Typing of Human Papillomavirus in Western Cape Women with Cervical Intraepithelial Neoplasia.

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I declare that this thesis is my own work.

It is being submitted for the degree of

Master Technologiae (Biomedical Technology)

to the Cape Technikon, Cape Town.

It has not been submitted before for any degree or examination

at any other Technikon or tertiary institution.

The work was undertaken at the Faculty of Applied Sciences, Cape Technikon

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University of Cape Town.

The opinions and conclusions drawn are not necessarily

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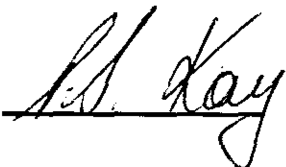

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Dedicated

to the women of Africa.

Nkosi sikelele i'Afrika

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Abbreviations

AIDS	acquired immune deficiency syndrome
β-gal	beta D-galactosidase
bp	base pairs
BPV	bovine papillomavirus
°C	degrees celsius
$CaCl_2$	calcium chloride
CANSA	Cancer Association of South Africa
CIN	cervical intraepethelial neoplasia
CRPV	cottontail papillomavirus
DNA	deoxyribonucleic acid
DNTPs	deoxynucleoside triphosphates
EDTA	ethylenediamine tetraacetic acid
EV	*Epidermodysplasia verruciformis*
G	gravitational accelaration
Hepes	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
IBSCC	International Biological Study on Cervical Cancer
kb	kilobase
LCR	long control region
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitres
mM	millimolar
NIH	National Institute of Health
ng	nanograms
O.D.	optical density
ORF	open reading frame
ORI	origin of replication
PAP	Papanicolaou
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmole	picomoles
PV	papillomavirus
Rb	retinoblastoma
TBE	Tris borate with EDTA
T_m	melting temperature
U	units of activity
μl	microlitre
μg	microgram

μM	micromolar
USA	United States of America
UV	ultraviolet
V/cm	volts per centimetre – potential difference
v/v	volume per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase
YT	yeast tryptone

Summary

Infection with specific high risk human papillomaviruses (HPV) has been shown to play a causal role in the development of cervical intraepithelial neoplasia (CIN) and cervical cancer in women. The development of a prophylactic vaccine to immunize women against HPV infection would play a vital role in protecting women against HPV infection and ultimately cervical cancer. Despite cancer of the cervix being the second most common cancer in South African women, a literature search reveals that few studies have been performed in South Africa on the types of HPV prevalent in women with CIN or cancer of the cervix.

HPVs that infect the anogenital tract have also been shown to infect the oral cavity. However, the HPV prevalence rates vary greatly between studies and the significance of the presence of HPV in the oral cavity is still not understood.

The primary objectives of this study were to establish the HPV prevalence rate infecting women with CIN lesions using a sensitive nested polymerase chain reaction (PCR) and to develop a novel restriction fragment length polymorphism (RFLP) method to type the high risk mucosal HPVs detected in these women. The secondary objective of this study was to establish the prevalence rate and HPV types infecting the oral mucosa of women with CIN lesions and to compare these HPV types with those detected in the cervix.

Cervical punch biopsies were taken from 163 women with CIN lesions and buccal cells were collected from 33 of these participants. DNA was extracted from the biopsies and buccal samples

and PCR using CCR5 primers performed to ensure sample adequacy. Nested PCR using consensus degenerate primers for HPV was performed on all samples showing sufficient amplifiable DNA. A novel restriction fragment length polymorphism (RFLP) method was developed to identify the 10 high risk mucosal HPVs considered human carcinogens of group 1 by the International Agency for Research on Cancer (IARC) as well as HPV 11 which is commonly found in the oral cavity. Samples suspected of containing more than 1 HPV type were cloned and nested PCR and RFLP repeated on the clones to provide proof of dual infections.

To establish the integrity of the novel RFLP, nested PCR and RFLP was performed on a sub group of biopsies and the results compared to those obtained by a published method. PCR using biotinylated PGMY09/PGMY11 primers was performed on the sub group of biopsies and HPV typing done by means of reverse line blot assay.

