratum spinosum shows ocassional benign dyskeratotic cells and koilocytes with contraction of the nucleus towards the cell membrane and prominent multiple nucleoli. BPV-1 positive, WS positive.

CASE NO. 165-79: Hyperparakeratotic, acanthotic stratified epithelium disposed in deep papillary folds and supported by a fibrovascular stroma. BPV-1 positive, WS negative.

CASE NO. 213-78: Hyperortho-parakeratotic, acanthotic, stratified squamous epithelium disposed in short papillary folds and supported by a fibrous stroma with mucous glands. The basal cell layer shows slight lymphocyte infiltration and hydropic degeneration. The basement membrane is thickened, but in some areas ill-defined due to lymphocytic infiltration. BPV-1 positive, WS negative.

CASE NO. 155-78: Hyperparakeratotic, acanthotic, stratified squamous epithelium disposed in sharp papillary folds with basal cell layer replication and a mature fibrovascular supporting stroma. Few koilocytes are present in stratum granulosum. BPV-1 positive, WS positive.

CASE NO. 315-75: Hyperorthokeratotic, acanthotic, stratified squamous epithelium disposed in papillary folds and supported by a mature, dense, fibrovascular stroma. BPV-1 positive, WS negative.

CASE NO. 557-70: Hyperparakeratotic, stratified squamous epithelium disposed in many papillary folds with basal cell

layer replication and a fibrous supporting stroma. Few
koilocytes are present in the stratum granulosum. BPV-1
positive, WS negative.

MATERIALS AND METHODS

I. AVIDIN-BIOTIN-PEROXIDASE COMPLEX FOR IMMUNOHISTOCHEMISTRY PRINCIPLE

The method is based on antigen-antibody reactions in
order to detect the location of viral proteins in the cells
of formalin-fixed, paraffin-embedded oral squamous papilloma
specimens mounted on microscope slides. The technique
involves the use of a primary antibody to react with the
tissue antigenic sites, a biotin-labeled secondary antibody
against immunoglobulin of the primary species (IgG or IgM),
followed by the addition of a freshly prepared complex of
biotin-labeled horseradish peroxidase (HRP) and modified
egg-white avidin.

The primary antibody is the rabbit anti-bovine
papillomavirus type 1 (BPV-1) which was obtained by the
immunization of a rabbit with bovine papillomavirus type 1
collected from cutaneous fibropapillomas of cattle. BPV-1 is
a readily available source of large quantities of
papillomavirus. The antiserum obtained when BPV-1 is used as
an antigen is less likely to give false reactions with
endogenous intranuclear proteins and DNA in human tissue
than is the antiserum derived from human papillomavirus

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(HPV). The virus was treated with 0.24M 2-mercaptoethanol and 1% sodium dodecyl sulfate (SDS), heated to 68°C for 2 minutes, diluted with three volumes of saline and mixed with an equal volume of Freund's complete adjuvant. A rabbit was cutaneously inoculated with the above purified, previously characterized BPV-1 with 480, 240 and 120 mg of virus protein at days 0, 15 and 28. The rabbit was bled at day 38. This hyperimmune serum (anti-BPV-1 [SDS]) is reactive with PV positive cutaneous and mucosal papillomas from a variety of species, (human, dog, cow, rabbit, deer, and horse). This cross reaction is due to the presence of genus-specific, common antigenic determinants in the major capsid proteins of papillomaviruses.[116]

The biotin-labeling of anti-rabbit IgG (secondary antibody) and peroxidase was accomplished with the incorporation of biotin moieties into immunoglobulin (Ig) or peroxidase (PO) molecules by the use of biotinyl-N-hydroxy-succinimide (BNHS). Biotinylation of Ig and PO improves sensitivity and reduces time by increasing the availability of biotin-binding sites and improving the ability to bind avidin molecules in the avidin-biotin-peroxidase complex. This is due to the great affinity of avidin, a 68,000 molecular weight glycoprotein found in egg white, for biotin, a small vitamin molecule, one mole of avidin binds up to four moles of biotin with a dissociation constant of 10-15M. During the formation of the avidin-biotin-peroxidase

biotin, a small vitamin molecule, one mole of avidin binds up to four moles of biotin with a dissociation constant of 10-15M. During the formation of the avidin-biotin-peroxidase complex, avidin acts as a bridge between biotin-labeled peroxidase and at the same time biotin-labeled peroxidase molecules, which contain several biotin moities, serve as a link between the avidin molecules. Consequently, a "lattice" complex containing several peroxidase molecules is likely formed. Binding of this complex to the biotin moities associated with the secondary antibody results in a high staining intensity.[117] Visualization of the viral proteins is accomplished by the histochemical peroxidation of diaminobenzidine.[118]

INTERPRETATION OF STAINING

Staining occured predominantly intranuclear in a focal or diffuse pattern. Perinuclear cytoplasmic staining of koilocytotic cells was also seen in the nuclei and cytoplasm. The positive signals appeared predominantly, as brown or dark brown staining of nuclei in koilocytotic cells, in a focal or diffuse pattern, although, cytoplasmic staining was also seen.

MATERIALS
SPECIMENS

One hundred lesions subjected to routine hematoxylin and eosin stain and diagnosed as "oral squamous papillomas", were retrieved from the files of the Oral Pathology Laboratory, Dental School, Loyola University Medical Center.

The specimens were formalin-fixed, paraffin-embedded, from the years of 1970 to 1992 and were categorized based on their size as:

a. Good size specimens, 3 or more mm.

b. Moderate size specimens, 2 or 3mm.

c. Poor sized specimens, less than 1 mm.

Only those categorized as good and moderate sized specimens were selected for the purpose of this investigation.

REAGENTS

1. Phosphate-buffered saline (PBS), 10X concentration

Sodium phosphate monobasic monohydrate	50 g
Sodium phosphate dibasic, anhydrous	240 g
Sodium chloride	280 g
Tween 20	4 mL
Deionized water, to make:	4 L

Diluted 1:10 with water to make the working solution.

2. 1% Albumin-saline (PBS-BSA)

Bovine serum albumin, fraction V	1 g
Tween 20	0.1 mL
PBS	100 mL
Mixed and added, as preservative:	
Sodium azide	50 mg

3. Primary antibody in PBS/BSA containing

1% normal goat serum Rabbit Anti-Bovine Papillomavirus (BPV-1)	30 μL
Normal goat serum	60 μL

PBS/BSA	6 mL
Sodium chloride	120 mg
Albumin, fraction V, source bovine blood	120 mg

Primary antibody dilution obtained was of 1:200.

The above concentration was used for a batch of 30 slides.

The amount of solution to be prepared depends on the amount

of slides being processed. Albumin and sodium chloride were

used in order to decrease background.

4. Streptavidin-horseradish peroxidase kits

    Obtain from Kirkegaard-Perry Laboratories

    Cat. No. 71-00-18 (mouse primary)

        71-00-19 (rabbit primary)

Each kit contained blocking serum, biotinylated secondary

antibody and streptavidin-HPR conjugate.

5. Diaminobenzidine (DAB) substrate

    a. Buffer

Ammonium acetate	3.85 g
Deionized water	900 mL

    Added citric acid, 10%, to obtain pH 5.5.

    Added water to make 1 L. Store at 4° C.

    b. Substrate solution

    Two tablets (10mg each) DAB (Sigma D-5905) were added

    for each 50 mL of buffer at room temperature, allowed

    to dissolve and filtered. Just before using, 90 μL 3%

    H^2O^2 per 50 mL was added. After use, substrate

    solution was poured into bottle containing sodium

hypochlorite solution, treated for 27 hours and then disposed via sink.

5. Hematoxylin

Harris-type hematoxylin solution (non-mercuric), was obtained from Newcomer Supply (Cat. No. 1201).

6. Pepsin

One packet of Dako pepsin (Cat No. S3002), was added to 200 mL of deionized water. When dissolved, 50 mL 1N HCL was added. Store at $-20°C$.

CONTROLS

Controls may be of several kinds:

1. A positive control (type 1), tissue specimen which is treated together with the patient specimen and monitored for typical appearance.

2. A negative control (type 2), for which the patient specimen is treated as usual, but omitting the primary antibody. Nonspecific positive reactions such as endogenous peroxidase would be revealed in such a control.

3. Dilution controls (type 3), in which a series of graded dilutions of primary antibody are applied to a control specimen to assess the performance of a particular brand or lot of antibody in comparison to previous or expected results.

Type 1 controls are performed, usually, with one control slide being included per antigen tested per case.

Representative samples are obtained, processed according to standard protocols and tested in the procedure before used.

Type 2 controls are included in instances where increased background might be expected to occur or in the case of tissues bearing pigment that might be confused with reaction product.

Type 3 controls are performed as needed and the results recorded in a log book showing the antibody, its dilution and any other pertinent information.

The type of control used in this investigation, were 4 micron thick sections of buffered 10% formalin-fixed, paraffin-embedded papilloma mounted on poly-L-lysine coated microscope slides, obtained from DAKO Corporation (Catalog No. T1211). Positive anti-papillomavirus antigen nuclei, were found more predominantly in stratum granulosum than in stratum basale. Also stained are pyknotic nuclei and nuclear breakdown products, as well as perinuclear tissue of stratum corneum. Any non-specific background staining of cytokeratins was ignored.

OTHER MATERIALS USED

DAKO Rabbit Anti-Bovine Papillomavirus (BPV-1), (cat. No.B580)

Albumin in powder

100%, 70%, 95% alcohol, and acid alcohol

Ammonia water

Concentrated normal goat serum

Deionized water

1N HCL

Sodium chloride in powder

Xylene

Absorbent wipes

Brushes of different sizes

Coplin Jars

Coverslips

Droppers and pump for droppers

Gloves

Humidified chamber for slide incubation

Mounting medium

37°C oven and 60°C oven

Permanent ink markers

2-8°C refrigerator

Silanized microscope slides

Staining racks

Standard light microscope

Tweezers

38°C water bath

PROCEDURE

1. 3 μm sections of paraffin-embedded tissue were cut and
    mounted on glass slides that have been coated with
    organosilane. Water bath temperature was monitored at
    38°C.

2. Sections were air-dried at 20-37°C.

3. All slides were pre-heated 20-30 minutes at 60°C., then transfered directly to xylene without cooling and treated with three changes of xylene, 5 minutes each.