The nested PCR proved to be a sensitive means of detecting a wide range of mucosal HPV types. The RFLP was shown to accurately identify HPV types 16, 18, 31, 33, 35, 45, 51, 52, 58 and 59 as well as HPV 11. Results were confirmed by performing RFLP on clones of known HPV types. The RFLP was also able to detect multiple HPV infections and variants as indicated by the detection of a variant of HPV 58 and the newly described HPV HAN 2294.

The HPV prevalence rate of the biopsies was 83% and twelve different HPV types were detected using RFLP. The four most common types were HPV 16, 33, 31 and 58. The buccal samples had

an HPV prevalence rate of 74%. Five different HPV types (HPV types 11, 13, 16 and 18) were detected in the buccal samples. There was no correlation between the HPV types detected in the cervix and the oral cavity.

When the HPV typing results obtained by the RFLP were compared to those obtained by the reverse line blot assay, it was shown that HPV types 16, 18, 31, 33 and 52 were the most common HPV types identified by the RFLP whereas HPV types 16, 18, 33, 45 and 52 were the most common HPV types identified with the reverse line blot assay. The RFLP therefore compared well to the reverse line blot assay. The lack of HPV 45 from the RFLP results proved to be a function of the PCR, which was shown not to amplify HPV 45 in clinical samples despite being able to amplify cloned HPV 45.

In conclusion, the nested PCR proved to be a sensitive means of amplifying HPV DNA from clinical samples and the RFLP was shown to accurately identify the 10 high risk HPV types declared as human carcinogens of group 1 by the IARC. HPV type 16 would need to be included in a prophylactic vaccine for South African women, however, further typing studies need to be performed to establish which HPVs are the next most common types in South African women with cervical disease.

Parts of this thesis have been published in peer reviewed publications:

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20th International Papillomavirus Congress held in Paris in 2002:

Poster presentation titled: High prevalence of HPV 16 in South African women with cancer of the cervix and cervical intraepithelial neoplasia.

Kay P and Williamson A-L

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Chapter 1

Introduction and Literature Review

1.1 Disease burden of cervical cancer

Internationally, cancer of the cervix is the second most common cancer in women (NIH Consensus Statement, 1996) with up to 50 % of patients dying from the disease (Fisher, 1994). In the United States, stage specific survival of cervical cancer patients has not improved since the 1960s (Schwartz *et al.*, 2001). Seventy to 85% of stage I and stage IIa cervical cancer patients are cured. In contrast, patients with more advanced non-metastatic cervical carcinomas only have a 40%-60% 5 year survival rate (NIH Consensus Statement, 1996).

The disease burden is even heavier in developing countries for reasons that have not yet been entirely elucidated. Inadequate or non-existent cervical screening programs within these countries along with poor nutrition and lack of access to health care facilities could be a contributing factor (Villa *et al.*, 2000). Statistics published by the Cancer Association of South Africa (CANSA, 2002) indicate that cancer of the cervix is the most common cancer in black South African women, accounting for 31,2% of all cancers within this group. Although South African women have a 1 in 41 lifetime risk of developing cancer of the cervix, important population differences exist, with black women having a 1 in 34 lifetime risk and white women having a 1 in 93 lifetime risk. This high rate of cervical cancer in black women is similar to that of other developing countries. However, the exact incidence of invasive cancer and its precursor lesions is unknown in South Africa as to date there is

no national cervical screening program (Michelow *et al.*, 1999). The reported cases are therefore likely to be an underestimation of the true figures.

1.2 Association of papillomaviruses and disease

In 1842 the Italian physician, Rigioni-Stern, noted that cervical cancer was common in prostitutes but rare in nuns. He therefore hypothesized that sexual activity was a risk factor for the development of cervical cancer. Just over century later an epidemiological study (Rotkin, 1967) identified early age of sexual activity and promiscuity as relevant factors for the development of cervical cancer which suggested the involvement of an infectious agent in the etiology of cervical cancer, but the nature of the etiological agent remained unknown at that time.

The first papillomavirus (PV) was described in 1933 (Shope and Hurst, 1933) with the recognition of the cottontail rabbit papillomavirus (CRPV) as the etiological agent responsible for cutaneous papillomas in cottontail rabbits. The first attempts to relate papilloma infections to cancer development were made by Rous and Beard (1935), who demonstrated the carcinogenic potential of CRPV in domestic rabbits and the syncarcinogenic activity of tar when jointly applied with the virus infection. The carcinogenic activity of CRPV was confirmed by Ito and Evans (1961) when they induced carcinomas in domestic rabbits with DNA extracted from CRPV papillomas and carcinomas. The induction of bladder tumours in cattle by bovine papillomavirus (BPV) was shown by Olsen *et al.* (1959), indicating that other members of the papillomavirus group could induce malignant tumours.

During the 1970s papillomavirus research was stimulated by the identification of novel human papillomaviruses (HPVs) detected in malignant lesions of patients suffering from the rare syndrome *Epidermodysplasia verruciformis* (Orth *et al.*, 1977), characterized by the development of skin carcinoma at sun exposed sites. The first study investigating the relationship between infection with HPV and cancer of the cervix was published in 1974 (zur Hausen *et al.,* 1974) and by the mid 1980s specific HPV types had been isolated from biopsies of cancer of the cervix (Durst *et al.,* 1983; Boshart *et al.,* 1984).

To establish the prevalence rate of HPV in cervical cancer, an international study was conducted by the International Biological Study on Cervical Cancer (IBSCC) (Bosch *et al.*, 1995). Biopsies from patients with invasive cervical cancer were collected at 32 hospitals from 22 countries in Europe, the Americas, Africa and South East Asia. The reported HPV prevalence rate was 93%. This study used the polymerase chain reaction (PCR), a highly sensitive technique for the detection of HPV. However the consensus PCR primers used in this study targeted the L1 gene which can be disrupted during integration events. Walboomers *et al.* (1999) re-tested the samples that were HPV negative, using PCR primer sets targeting the E7 gene which is retained during integration events and two different sets of consensus primers targeting the L1 gene. By combining the data from this study and the previous study the HPV prevalence rose to 99.7% indicating that infection with HPV is a necessary cause of invasive cervical cancer worldwide. This study confirmed the consensus statement issued by the National Institute of Health in 1996 that carcinoma of the cervix is causally related to infection with HPV.

In addition to the reports of HPV prevalence in cancer of the cervix, there have been reports of recurrent tumours in women who have been treated for cervical cancer (Park *et*

al., 1996; Nagai *et al.*, 2001). These tumours have occurred at sites of histologically benign lymph nodes. It has been suggested that the presence of HPV in these nodes could be of importance for the development of metastatic tumours (Park *et al.*, 1996). However other studies refute this (Czegledy *et al.*,1998). The pathogenesis of HPV is based on the paradigm that HPV infection is a result of the virus entering the basal layers of the epithelium through microabrasions and that viral replication can only take place in terminally differentiating epithelial cells. Therefore the clinical significance of the presence of HPV in tissue other than epithelium is not clear.

Infection with HPV has also been implicated as a risk factor for the development of oropharyngeal cancers (Snijders *et al.*, 1996; Schwartz *et al.*, 1998) but the HPV prevalence rates vary greatly between studies even when PCR methods are used. Shroyer and Greer (1991) reported a 10% prevalence of HPV in malignant and premalignant lesions of the oral cavity while Watts (1991) reported a 100% HPV prevalence in carcinomas of the oral cavity.

The presence of HPV has also been shown in adjacent non-neoplastic tissue of oral mucosa (Ostwald *et al.*, 1994; Snijders *et al.*, 1996) with a decrease in HPV PCR positivity with increasing distance from the site of the tumour. This suggests a focal point of HPV infection at the site of the tumour. However, HPV has also been detected in the oral mucosa of patients without tumours or papillomas. The HPV prevalence rates in these studies varies from 1% to 81.1% (Kelloski *et al.*, 1992; Ostwald *et al.*, 1994; Badaracco *et al.*, 1998 and Terai *et al.*, 1999) even though PCR based assays were used to detect HPV DNA in all the studies. Oral samples for HPV testing are collected by biopsy, scrape or mouthwash and as the copy numbers of HPV in mucosal tissue is reportedly low, even in oral neoplasias (Lawton *et al.*, 1992; Sugarman and Shillitoe, 1997), the method of sample

collection could have played a role in the differences of HPV prevalence in these studies. In addition, the sensitivity of the PCR assays employed in the different studies could have contributed to the discrepant results.