4. Slides were rinsed in two changes of absolute alcohol. In order to inactivate/extract endogenous peroxidase and hemoprotein, slides were then immersed in absolute ethanol with 0.075% HCL for 15 minutes, (0.1 mL conc. acid/50 mL).

5. Sections were brought through graded alcohols (95%-70%) to water and then to PBS. No enzyme treatment was required.

6. In a moist chamber, slides were covered with the blocking reagent (normal goat serum), provided in the kits and left at room temperature for 30 minutes.

7. Sections were blotted quickly on filter paper and covered with a solution of the primary antibody in PBS/BSA containing 1% normal goat serum. Slides were placed back in the moist chamber and placed in the refrigerator, the level of the chambers checked and left incubated for 15-18 hours (or longer if needed).

8. Incubation in antibody solutions at this and subsequent stages was followed by washing in three changes of PBS, 5 minutes each, to remove unreacted antibody.

9. Biotinylated antibody against IgG of the species in which the primary antibody was prepared (prediluted or prepared from concentrated antibody) was applied to the

sections for 30 minutes at ambient temperature. In this

particular case goat anti-rabbit IgG was used.

10. After washing as before, sections were covered with

streptavidin-peroxidase reagent in the moist chamber

for 30 minutes at ambient temperature.

11. Sections were washed in PBS as before, then rinsed with

deionized water and placed in peroxidase substrate

solution (DAB) for 3 minutes in a dark chamber.

12. After thorough washing in water, the sections were

counterstained with Harris hematoxylin for 4 minutes,

differentiated in acid alcohol (1% HCl in 70% ethanol),

blued in 0.1% ammonia water, washed in running tap

water, dehydrated, cleared and mounted in AccuMount.

DEHYDRATION PROCEDURE: 70%-95%-95%-100%-100% alcohol for 3

minutes each and three changes of xylene for 5 minutes each.

Note: The entire procedure was performed according to
protocol prepared by Robert Martinez,
(Immunohistochemistry Laboratory, Loyola University
Medical Center) and adapted to this investigation.

II. IN SITU HYBRIDIZATION FOR HPV TYPE WIDE SPECTRUM PROBE, HPV TYPE SPECIFIC PROBES, POSITIVE AND NEGATIVE CONTROL PROBES.
PRINCIPLE

The "in situ" hybridization technique is used to detect the presence of human papillomavirus (HPV) DNA sequences within the nuclei the epithelial cells of formalin-fixed, paraffin-embedded, tissue sections of oral squamous papilloma specimens, mounted on slides. The morphology of the tissue is preserved with this technique making it possible to relate the presence of HPV with pathologic changes and at the same time enables the investigator to visualize the exact location of viral DNA.

Tissue sections are first de-paraffinized and then digested with a proteolytic enzyme, pepsin, in order to remove proteins that mask DNA, to increase the accessibility of HPV DNA sequences and to allow probes to permeate the tissue sections more effectively. Next, the double-stranded HPV DNA sequences and biotinylated probes are simultaneously denatured at a temperature of 90°C, by placing the slides on a heating block, the probe is allowed to hybridized the HPV DNA sequences within the tissue specimen. A stringent wash solution is then used to remove excess probe, and this stringent wash solution contains a blocking agent that blocks nonspecific binding sites on the tissue which may otherwise react with the detection reagents. After the stringent wash, the streptavidin alkaline phosphatase

conjugate is used, which binds to the biotin groups on the hybridized probe molecules. The site of hybridization is visualized by the colorimetric reaction of the alkaline phosphatase enzyme conjugate with its substrate, BCIP (5 bromo-4 chloro-3 indolyl phosphate) and the concomitant reduction of NBT (nitro blue tetrazolium), this reaction results in the deposition of an insoluble blue-purple product at the site of hybridization.

PROBES

HPV types 6, 11, 16, 18, 31, 33, wide spectrum HPV, positive control (human DNA) and negative control (plasmid DNA), biotinylated DNA probes were used. Biotinylation of the probe is accomplished through the non-radioactive labeling of the nucleic acids with biotin (vitamin H). The biotinylated nucleotide are enzymatically incorporated into the probe molecule by the nick translation DNA labelling method.[119] The biotin molecule is covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm in the nucleotide analogs of deoxyuridine triphosphate and 5-(3-amino) allyl uridine triphosphate. These analogs function as substrates for a series of RNA or DNA polymerases in vitro. The interactions of biotin and avidin have one of the highest binding constant ($K_{dis}=10^{-15}$) known. The use of streptavidin (a modified avidin tetrameric protein) coupled (or conjugated) to the enzymes alkaline phosphatase and peroxidase which act

as indicator molecules, enables the detection of minute quantities of biotin[120] in the biotinylated probes. Thus, the highest sensitivities for the detection of biotinylated probes are achieved by using streptavidin, which unlike avidin exhibits very low nonspecific binding.[121]

WIDE SPECTRUM HPV BIOTINYLATED DNA PROBE

This double-stranded DNA probe is a nick-translated probe prepared from human papillomavirus (HPV) DNAs which are cloned into plasmid DNA vectors from phage vectors. The targets for this probe are the genomic DNAs of HPV types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52. When tested at intermediate stringency (Tm -20°C.) in a non-radioactive Southern blot hybridization using 50 ng of cloned HPV DNA's as targets, this probe hybridizes to the DNA of HPV types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52. The probe is used for the detection of HPV DNA in tissue specimens by "in situ" hybridization (ISH) and when used in the recommended ISH procedure, the probe demonstrates reactivity only with tissues infected with HPV.[122]

HPV TYPE 6 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA probe is a nick-translated probe prepared form sub-genomic clones of human papillomavirus (HPV) type 6 which are cloned into plasmid vectors. The targets for this probe include regions of the E5 and L2 ORF's (open reading frames) of HPV type 6. When tested by non-radioactive Southern blot hybridization at

high stringency (Tm -10°C.) against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52 this probe is specific for HPV-6.[123]

HPV TYPE 11 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA nick-translated probe is prepared from sub-genomic clones of human papillomavirus (HPV) type 11 which are cloned into plasmid vectors. The targets for this probe include regions of the E5 and L2 ORF (open reading frames) of HPV 11. When tested by non-radioactive Southern blot hybridization at high stringency (Tm -10°C.) against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52, this probe is specific for HPV 11.[124]

HPV TYPE 16 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA nick-translated, is prepared from sub-genomic clones of human papillomavirus (HPV) type 16 which are cloned into plasmid vectors. The targets for this probe include regions of the E1, E2, E4, E5, E6 and E7 ORF's (open reading frames) as well as the URR (upstream regulatory region), of HPV type 16 when tested by non-radioactive Southern blot hybridization at high stringency (Tm - 10°C.) against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52 this probe is specific for HPV 16.[125]

HPV TYPE 18 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA nick-translated

probe is prepared from sub-genomic clones of human papillomavirus (HPV) type 18 which are cloned into plasmid vectors. The targets for this probe include regions of the E2, E5, E6, E7 and L2 ORF's (open reading frames) as well as the URR (upstream regulatory region) of HPV type 18. When tested by non-radioactive Southern blot hybridization at high stringency (Tm - 10°C.) against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52, this probe is specific for HPV 18. The probe is used for the detection of HPV type 33 DNA in tissue specimens by "in situ" hybridization and when used in the recommended ISH procedure the probe demonstrates reactivity only with tissues infected with HPV 18.[126]

HPV TYPE 31 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA nick-translated probe prepared from sub-genomic clones of human papillomavirus (HPV) type 31 which are cloned into plasmid vectors. The targets for this probe include regions of the E2, E5 and L2 ORF's (open reading frames) as well as the URR (upstream regulatory region) of HPV type 31. When tested by non-radioactive Southern blot hybridization at high stringency (Tm - 10°C.) against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51 and 52, this probe is specific for HPV 31. The probe is used for the detection of HPV type 31 DNA in tissue specimens by "in situ" hybridization (ISH) and when used in the recommended

ISH procedure, the probe demonstrates reactivity only with tissues infected HPV 31.[127]

HPV TYPE 33 BIOTINYLATED DNA PROBE

This type-specific double-stranded DNA nick-translated probe prepared from sub-genomic clones of human papillomavirus (HPV) type 33 which are cloned into plasmid vectors. The targets for this probe include regions of the E2, E5, E6 and L2 ORF's (open reading frames) as well as the URR (upstream regulatory region) of HPV type 33. When tested by non-radioactive Southern blot hybridization at high stringency (Tm -10°C.), against 100 ng of cloned HPV DNA from types 6, 11, 16, 18, 30, 31, 33, 45, 51 and 52, this probe is specific for HPV 33. The probe is used for the detection of HPV type 33 DNA in tissue specimens by "in situ" hybridization and when used in the recommended ISH procedure the probe demonstrates reactivity only with tissues infected with HPV 33.[128]

The detection sensitivity of wide spectrum and type-specific HPV probes is approximately 100 molecules of target sequences as measured by "in situ" hybridization and as measured with an analogous probe on HPV-infected, cultured cells. When assayed under recommended protocol none of the probes reacted with normal human nuclear DNA when probed by "in situ" hybridization, or with E. Coli DNA when tested by blot hybridization.

POSITIVE CONTROL (HUMAN DNA) BIOTYNILATED DNA PROBE

Positive control (human DNA) biotinylated DNA probe is a double stranded human DNA nick-translated probe prepared from total human DNA which has been purified from cultured human cells. This probe targets total genomic human DNA. The probe is used for the detection of human DNA in the nuclei of tissue specimens by "in situ" hybridization, it is only intended for use on human tissues. The detection sensitivity of this probe when assayed by "in situ" hybridization when using the recommended protocol, shows most normal diploid nuclei stained positively.[129]

NEGATIVE CONTROL (PLASMID DNA) BIOTINYLATED DNA PROBE

Negative control (plasmid DNA) biotinylated DNA probe is a double-stranded plasmid DNA nick-translated probe prepared from pUC18 plasmid DNA which has been purified from cultured E. Coli cells. This probe targets total plasmid DNA from pUC18. This probe is used as a negative control to asses the levels of background staining and non-specific probe binding on formalin-fixed, paraffin-embedded tissue specimens during "in situ" hybridization. This probe is intended for use on human tissues only. When assayed by "in situ" hybridization using the recommended protocol, no staining was observed on normal formalin fixed, paraffin-embedded tissues.[130]

MATERIALS
REAGENTS

The following materials, sufficient for processing 50

slides, are included in the DAKO® In Situ Hybridization Detection System K601 and K602.

Quantiy	Description
2 Bottle 2:	15 ml Stringent Wash concentrate (50X) containing 0.015m NaN_3.
1 Bottle 3:	15 ml Streptavidin, Alkaline Phosphatase conjugated, in Tris buffer (1X) containing 0.015m NaN_3.
1 Bottle 4:	15 ml BCIP/NBT substrate/chromogen solution (1X)

1 Plastic pipette for Stringent Wash dilution

1 5 ml calibrated test tube for Stringent Wash dilution

Pepsin/HCL Digestion reagent

Tris-buffered Saline (TBS)

OTHER MATERIALS USED

Absorbent wipes

Alcohol solutions (95 and 100%)

Biotinylated probes

Coverslips

Deionized or distilled water

Gloves

90°C heating plate or oven

Humidified chamber for slide incubation

37°C incubator

40-60°C incubator or water bath

Mounting medium

60°C oven

Silanized-treated microscope slides

Staining racks or coplin jars

Standard light microscope

Xylene

PREPARATION OF REAGENTS

The following reagents required preparation prior to
beginning the procedure.

A. Pepsin/HCI Digestion reagents.

This reagent is required for "in situ" procedures using
DNA hybridization on formalin-fixed tissues. Pre-measured
pepsin packets, Code No. S3002 are available from DAKO.
The entire contents of one of the pepsin packets were
dissolved in 250 ml on 0.2N hydrochloric acid. The
digestion reagents were heated at 37° C using a water
bath just prior to use. Excess solution was stored
immediately at 20° C.

B. Tris-buffered Saline (TBS).

Pre-measured TBS packets, Code No. S3001 are available
from DAKO. The entire contents of one TBS powder were
dissolved package in 1 liter of deionized or distilled
water. The diluted solution is stable for one month a
4°C.

C. Stringent Wash Solution.

Stringency requirements will vary depending on probe

composition (%G + C) and the degree of homology between probe and target sequences. Generally, a wash of intermediate stringency should be tried first. If the resultant background is too high, stringency should be increased by raising the temperature of the wash; if signal intensity is too weak, stringency should be lowered by decreasing the wash temperature.

The chart below was used to select stringency conditions when using nick-translated DNA probes of approximately 50 % G + C content on formalin-fixed, paraffin-embedded tissues.

Stringent wash temp.	Stringency
40°C	low
50°C	medium
60°C	high

The stringent wash temperature used for the Wide Spectrum Probe was 48°C, the same as for the positive and negative controls that accompanied the procedure.

The stringent wash temperature used for each of the Type Specific Probes was 58°C, the same as for the positive and negative controls that accompanied the procedure.

The dropper tip from Bottle 2 was removed. With the use of the plastic pipette and calibrated test tube, 1 ml of the 50X Stringent Wash concentrate (Bottle 2), was dispensed into each 50ml of deionized water. Any unused diluted solution was discarded. The stringent wash concentrate provided made 1500 ml of diluted solution.

SPECIMEN COLLECTION AND PREPARATION

The reagents and procedures used in this kit were optimized for DNA-DNA hybridization using tissues that have been routinely processed using buffered formalin and embedded in paraffin.

A. Tissue processing

Fixation times in buffered formalin should be 24 hrs or less; prolonged fixation can result in decreased specific staining and elevated background staining. It is recommended that fixation times be standardized to minimize tissue variability.

In this case, tissues were formalin-fixed, paraffin-embedded, but fixation times were unknown.

B. Tissue sectioning and mounting

Tissue sections were cut 3 microns thick. The aggressive digestion and heating steps used in the DNA "in situ" procedure require that tissue sections be well adhered to the glass slides; slides were coated with organosilane. The procedure for the coating of normal slides with organosilane was as followed:

Clean slides were immersed in 2% solution of 3-aminopropyl-triethoxysilane in acetone for 2 minutes. Rinsed twice in distilled water and air dried. After dried, coated slides were baked for 30 minutes at 60°C, to ensure optimal adhesion of the sections to the slides and stored at room temperature in a dust-free container.

PROCEDURE

In Situ hybridization procedure for double-stranded DNA probes and formalin-fixed, paraffin-embedded tissues:

Prior to beginning the procedure, the pepsin/HCI reagent was prepared and pre-warmed to 37°C.

TBS was prepared and dispense into 4 baths of 250 ml each.

A heating block was pre-warmed to 90°C.

All instructions were read thoroughly, with particular attention to incubation times and temperature.

A. De-paraffinization

Slides with tissue sections were warmed in a 60°C oven for 30 minutes.

Sections were immersed in two changes of xylenes, for 5 minutes. Sections were immersed in two changes of 100% alcohol, followed by three changes of 95% alcohol, for one minute each. Sections were then rinsed in two changes of deionized water for 3 minutes each.

B. Tissue Digestion

The conditions listed below were optimized for tissues that have been fixed according to DAKO recommendations. Over-fixed tissues may require longer digestion times in order to achieve adequate staining.

Sections were immersed in pre-warmed pepsin-HCI for 15 minutes at 37°C, (digestion conditions may need to be tailored to the particular tissue used).

Sections were washed in two changes of distilled water

for 5 minutes.

Slides were removed from water and excess water wiped from around each tissue section.

C. Denaturation and Hybridization

One drop from each probe of hybridization solution was applied to the tissue section. A coverslip was placed over the tissue and hybridization solution, taking care not to introduce bubbles under the coverslip.

The slides were laid on a flat heating block which had been pre-warmed to 90°C. The slides were oriented with the coverslips facing up and with the tissue sections directly over the heating surface. Sections were heated for 5 minutes at 90°C to denature the probe and target DNA. A 90°C oven may also be used for this step; resting the slides on a metal tray or shelf is recommended to ensure efficient heat transfer to the slides when using an oven.

After denatured, the slides were transfered from the heating block to a pre-warmed humid chamber, and incubated at 37°C to hybridize the probe to the target DNA. The required incubation period will depend on the probe concentration used, and may vary from one to twelve hours. At this point the stringent wash was prepared and pre-warmed to the appropriate temperature in a water bath.

D. Detection Procedure

The tissues can not be allowed to dry during the detection procedure, since this would alter the hybridization signal. The slides were removed from the humid chamber and immersed in a TBS bath for 5 minutes. Slides were soaked in the TBS solution in coplin jars, until the coverslips slided off of the slides. The slides were immersed in a fresh TBS bath for 2 minutes in coplin jars.

The slides were immersed in the pre-warmed stringent wash solution in coplin jars and incubated at the appropriate temperature, (refer to page 70: Reagent Preparation) for 30 minutes. The slides were immersed in a fresh TBS bath in coplin jars for 1 minute. The slides were removed from the TBS and carefully excess fluid wiped off from around the sections. The slides were placed on a level surface and enough Streptavidin-AP reagent was applied to each section to completely cover the tissue and incubated for 20 minutes at room temperature. The Streptavidin-AP reagent was poured off from each slide and slides were immersed in a fresh TBS bath for 5 minutes, in coplin jars. The slides were removed from the TBS and carefully excess fluid was wiped off from around the sections. The slides were placed on a level surface and enough of the BCIP/NBT substrate solution was applied to each section to completely cover the tissue and the slides were incubated in the dark at room temperature. The time

required for substrate development will vary with target
concentration. High copy targets are usually
visualized in 60 minutes or less; low target
concentrations may necessitate the use of longer
substrate incubations. If long incubations are used, it
is recommended that the reaction be monitored each hour
to avoid over-staining. In this case the slides were
incubated for 60 minutes. The substrate solution was
poured off from each slide and slides were immersed in
deoionized water bath for 5 minutes.

In this case, slides were not counterstained or mounted in
permanent media to avoid any possibility of loss of the
positive signal and/or misinterpretation of the signal. If
desired, tissues may be counterstained by immersing slides
in Nuclear Fast Red for 1-2 minutes, followed by a rinse in
distilled water for 1 minute. And mounted with permanent
(non-aqueous) media, by immersing the slides for one minute
each in 95% and 100% alcohols followed by 1 minute in
Histoclear or xylenes. When using aqueous mounting media,
slides should be mounted directly without dehydration.

NOTES ON THE PROCEDURES

Tissue sections should not be allowed to dry out once the
hybridization procedure has been started. Sections that are
allowed to dry will exhibit elevated levels of background
staining.

The incubation times and temperatures specified in the kit

instructions are critical for successfully using this procedure. Any change in the procedure may result in decreased performance of the assay.

The color of the positive signals produced by the BCIP/NBT reaction had a purple hue, and background may be more prominent when tissues are mounted with aqueous mounting media.

Mounting with permanent media will result in bluer reaction signals and very low background levels, but some signal may be lost during the tissue dehydration and clearing steps.

In order to determine the specificity of the hybridization reaction, a biotinylated probe of irrelevant specificity may be used as a negative control (i.e. DAKO Negative Control [plasmid DNA] Biotinylated DNA Probe, Code No. X1102 was used).

A positive control probe is useful for determining the suitability of tissue fixation for "in situ" hybridization. DAKO Positive control (Human DNA) Biotinylated DNA probe, Code No. X1101, was used to demonstrate genomic DNA in the nuclei of human tissues. The specificity of the detection reagents can be demonstrated by carrying out the hybridization procedure in the absence of probe; the presence of background using this control is indicative of either high levels of endogenous biotin in the tissue, or nonspecific binding of the detection reagents. The latter problem may occur if the sections are allowed to dry out

during the detection procedure.

LIMITATIONS OF THE ASSAY

Negative results may be caused by a variety of procedure factors, such as improper tissue fixation, mishandling of reagents or performing incubations at improper temperatures. Target sequences which exist only at very low copy numbers may go undetected. In some instances, binding of streptavidin to endogenous biotin within tissues may contribute to high background. Liver, kidney, and highly proliferative tissues or cells often exhibit high levels of endogenous biotin.

The stringency of hybridization reactions is dependent upon many parameters, including probe specificity and the temperature and ionic strength of both hybridization and wash solutions.

INTERPRETATION OF STAINING

Positive signals, corresponding to areas of hybridization, appeared as blue or blue-purple regions within individual cells of the tissue. Overdevelopment of the substrate chromogen may result in black signals.