Despite the differences in prevalence rates between the above studies, there was consensus between the HPV types detected, with all studies reporting the presence of anogenital HPVs in both normal and neoplastic oral mucosa. HPV 6, 11, 16, 18, 31, 33 were the most common HPV types detected in the above studies. Other anogenital HPVs detected in the above studies were HPV 44, 56, 57, 59 and 61 as well as HPV types not identified by the methods employed in the studies.

1.3 Biology of the HPVs

1.3.1 Life cycle of the HPVs

Papillomaviruses belong to the *Papillomavirinae*, a subfamily of the *Papovaviridae* (Pfister and Fuchs, 1994). The PVs infect a variety of different vertebrates including humans, horses, dogs, sheep, elk, deer, non human primates and some avian species. The PVs are highly species specific and do not cross species barriers in nature, except in the case of bovine PVs which have been shown to cause sarcoid lesions in horses (Shah and Howley, 1996). However these sarcoid lesions do not produce infectious virions.

Infection with PV results in either benign or malignant cellular proliferation with individual PV types having a tropism for specific epithelial tissues (Shah and Howley, 1996). Benign lesions of the host cell are characterised by the viral DNA remaining in an episomal form whereas in malignancy the viral genome is integrated (Chow and Broker, 1994) although not all HPV positive malignant lesions are characterised by the integration of the viral

genome. Malignant tumours can contain purely integrated HPV DNA, episomal DNA or a combination of both (Badaracco *et al.*, 2002)

The life cycle of HPV is closely linked to the differentiation of epithelial cells. In squamous epithelium, the basal cells are the only cells capable of dividing. Therefore, to induce a lesion that can persist, the virus needs to be introduced into the basal layers (Howley, 1996). This takes place through microabrasions of the epithelium (Ponten and Guo, 1998) resulting in either a productive or non productive infection.

In a productive infection the transcription and translation of the viral early genes takes place in the basal layers of the epithelium resulting in steady state viral DNA replication. As the basal cell progresses up through the epithelial layers the late genes are expressed leading to the synthesis of capsid proteins, vegetative viral synthesis, assembly of virions and shedding of virus from the surface of the epithelium. This takes place only in the terminally differentiated squamous epithelial cells (Howley, 1996).

A nonproductive infection takes place in cells that fail to differentiate. Only the early genes are expressed (Howley, 1996) and viral replication does not take place. However transformation of the host cell can occur whereby the viral genome integrates into the host genome resulting in a change of the cellular phenotype.

1.3.2 Virion structure

The PVs are small, icosahedral DNA viruses measuring about 52-55 nm in diameter. The virus is non-enveloped and encapsidated by 72 pentameric capsomeres (Pfister and Fuchs,

1994) (Figure 1). The capsid consists of two structural proteins with the major capsid protein encoded by the L1 gene and the minor capsid protein encoded by the L2 gene.

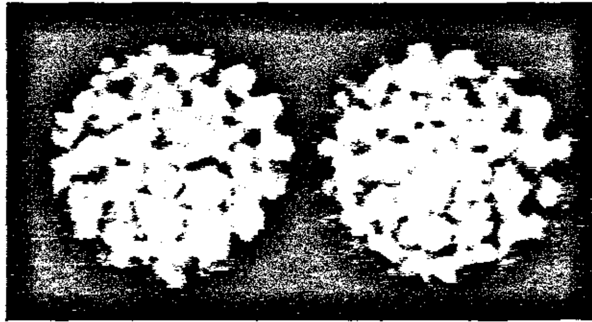

Figure 1. Electron micrograph image of the icosahedral papillomavirus showing the 72 pentameric capsomeres. Reproduced with permission from Dr. L. Stannard

1.3.3 Genomic organisation

All PVs have a similar genomic organisation (Figure 2). The genome consists of a single molecule of circular double stranded DNA of approximately 8,000 base pairs (bp) in size (Howley, 1996).

The coding strand for all of the PVs contains 10 open reading frames (ORFs). There is approximately 1kb of coding strand that contains no ORFs and this is referred to as the long control region (LCR). All of the ORFs are located on one strand ie only one strand is transcribed and transcription occurs in one direction only.