Counterstaining with nuclear counterstains may make the interpretation of positive signals difficult if too much counterstain is used. The use of hematoxylin is not recommended, since the blue color of the positive reaction is easily obscured by the blue counterstain. A light counterstain of the tissue section using Nuclear Fast Red is

recommended if additional morphological detail is desired.

Note: The entire procedure was performed according to DAKO In Situ Hybridization Detection System for Biotinylated Probes K601 and K602 and adapted to this investigation.

RESULTS

As indicated in TABLE A, 63 of 100 papillomas evaluated by the Avidin-Biotin-Peroxidase Complex method, which showed either nuclear or nuclear and cytoplasmic staining, were considered as positive.

Papilloma virus antigen was detected in stratum corneum in 1 case; in stratum granulosum in 35 cases; in stratum corneum and granulosum in 7 cases; in stratum corneum and granulosum and upper stratum spinosum in 3 cases; in stratum granulosum and upper stratum spinosum in 17 cases.

Positivity of the staining reaction was noted as a brown to dark brown precipitate in the nuclei and cytoplasm, but cytoplasmic staining without nuclear staining was not taken as positive. Labelling of the cytoplasm can occur due to fragmentation of infected nuclei and release of virus particles and virus-associated proteins into the cytoplasm, or due to non-specific reactivity of cytoplasmic substances with HPV antibodies.[131]

The 63 cases that stained positively for the papilloma virus antigen were tested by "in situ" hybridization. A wide spectrum probe (WSP) which targets HPV DNA of types 6, 11, 16, 18, 31, 33, 30, 35, 45, 51 and 52 was used. Of these 63 specimens 39 yielded positive results under intermediate

stringency (48° C).

The 39 cases that were positive with the WSP were, in turn, tested individually, each with the type specific probes (TSP), under conditions of high stringency (58° C), yielding the following results, (APPENDIX C):

HPV type 6 positive, 7 papillomas.

HPV type 11 positive, 2 papillomas.

HPV type 16 positive, 0 papillomas.

HPV type 18 positive, 2 papillomas.

HPV type 31 positive, 10 papillomas.

HPV type 33 positive, 3 papillomas.

HPV types 6 and 11 positive, 2 papillomas.

HPV types 6 and 31 positive, 5 papillomas.

HPV types 6 and 33 positive, 1 papilloma.

HPV types 11 and 31 positive, 2 papillomas.

HPV types 18 and 33 positive, 1 papilloma.

HPV types 11, 31 and 33 positive, 1 papilloma.

HPV types non-conclusive results, 3 papillomas.

As indicated in TABLE B and APPENDIX B, the following reactivity were seen with the TSP:

HPV type 6 probe, 15 cases were strongly positive, 5 were moderately positive and 8 weakly positive.

HPV type 11 probe, 6 cases were strongly positive, 2 were moderately positive and 1 weakly positive.

HPV type 16 probe, none of the cases were strongly positive, 2 were moderately positive and 4 weakly positive.

HPV type 18 probe, 2 cases were strongly positive, 1

moderately positive and 6 weakly positive.

HPV type 31 probe, 17 cases were strongly positive, 5 moderately positive and 2 weakly positive.

HPV type 33 probe, 5 cases were strongly positive, 3 moderately positive and 7 weakly positive.

Multiple strong hybridization signals with the following HPV type probes:

HPV type 6 and 11 probes, 2 cases.

HPV type 6 and 31 probes, 5 cases.

HPV type 6 and 33 probes, 1 case.

HPV type 11 and 31 probes, 2 cases.

HPV type 18 and 33 probes, 1 case.

HPV type 11, 31, and 33 probes, 1 case.

Two of the cases belonged to a same patient, being the two papillomas strongly positive with HPV type 11 probe and moderately positive with HPV type 31 probe.

Multiple moderate signals were seen in 1 case which reacted with probe 18. Multiple weak signals were seen in 2 cases, one reacted with probes 6, 31, 18 and others with probes 31 and 33. The results for these cases were recorded as non-conclusive (NC).

Positivity of the staining reaction was noted as a blue-purplish or black precipitate in the nuclei of the infected cell. In some cases of tissues that contained high amounts of DNA, the stain spread from the nuclei into the surrounding spaces. However, cytoplasmic staining in the absence of nuclear staining was not interpreted as positive.

This was seen in 6 cases.

Positive staining occurred in cells with pyknotic ovoid nuclei, crescent-shaped or flat elongated nuclei which could be compressed against the cell membrane and small or enlarged centered or wrinkled nuclei, but these characteristic could also be seen in negative cells.

Staining intensity varied from weak (pale blue-purple) to strong (dark blue-purple or black).

Staining patterns of nuclei were based on the following criteria, according to Enzo PathoGene DNA Probe Assay for Identification of Human Papillomavirus (Catalog No. PG-877), Interpretation Guide:

a. Full nuclear staining, nucleus appeared completely filled with stain.

b. Granular or stippled nuclear staining, nucleus appeared filled with granules or dots.

c. Mixed nuclear staining, the nuclei of some cells appeared fully stained, while the nuclei of others showed a granular or stipped staining.

Reactions with the WSP were either considered positive or negative. A weak, moderate or strong nuclear staining was considered as positive.

Reactions for TSP were considered as positive, inconclusive or negative based on the intensity of the hybridization signal:

1. Positive, a strong hybridization signal, when nuclear

staining was dark, same as control slide and a moderate
hybridization signal, when the signal was lighter than
control slide but darker than the weaker hybridization
signal.

2. Non-conclusive, only weak or moderate hybridization
signals with more than one probe.

3. Negative, absence of nuclear staining.

Nuclear staining was confined to single cells localized in
certain areas of the epithelium or clusters of cells
localized or diffused throughout the epithelium.

Weak multiple hybridization signals of the same
papilloma with different specific probes were considered
negative since according to the manufacturer (DAKO
Corporation), this may be indicative of cross reactivity
with other HPV types in the tissues which contains very high
amounts of HPV DNA. HPV type is determined in these cases by
comparison of the signal intensities resulting from the
individual probes. The strongest hybridization signal was
the one considered as positive.

Positive and negative controls showed no equivocal
results and no signs of cross hybridization.

All positive controls were strongly positive for each of the
probes.

Specimens that were strongly positive for HPV types 6,
11 and moderately positive for HPV type 16 showed nuclear
staining in cells of stratum granulosum and upper stratum

spinosum.

Specimens that were strongly and moderately positive for HPV type 18, staining of nuclei was confined to stratum granulosum and spinosum in 2 cases and one case also showed reactivity in the stratum basale.

Specimens that were positive for HPV types 31 and 33 exhibited a multi-layered pattern of reactivity, in these cases staining was seen in stratum granulosum, spinosum and basale.

Based on the histomorphological description, a comparison of papillomas positive and negative with the wide spectrum probe (WSP) showed the following results:

FEATURE	WSP POSITIVE	WSP NEGATIVE
Hyperorthokeratin	3 cases	1 cases
Hyperparakeratin	8 cases	9 cases
Hyperorthoparakertin	0 cases	2 cases
Hyperpara/parakeratin	2 cases	0 cases
Hyperortho/orthokeratin	0 case	1 case
Orthokeratin	2 cases	4 cases
Orthoparakeratin	8 cases	0 cases
Parakeratin	16 cases	7 cases
Acanthosis	35 cases	21 cases
Basal cell replication	34 cases	16 cases
Dyskeratosis	2 cases	0 cases
Hyperchromatic nuclei	1 case	0 cases
Koilocytosis	34 cases	14 cases

Mitotic activity	5 cases	2 cases
Multiple nuclei	1 case	0 cases
Prominent nucleoli	1 case	1 case

Koilocytosis varied from huge koilocytic cells to small koilocytic cells which could either be localized or sparsed through the epithelium.

DISCUSSION AND CONCLUSIONS

The main objective of this study was to detect the presence of HPV types 6, 11, 16, 18, 31 and 33 in oral squamous papillomas. This was accomplished through the use of two different methods, the Avidin-Biotin-Peroxidase Complex for the immunohistochemistry technique and "in situ" hybridization.

Papillomavirus antigen was detected in 63% (63/100) and HPV DNA in 61% (39/63) of the papillomas which were tested with the Wide Spectrum Probe, while 38% (24/63), were negative for HPV DNA.

The majority of the papillomas, were infected with HPV type 31 DNA, 25% (10/39); followed by HPV type 6 DNA, 17% (7/39); HPV types 6 and 31 DNA, 12% (5/39); HPV type 33 DNA, 7% (3/39), HPV type 11, 5% (2/39); HPV types 6 and 11 DNA, 5% (2/39); HPV types 11 and 31 DNA, 5% (2/39); HPV type 18 DNA, 5% (2/39);HPV types 6 and 33 DNA,(1/39); 2% HPV types 18 and 33 DNA, 2% (1/39); HPV types 11, 31 and 33 DNA, 2% (1/39). None of the cases expressed HPV type 16 DNA and 3 of the cases showed non-conclusive results, (APPENDIX C).

Oral lesions with no evidence of viral antigen and/or DNA may:

1. Be a non-viral associated tumor, a normal anatomic

variant or a papillary hypertrophy resulting from long standing inflamatory disease.[132]

2. Be a new HPV genotype with a nucleotide sequence that does not cross hybridize with the probes employed in this study.

3. Represent HPV-associated lesions with a viral copy number below that detectable by the "in situ" hybridization technique.