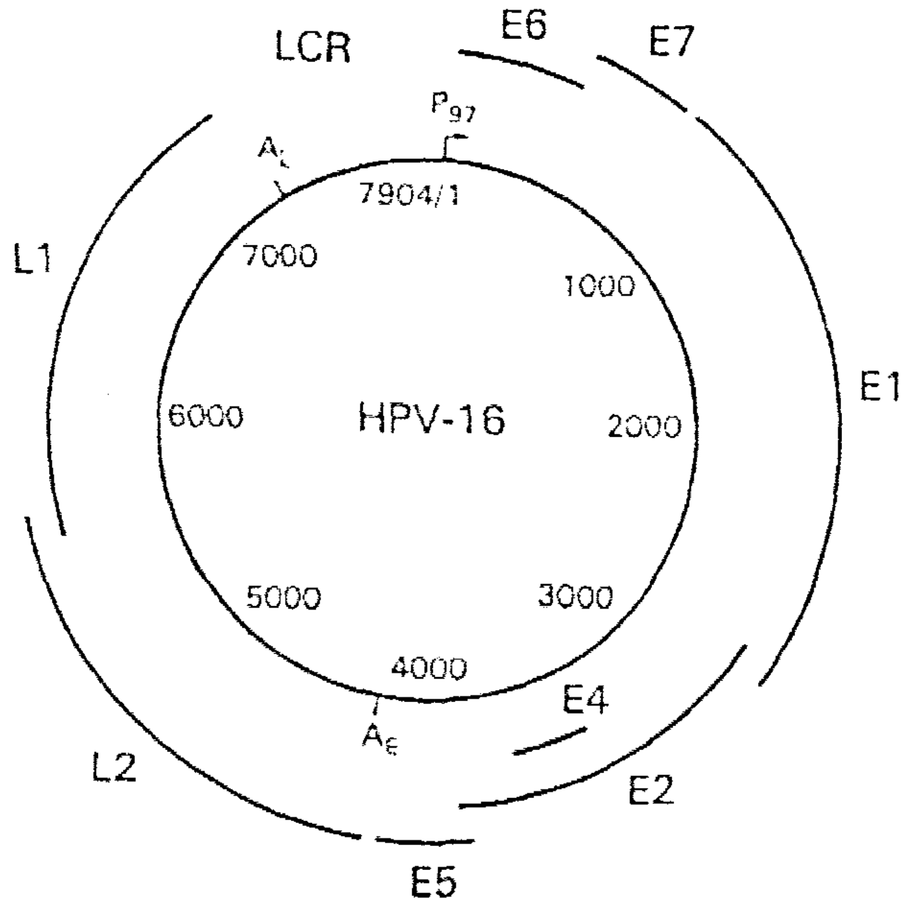

Figure 2. Genomic map of HPV 16. The genome is a double-stranded circular DNA molecule of 7904 bp. The transcriptional promoter is designated P97. The ORFs are designated E1 to E7. L1 and L2 are indicated outside of the circular genome. A_E and A_L represent the early and late polyadenylation sites. The long control region contains transcriptional and regulatory elements. (Fields Virology 3rd. edition).

1.3.4 Functions of the viral proteins

L1 and L2 proteins

The L1 gene codes for the major capsid protein which contains reactive epitopes for type-specific neutralization. The L1 gene is the most conserved of all the HPV genes. The L2 gene codes for the minor capsid protein and is responsible for group-specific reactivity of antisera (zur Hausen, 1996).

Long control region

The LCR makes up about 10% of the HPV genome and varies substantially in nucleotide composition between HPV types. The regulation of viral gene expression is controlled by both cellular and viral transcription factors with most of these regulations occurring within the LCR (zur Hausen, 1996). Cellular transcription factors bind to the *enhancer*-region of the LCR thereby regulating the transcription of the E6 and E7 promoter which is located at the 3'-terminus of the LCR (Bauknecht *et al.*, 1992). The LCR also contains binding sites for the E2 protein. One of these E2 binding sites plays a role in replication initiation but also modulates E6 and E7 transcription (Thierry *et al.*, 1992). Two additional E2 binding sites modulates the promoter of E6 and E7 by displacing the transcription complex (Tan *et al.*, 1994).

E1 and E2 proteins

E1 is highly conserved among HPV types and is the largest ORF in the PV genome. Both E1 and E2 contain site specific DNA binding motifs. E1 is essential for PV replication (Ustav and Stenland, 1991) and has ATPase and DNA helicase activity. E2 proteins interact with E1 to stimulate viral DNA replication by facilitating the binding of E1 to the origin of replication (ORI) (Seo *et al.*, 1993). In addition to its role in viral replication E2

acts as a transcriptional activator through E2 responsive elements within the LCR (Spalholz, *et al.*, 1987).

E6 and E7 proteins

Expression of the E6 and E7 oncogenes is required for the maintenance of the malignant phenotype and it has been shown that human keratinocytes transfected with DNA from high risk HPV types become immortal and carry the viral genome in an integrated state (Durst *et al.*, 1987). The E6 protein complexes with the cellular p53 targeting its degradation through ubiquitin-dependant proteolysis (Scheffner *et al.*, 1990). E7 binds to the product of the retinoblastoma (Rb) tumour suppressor gene (Munger *et al.*, 1992) which influences the expression of genes involved in cell cycle progression leading to uncontrolled cell growth. E2 plays an important role in activating or suppressing the expression of E6 and E7 genes by the binding of E2 products to specific sites within the LCR (Cripe *et al.*, 1987). The functions of the proteins encoded by the HPV genes are summarised in Table I.

Table I. Functions assigned to the open reading frames of mucosal human papillomavirus

ORF	Function
E1	Initiation of viral DNA replication
E2	Transcriptional regulatory protein
E4	Late protein, interacts with cell cytoskeleton
E5	Membrane transforming protein
E6	Transforming protein; targets p53
E7	Transforming protein; binds Rb
L1	Major capsid protein
L2	Minor capsid protein

1.4 Classification of the HPVs

In the absence of serological assays the PVs are typed on their genomic homology and are not referred to as serotypes but as PV types (Howley, 1996). The availablility of large data bases of PV genome sequences has greatly aided the typing of PVs. Phylogenetetic analysis of PVs is possible due to their genomic conservativeness ie. they all have similar genome sizes, organisation ORFs and protein functions. This genomic conservativeness also provides evidence for their monophyletic origin (Chan *et al.*, 1992).

Initially the PVs were typed on the basis of DNA hybridisation. Any PV showing less than 50% homology to any previous PV by liquid hybridisation experiments under stringent conditions, was deemed a new type (Coggin and zur Hausen, 1979). However due to the scattered distribution of short sequence homologies, PVs with greater than 50% sequence homology could show little or no relatedness by hybridisation. As the L1 gene has shown to be the most conserved of all the HPV ORFs it was decided at the 1995 Annual Papillomavirus Conference to define a new HPV type by means of sequence homology of the L1 gene. HPV types are defined as having more than 10% sequence difference in the L1 ORF as compared to established prototypes. A subtype has more than 2% variance but less than 10% difference and a variant has less than 2% difference in the L1 ORF.

Chan *et al.* (1995) adopted a cladistic approach for the taxonomic classification of the PVs. A cladisitc approach uses evolutionary relationships derived from shared characteristics and establishes taxonomic groups that are monophyletic (clades). By using this approach the PVs are grouped into supergroups A, B, C, D and E (Figure 3).