4. Have been virally initiated with subsequent shedding of infected cells through epithelial proliferations and maturation.[133]

5. Have been originally present but lost during the harsh treatments of the procedure.

Based on the findings of this investigation, a histomorphological description of the papillomas which express HPV DNA and those papillomas which do not express HPV DNA was performed, in order to establish a correlation between histology and molecular hybridization findings. It is believed, that papillomavirus (PV) induce changes in the epithelium which reflect their biological behavior. PV induced lesions display features of acanthosis, dysplasia, dyskeratosis, hyperchromasia, hyperkeratosis, koilocytosis and basal cell replication. These cytologic changes are associated with virus production, which result in the death of the infected cell as the virus multiply in the nucleus and the level where these cytopathic changes begin is

determined by the virus type.[134]

PV affects the keratinocyte metabolism, changes in keratin composition are seen in a variety of epidermal disorders and these changes seem to correlate with the degree of hyperplasia in the lesion. The most common finding is the decrease in the relative amount of 67K keratin.[135] Acanthosis and basal cell proliferation, is thought to occur due to the presence of HPV DNA in the basal cell layer. In common warts, autoradiography with tritiated thymidine has shown an increase in DNA synthesis in basal and parabasal cells, two to three layers above the basement membrane[136] and juvenile laryngeal papillomas in parabasal cells extending nearly to the surface.[137] Also, basal cell hyperplasia, is thought to be due to an early gene expression of the HPV ocurring prior to vegetative viral DNA synthesis. In contrast, the synthesis of viral capsid proteins appear to be a function of late gene expression since it occurs only in terminally differentiated squamous cells in the superficial layers.[138]

PVs seem to have a predilection for specific sites of the epithelium rather than being specific for a particular lesion. This tissue-specificity may reflect a requirement for cellular factors present in keratinocytes. PV subtypes may have evolved by adapting to different patterns of keratinocyte differentiation found in specific epithelia.[139]

HPVs 6, 11, 13, 16, 18 and 32, the types that most commonly infect the oral mucosa, share a degree of sequence homology with each other and these homologous regions might play a role in tissue-specific infection. The above might explain the fact, that similar HPV types have been detected in clinically and histomorphologically different lesions, except for types 13 and 32. These types are mainly seen in Focal Epidermal Hyperplasia, (FEH) and have only been detected in oral mucosa and perioral skin, as well as in a small percentage of Condyloma Acuminatum, (CA) and Squamous Cell Papillomas (SCP). Lesions which contain HPV types 13 and 32 show FEH-like characteristics, such as hyperpara-or hyperorthokeratosis, increased cellular density, acanthosis, unusual rete pegs that demonstrate clubbing and horizontally anastomosing outgrowths, koilocytes, mitosis-like nuclear degeneration, swollen cells with ballooning type of nuclear degeneration.

Immunoregulation plays a role in the pathogenesis of HPV induced lesions, since they predispone the individual to uncommon types and to develop lesions with an unusual clinical apperance.[140]

In the genital tract, proliferations induced by viral types 6, 11 and 31, preserve characteristics of regular differentiation pattern, the nuclei are regularly shaped and the DNA content is tetraploid or diploid.[141] While, proliferations caused by types 16 and 18, are characterized

by marked nuclear atypia, regular and atypic mitotic figures even in the upper third of the epithelial layer, an aneuploid karyotype and a low number of koilocytotic cells which may be entirely absent.[142] Also, they seem to regress less and recurr more frequently than those proliferations cause by HPV types 6 and 11. Common warts regressing more frequently than condyloma acuminatum. In general, PV-induced proliferations of the genital tract are caracterized by long duration and frequent recurrence, even after surgical removal.[143]

The majority of HPV induced lesions in skin and mucosa regress spontaneously but a certain number progress to malignancy. HPV types 6 and 11 are considered to cause benign lesions, while HPV types 16 and 18 are considered to have oncogenic potential. Thus, lesions caused by these types are considered malignant, or at least pre-malignant. HPV type 18 seems to produce a more invasive type of lesion than HPV type 16. Their role in neoplastic transformation is clearly seen in Epidermodysplasia Verruciformis (EV) and ano-genital carcinomas.[144] In the oral cavity, there is a report of leukoplakias transforming into squamous cell carcinomas.[145]

The ability of certain HPV types for malignant transformation seems to be related to two transforming proteins encoded in their genome, which bind to the tumor suppressor gene of the cell, providing a mechanism for HPV

induced cervical neoplasm.[146] Or, there could be a genetic
lack or malfunction of the tumor suppressor gene, thus
allowing the virus to control the replication mechanisms of
the epithelial cells and start replicating itself. Repeated
studies have demonstrated that the majority of cervical
cancers and derived cell lines contain HPV DNA. But not all
cells infected with the virus undergo malignant
transformation because the malignant tumors are monoclonal,
while the HPV lesions are not.[147,148]

In the etiology of cancer, there seems to be a need for
cofactors which act with the virus in order to produce the
malignant transformation, because usually the time elapsed
since the time of infection and the onset of cancer could be
decades.[149] In the oral cavity, such co-factors could be
alcohol, chewing tobacco, cellular mutations, heredity,
radiation, smoking and in the skin, also, sunlight. Such is
the case in EV, where skin lesions containing HPV types 5
and 8 become malignant after being exposed to sunlight.

HPV infection is transmissible and the virus is highly
contagious. Since it has no envelope, the virus is resistant
to either inactivation, freezing and dessication, therefore
remains infectious after drying.[150]

The majority of the papillomas which expressed HPV DNA
with the Wide Spectrum Probe (WSP), showed parakeratin 41%
(16/39), followed by hyperparakeratin 20% (8/39) and ortho-
parakeratin 20% (8/39), hyperorthokeratin 7% (3/39),

combination of hyperpara/parakeratin 5% (2/39) and orthokeratin 5% (2/39).

Of the papillomas which did not expressed HPV DNA with the WSP, the majority showed hyperparakeratin 37% (9/24), followed by parakeratin 29% (7/24), orthokeratin 16% (4/24), hyperorthoparakeratin 8% (2/24), hyperorthokeratin 4% (1/24) and combination of hyperortho/orthokeratin 4 % (1/24).

Acanthosis was seen in 89% (35/39) of the papillomas which expressed HPV DNA and in 87% (21/24) of the papillomas which do not expressed HPV DNA.

Basal cell layer replication was seen in 87% (34/39) of the papillomas which expressed HPV DNA and in 66% (16/24) of the cases which did not expressed HPV DNA.

Dyskeratosis (benign) was only seen in 2 of the papillomas which expressed HPV DNA 5% (2/39).

Koilocytosis was seen in 87% (34/39) of the papillomas which expressed HPV DNA and in 58% (14/24) of the papillomas which did not expressed HPV DNA.

Basal cell mitotic activity was seen in 12% (5/39) of the papillomas which expressed HPV DNA and in 8% (2/24) of the papillomas which did not expressed HPV DNA. Suprabasal cell mitotic activity was seen in 5% (2/39), of the papillomas which expressed HPV DNA and in 12% (3/24) of the papillomas which did not expressed HPV DNA.

Nuclear hyperchromasia in basal cells was seen in 2% (1/39), prominent nucleoli in basal cells were seen in 2%

(1/39), multiple nucleoli in basal and suprabasal cells were seen in 2% (1/39), of the papillomas which expressed HPV DNA. Prominent nucleoli in basal cells were seen in 4% (1/24) of the cases which did not expressed HPV DNA, (APPENDIX D and E).

Nuclear shape varied in cells which expressed HPV DNA. Positive staining occured in cells with pyknotic ovoid nuclei, crescent-shaped or flat elongated nuclei which could be compressed against the cell membrane, wrinkled nuclei and small or large centered nuclei.

Varying degrees of papillomatosis, were seen in all papillomas.

Thus, different forms of keratinization, acanthosis, koilocytosis, papillomatosis, basal and suprabasal cell replication, basal and suprabasal mitotic activity were seen in papillomas whether or not they expressed HPV DNA.

As shown in this study, hyperkeratinization is not pathognomonic of HPV infection. But in the oral cavity, keratinization present in papillomas located in areas of normally non-keratinizing epithelium such as ventral surface of the tongue, floor of the mouth, vestibular mucosa and tonsils, is abnormal.

Koilocytosis and parakeratinization were the most prominent features of HPV infected papillomas. Thus, in the oral cavity koilocytosis and parakeratinization are caracteristic of the presence of HPV DNA, according to the results of this

investigation.

The type of HPV determines its location in the epithelium. HPV types 6, 11, 16 DNA were only detected in the most superficial layers of the epithelium , while HPV types 31 and 33 were also detected in stratum basale. HPV type 18 DNA, was detected in one papilloma in the stratum granulosum, spinosum and basale.

A papilloma can be infected with more than one type of HPV based on the most recent investigation using an improved technique for Polymerase Chain Reaction (PCR).[151] Single infection was seen in 24 papillomas which reacted strongly with only one probe (6, 11, 18, 31, 33). Dual infection was seen in 11 papillomas where there was a strong reaction with two different probes (6 and 11; 6 and 31; 6 and 33; 11 and 31; 18 and 33). A triple infection was seen in a papilloma which reacted strongly with probes 11, 31 and 33, (APPENDIX A).
There were two cases of multiple weak positive signals and one with multiple signals which were regarded as non-conclusive.
The multiple weak or moderate signals present in the same papilloma could have been caused by:

    a. Cross-hybridization between the probe and viral DNA sequences.[152]

    b. Very low DNA number copies present in the specimen weakly detected by the probe.

    c. Presence of a diferent type of virus from the type
being tested by the type specific probe (TSP) but
target by the wide spectrum probe (WSP).

    d. Type of solution used to fix the tissue and fixation
times will affect the intensity of the hybridization
signal, reducing it even to undetectable levels.[153]

    e. Strong homology between HPVs, such in the case of
HPV types 6 and 31 that causes cross hybridization
between these two types.[154]

A risk profile for malignancy could be established
according to the type of HPV present.
Papillomas infected with HPV types 6 and 11 are considered
"low risk". Although, there has been reports of laryngeal
papillomas positive for HPV type 6, progressing to
pharyngeal carcinomas,[155] but it could have been caused by
an undetected HPV, belonging to the types considered
oncogenic.
Papillomas infected with HPV types 31 and 32 are considered
"intermediate risk", and papillomas infected with HPV 16 and
18, are considered "high risk".

Typing should be done of all oral squamous papillomas
because of the oncogenic potential of some HPV types and
patients should be closely monitored. And also, because of
statitical and histopathological evidence suggesting that
condylomata which may exist as a sub-clinical PV infection
and cervical intraepithelial neoplasm (CIN), represent the

extremes of a single disease spectrum.[156]

The above could also be applied to the oral cavity. Oral squamous papillomas (including oral condyloma acuminatum and oral verruca vulgaris)-leukoplakia-oral verrucous carcinomas-oral squamous carcinomas could all be part of a same disease spectrum, since the same HPV DNA types have been detected in these lesions.

In the oral cavity, all papillomas, whether they exhibit the same clinical and histomorphological caracteristics of its counterpart in the ano-genital region (Condyloma Acuminatum) or its counterpart in the skin (Verruca Vulgaris), should be considered variants of the same disease entity. Therefore, base on the findings of this investigation and in the characteristics observed in the majority of the cases assayed, oral squamous papillomas could be re-defined as:

Oral squamous papillomas are composed of keratinized, acanthotic, stratified squamous epithelial ridges, disposed in papillary projections with basal cell layer replication and a fibrous or fibrovascular supporting stroma. The epithelial ridges could be elongated, short, broad, fused or not. The papillary projections could be sharp or blunt. The overlying epithelium is mainly parakeratinized, but it could also be hyperparakeratinized, orthokeratinized, hyperorthokeratinized, or a combination of para-orthokeratin. Koilocytosis, dyskeratosis, dysplasia might be

present or not.

The diagnosis of Oral Squamous Papillomas, would be
made based on whether or not they express viral DNA and
whether or not they exhibit characteristics of Condyloma
Acuminatum or Verruca Vulgaris:

HPV DNA positive papillomas, with caracteristics of
Condyloma Acuminatum, would be diagnosed as "Oral
Squamous Papillomas of viral origen, compatible with
Condyloma Acuminatum."

HPV DNA positive papillomas, with characteristics of
Verruca Vulgaris, would be diagnosed as "Oral Squamous
Papillomas of viral origen, compatible with Verruca
Vulgaris."

HPV DNA positive papillomas, which do not present
characteristics of Condyloma Acuminatum or Verruca
Vulgaris would be diagnosed as "Oral Squamous
Papillomas of viral origin".

HPV DNA negative papillomas, would be diagnosed as
"Oral Squamous Papillomas of non-specific origin."

Based on the above re-definition, the present confusion
that exists regarding the diagnosis of oral squamous
papilloma, condyloma acuminatum, and/or verruca vulgaris
could be eliminated if all these lesions were considered as
papillomas in the oral cavity, whether they exhibit
characteristics of condyloma acuminatum or verruca vulgaris.

HPV types 6, 11, 16, 18, 31, 33 infect the oral mucosa

as have been shown in this investigation. Whether, transient or permanent hosts of the oral cavity, they cause papillary lesions of the epithelium. And, as DNA hybridization techniques for detection of HPV improve and other more sensitive techniques develop, more oral squamous cell papillomas will be found to harbor HPV DNA.

TABLE A

PAPILLOMAS CASE SELECTION AND RESULTS

CASE NO.	AGE/SEX	LOCATION	BPV-1	WSP	TSP
1335-92	31/M	Hard Palate	−		
1277-92	29/F	Hard Palate	+	−	
1227-92	36/F	Soft Palate	+	+	33
1093-92	40/F	Hard Palate	+	−	
1090-92	50/M	Lower Lip	+	−	
996-92	40/F	Gingiva # 8	−		
717-92	12/F	Lower Lip	+	−	
689-92	40/F	Soft Palate	−		
567-92	41/M	Vermillion Lip	+	−	
564-92	32/M	Lower Lip	+	+	6
492-92	28/M	Lingual Frenum	+	+	31
461-92	44/M	Buccal Mucosa	+	−	
236-92	36/M	Buccal Mucosa	+	+	6
214-92	57/M	Floor Mouth	+	+	6,11
1040-90	36/M	Submax. Duct	−		
1065-90	32/M	Uvula	+	−	
799-90	52/F	Vermillion Lip	+	+	18,33
670-90	25/M	Buccal Mucosa	+	+	NC*
464-90	64/F	Tip Tongue	+	−	
424-90	64/M	Alveolar Ridge	−		
242-90	55/F	Lateral Tongue	+	+	18
1067-1-89	65/F	Buccal Mucosa	+	+	31
1060-89	29/M	Soft Palate	−		

869-89	63/M	Lingual Frenum	+	+	6,33
780-89	52/M	Ventral Tongue	+	+	11,31
734-89	54/M	Labial Mucosa	+	+	18
713-89	81/F	Buccal Gingiva	+	−	
674-89	17/M	Gingiva	+	+	31
657-89	30/M	Soft Palate	+	+	NC
656-89	77/M	Hard Palate	−		
645-89	17/M	MGJ*	+	+	11,31
637-89	38/F	FM-BT*	+	+	11,31,33
377-89	8/F	Unknown	−		
285-89	28/F	Area # 13	+	+	33
174-89	NK*/M	Soft Palate	+	−	
191-89	47/F	Tongue	−		
131-89	73/M	Tip Tongue	+	+	31
129-89	NK/M	Lower Lip	−		
71-89	65/F	Soft Palate	+	+	31
1050-88	32/M	Dorsal Tongue	−		
980-88	62/F	Ventral Tongue	−		
970-88	50/M	Soft Palate	+	+	6
934-88	53/F	Hard Palate	+	+	NC
834-87	10/F	Retromolar Pad	−		
735-87	45/M	Dorsum Tongue	+	+	31
628-87	NK/F	Buccal Gingiva	−		
189-87	51/F	Tip Tongue	+	−	
13-87	53/M	Floor Mouth	−		
1106-86	57/M	Buccal Mucosa	+	+	6,31
843-86	41/M	Buccal Mucosa	−		
798-86	51/F	Lateral Tongue	+	+	31
789-86	56/M	Tonsil Pillar	−		
765-86	55/M	Lingual Gingiva	+	+	6

798-86	51/F	Lateral Tongue	+	+	31
789-86	56/M	Tonsil Pillar	-		
765-86	55/M	Lingual Gingiva	+	+	6
578-86	62/M	Uvula	+	-	
524-86	25/F	Buccal Mucosa	-		
485-86	25/F	Tip Tongue	+	+	31
356-86	36/M	Tuberosity	-		
287-86	67/F	Soft Palate	-		
1065-85	33/M	Unknown	+	-	
962-85	41/M	Buccal Mucosa	+	+	6
902-85	NK/M	Comissure Lip	+	+	6,31
892-85	NK/M	Retromolar Pad	+	-	
816-A-85	NK/M	Alveolar Ridge	+	+	11
816-B-85	NK/M	Lingual Frenum	+	+	11
782-85	49/M	Dorsum Tongue	+	+	6,31
627-85	32/F	Tip Tongue	+	-	
479-85	26/M	Dorsal Tongue	+	+	6,31
481-85	63/M	Tonsilar Pillar	+	-	
228-85	47/F	Tongue	+	-	
341-85	41/F	Ventral Tongue	-		
268-85	37/M	Hard Palate	+	-	
160-85	33/M	Ventral Tongue	+	+	6
333-84	27/M	Buccal Mucosa	-		
240-84	63/F	Gingiva # 2	-		
698-83	54/F	Lateral Tongue	-		
682-83	58/M	Dorsum Tongue	-		
655-83	38/M	Mucosa Lip	+	+	6,11
623-82	60/M	Palate	+	+	31
244-82	62/F	Upper Lip	+	+	6,31
216-82	63/F	Buccal Mucosa	-		

153-82	NK/F	Hard Palate	+	+	33
143-82	NK/F	Alveolar Ridge	+	-	
142-82	53/F	Alveolar Ridge	-		
728-81	30/M	Mucosa Cheeck	+	-	
620-81	NK/M	Lateral Tongue	-		
387-81	35/M	Lower Lip	-		
200-80	60/F	Palate	+	+	31
202-79	30/F	Hard Palate	-		
165-79	38/M	Hard Palate	+	-	
213-78	6/F	WDP*	+	-	
193-78	42/F	Vermillion Lip	-		
155-78	23/M	Lingual Frenum	+	+	6
532-77	56/M	Hard Palate	-		
454-77	27/M	Retromolar Pad	-		
410-75	25/M	FM-BT	-		
315-75	NK/F	Papilla Max.	+	-	
823-74	NK/M	Unknown	-		
1002-70	50/M	Lower Lip	-		
785-70	5/F	Gingiva	-		
557-70	56/M	Soft Palate	+	-	

* NC: Non-conclusive

* MGJ: Muco-Gingival Junction

* FM-BT: Floor Mouth-Base Tongue

* NK: not known

* WDP: Wharton's Duct Papilla

TABLE B

HPV TYPE SPECIFIC PROBE REACTIVITY

CASE	HPV-6	HPV-11	HPV-16	HPV-18	HPV-31	HPV-33
1227-92	+		+			+++
564-92	+++					
492-92	+			+	+++	
236-92	+++					
214-92	+++	+++				
799-90	+			++		++
670-90					+	+
242-90	+			+++		
10671-89					+++	
869-89	+++				+	+++
780-89	++	+++			+++	++
734-89	+	+		+++	++	+
674-89	++				+++	
657-89	++	++		+	++	++
645-89		++			++	
637-89	++	+++	+	+	+++	+++
285-89			++			+++
131-89					+++	
71-89			+	+	+++	+
970-88	+++					
934-88	+		'	+		+
735-87	+		++		+++	+
1106-86	+++				+++	

962-85	+++					
902-85	+++			+	+++	+
816-A-85		+++			++	
816-B-85		+++			++	
782-85	+++				+++	
479-85	+++				+++	
160-85	+++					
655-83	+++	+++				
623-82			+		+++	+
244-82	+++				+++	
153-82						+++
200-80	++				+++	
155-78	+++					

+   Weakly reactive

++ Moderate reactive

+++ Strongly reactive
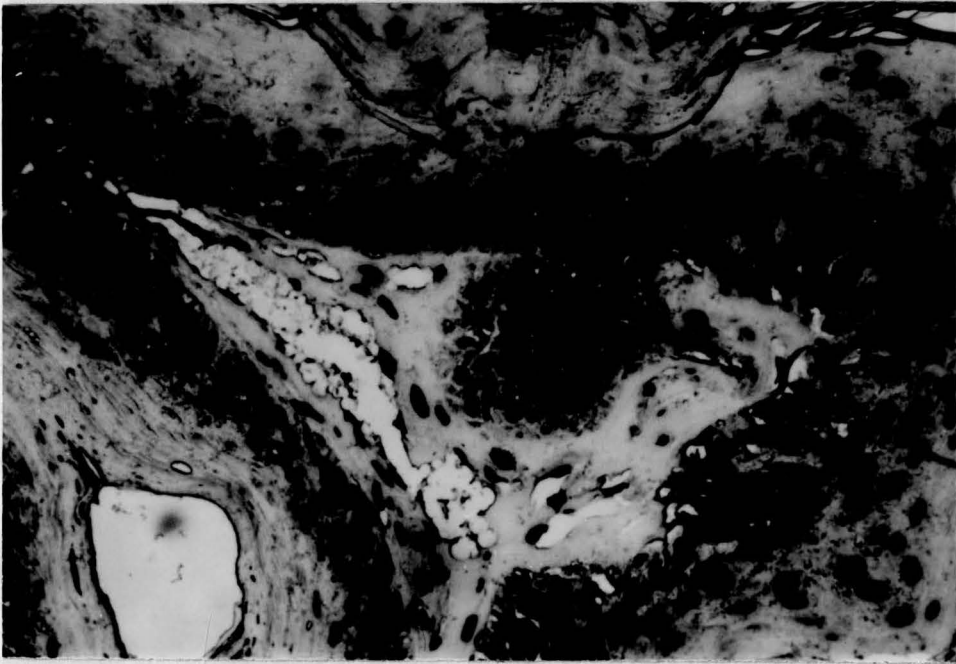
FIGURE 1: HPV Type Wide Spectrum Positive Control

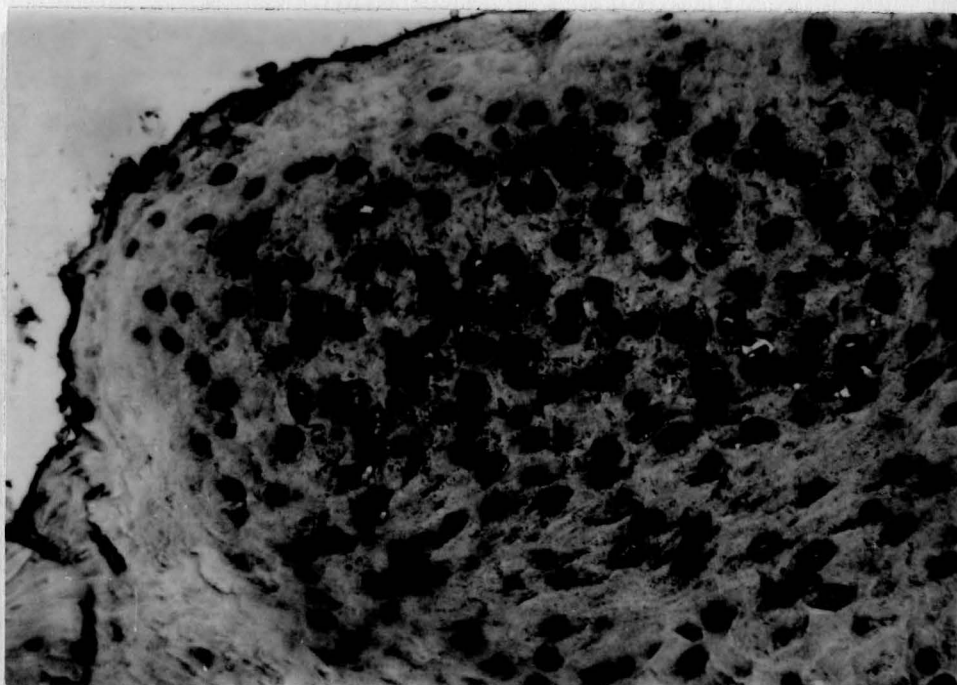FIGURE 2: HPV Type Wide Spectrum Positive Papilloma
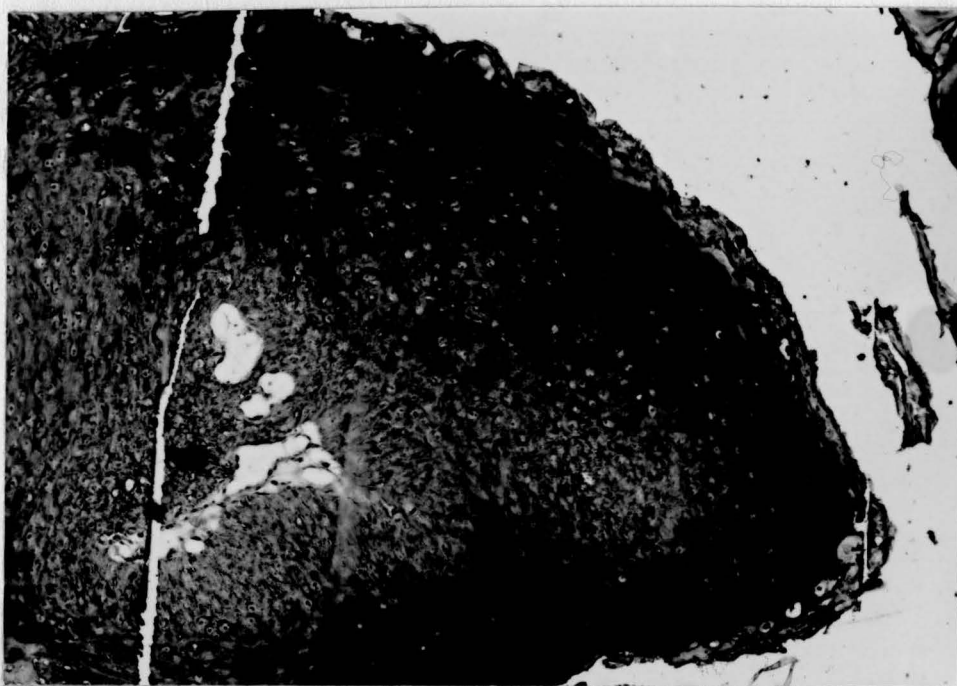
FIGURE 3: HPV Type 6 Positive Control

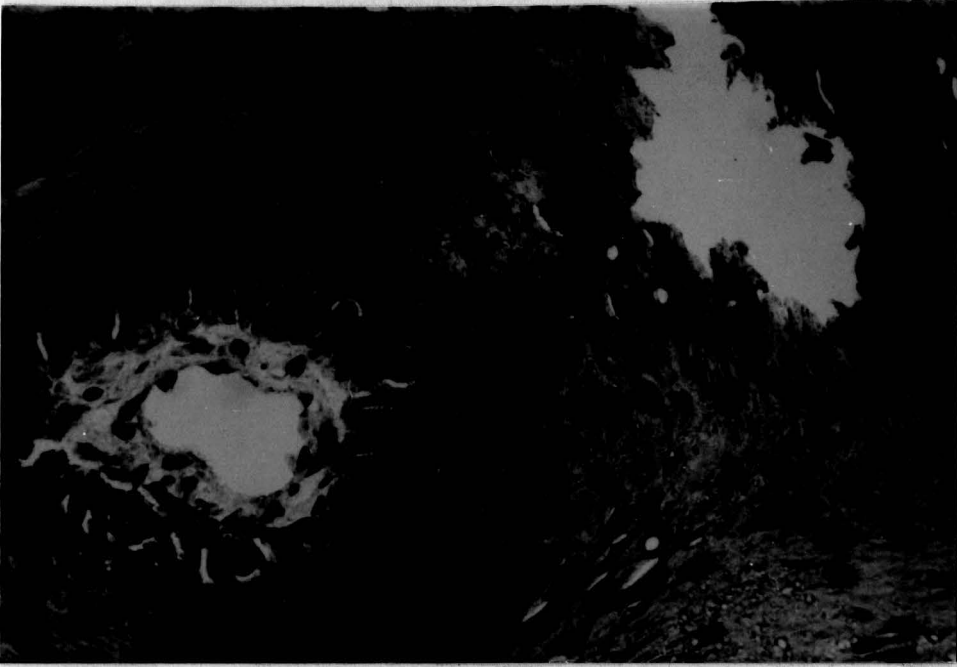FIGURE 4: HPV Type 6 Positive Papilloma

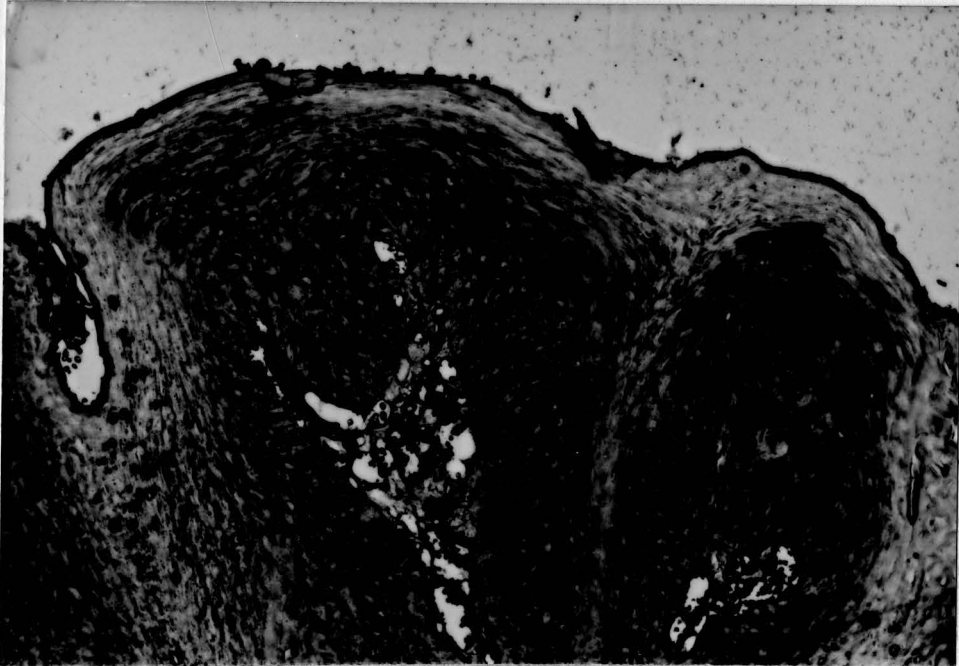FIGURE 5: HPV Type 11 Positive Control

FIGURE 6: HPV Type 11 Positive Papilloma
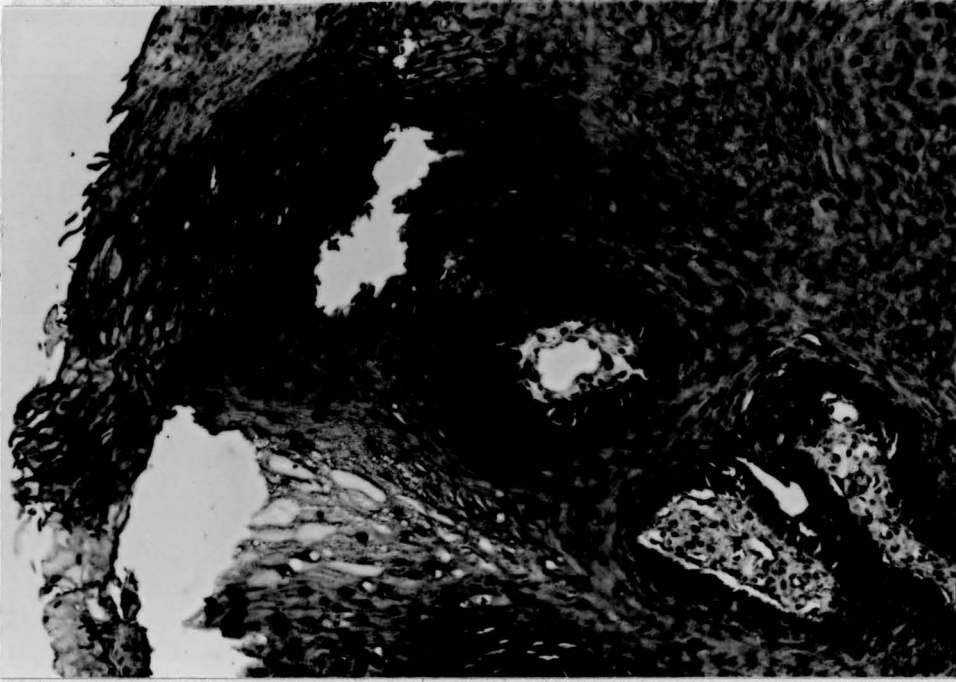
FIGURE 7: HPV Type 18 Positive Control
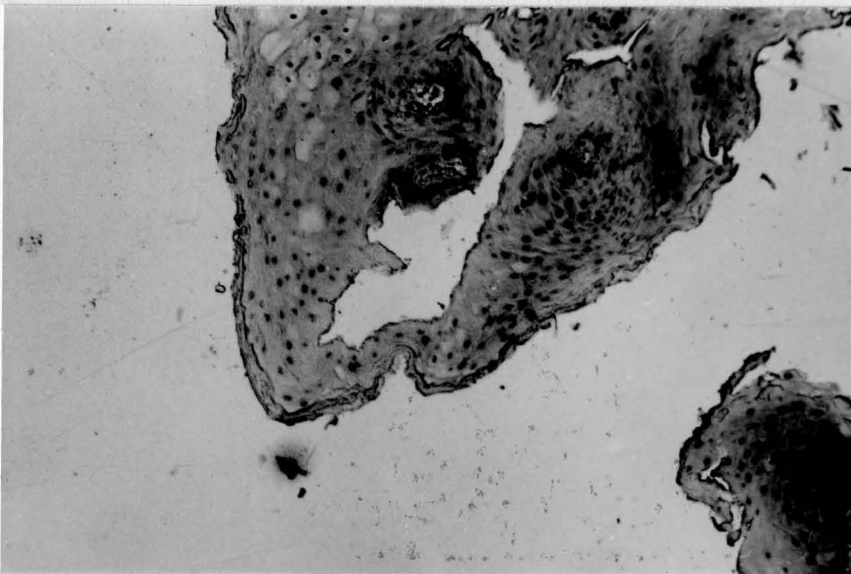
FIGURE 8: HPV Type 18 Positive Papilloma
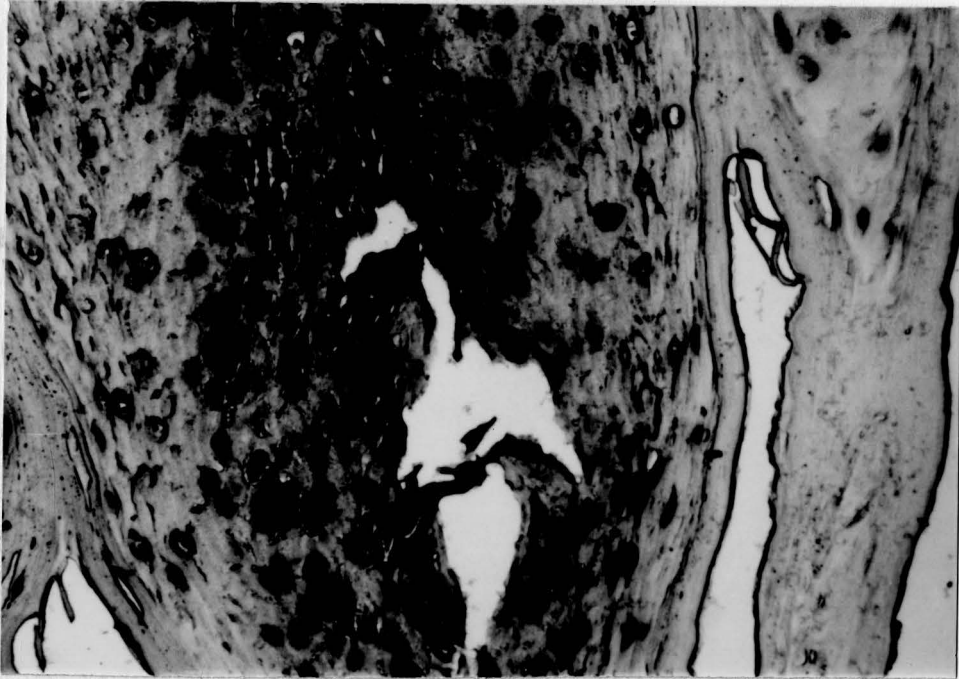
FIGURE 9: HPV Type 31 Positive Control
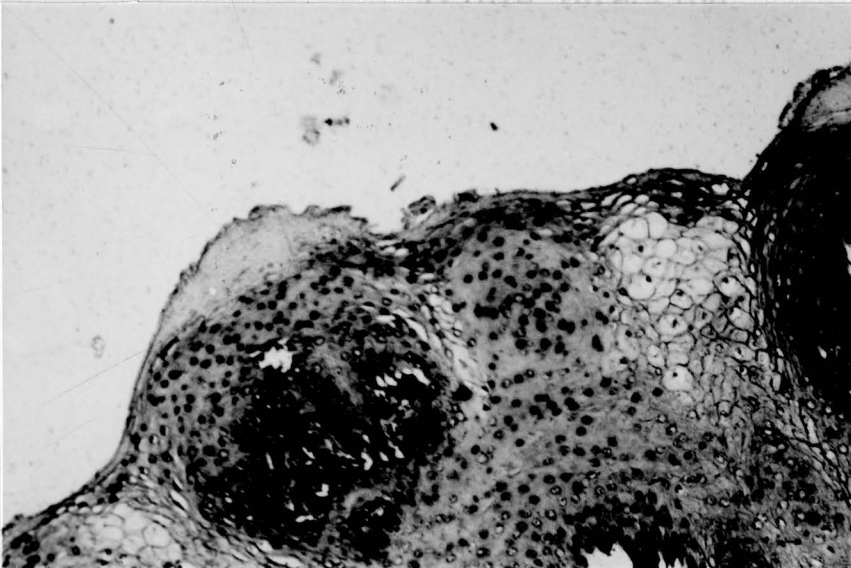
FIGURE 10: HPV Type 31 Positive Papilloma

APPENDIX A

HPV INFECTED PAPILLOMAS

Single Infection

HPV TYPE	NO. PAPILLOMA
6	7
11	2
16	0
18	2
31	10
33	3

Dual Infection

HPV TYPE	NO. PAPILLOMA
6, 11	2
6, 31	5
6, 33	1
11, 31	2
18, 33	1

Triple Infection

HPV TYPE	NO. PAPILLOMA
11,31,33	1

Inconclusive Infection

HPV TYPE	NO. PAPILLOMA
NC	3

APPENDIX B

TYPE SPECIFIC PROBE REACTIVITY

HPV Type	Weak	Moderate	Strong
6	8	5	15
11	1	2	6
16	4	2	0
18	6	1	2
31	2	5	17
33	7	3	5

APPENDIX C

HPV DNA POSITIVE PAPILLOMAS

HPV Type	Percentage	Cases
31	25%	10/39
6	17%	7/39
6, 31	12%	5/39
33	7%	3/39
NC	7%	3/39
11	5%	2/39
6, 11	5%	2/39
11, 31	5%	2/39
18	5%	2/39
6, 33	2%	1/39
18, 33	2%	1/39
11,31,33	2%	1/39

APPENDIX D

HISTOMORPHOLOGY OF HPV DNA POSITIVE PAPILLOMAS

Feature	HPV DNA Positive	Cases
Acanthosis	89%	35/39
Koilocytosis	87%	34/39
Basal Cell Replication	87%	34/39
Parakeratin	41%	16/39
Hyperparakeratin	20%	8/39
Ortho/parakeratin	20%	8/39
Basal Cell Mitosis	12%	5/39
Hyperorthokeratin	7%	3/39
Hyperpara/parakeratin	5%	2/39
Orthokeratin	5%	2/39
Dyskeratosis (benign)	5%	2/39
Suprabasal Cell Mitosis	5%	2/39
Basal Cell Nuclear Hyperchromasia	2%	1/39
Basal Cell Prominent Nucleoli	2%	1/39
Multiple Nucleoli Supra/Basal Cell	2%	1/39

APPENDIX E

HISTOMORPHOLOGY OF HPV DNA NEGATIVE PAPILLOMAS

Feature	HPV DNA Negative	Cases
Acanthosis	87%	21/24
Basal Cell Replication	66%	16/24
Koilocytosis	58%	14/24
Hyperparakeratin	37%	9/24
Parakeratin	29%	7/24
Orthokeratin	16%	4/24
Suprabasal Cell Replication	12%	3/24
Basal Cell Mitosis	8%	2/24
Hyperortho/parakeratin	8%	2/24
Hyperorthokeratin	4%	1/24
Hyperortho/orthokeratin	4%	1/24
Basal Cell Prominent Nucleoli	4%	1/24

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128. DAKO Specifications. HPV type 33 biotinylated DNA probe (prediluted for in situ hybridization). <u>Code No: Y1407</u>.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master in Oral Biology.

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