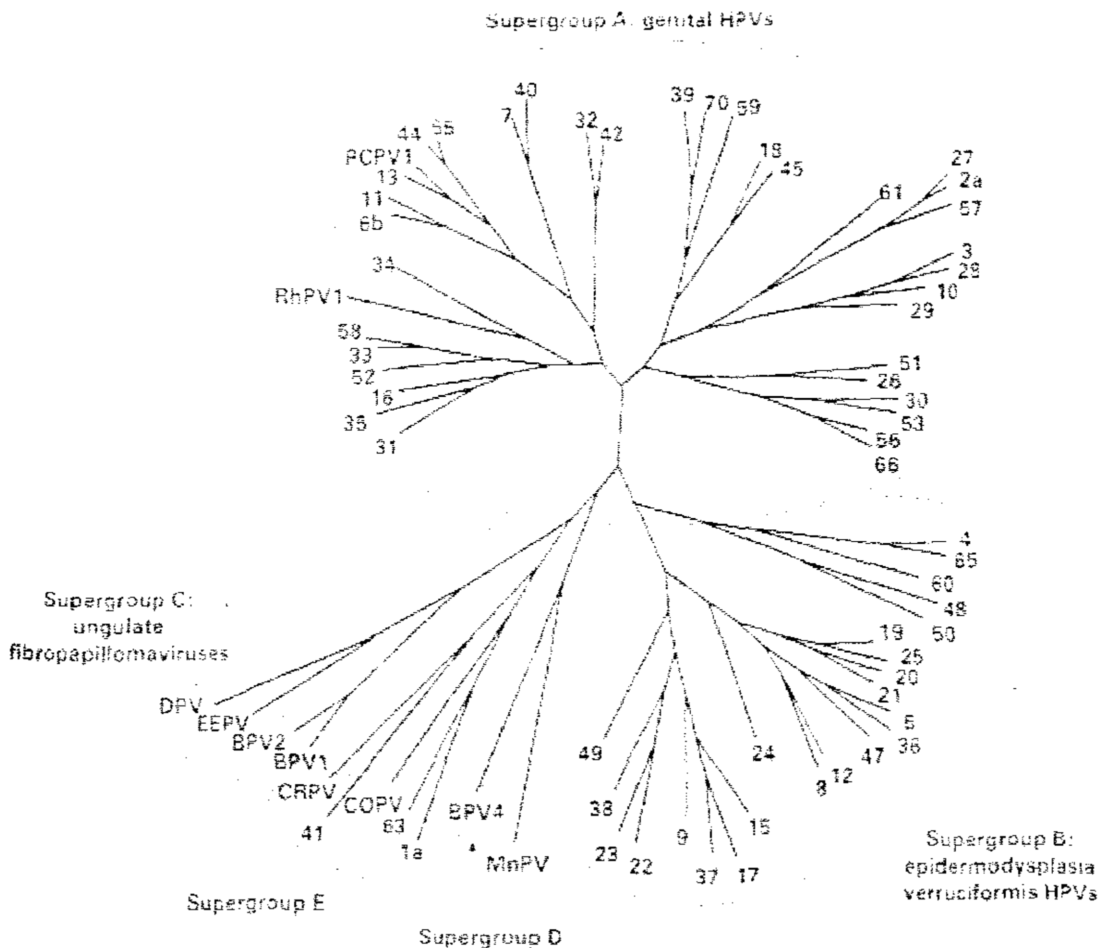

Figure 3. A phylogenetic tree of 92 papillomavirus types based on a maximum-likelihood evaluation of a 291 bp L1 segment. (Taken from Chan *et al.*, Journal of Virology. 1995. 66[10]).

Found within these supergroups are defined groups based on their bootstrap scores, monophyly and biological similarity and these groups are in turn made up of different HPV types.

All HPV types fall into Supergroups A, B or E. Supergroup E unites distantly related HPV types 1, 41 and 63. Supergroup B comprises of group B1, consisting mainly of HPVs associated with *epidermodysplasia verruciformis* (EV) lesions, and group B2 consisting of HPVs associated with non-EV lesions, common warts, flat warts and cutaneous squamous cell carcinomas. Supergroup A is the largest and most significant as it comprises those

HPVs associated with mucosal or genital neoplasias. There are 11 groups within supergroup A. Groups A6, A7 and A9 comprise of the most commonly detected high risk genital HPV types while group A10 contains the most commonly detected low risk genital HPV types (Table II).

Table II. List of HPV types in supergroup A. Cladistic Relationship as determined by the phylogenies based on the L1 segment using maximum likelihood evaluation and weighted parsimony evaluation. (Adapted from Chan *et al.*, 1995)

Supergroup A	
Group	**HPV types**
A1	32, 42
A2	3, 10, 28, 29
A3	61, 62, 72, CP6108, CP8304, MM8
A4	2, 27, 57
A5	26, 51, 69, IS039, MM4
A6	30, 53, 56, 66
A7	18, 39, 45, 59, 68, 70
A8	7, 40, 43
A9	16, 31, 33, 35, 52, 58, 67, RhPV
A10	6, 11, 13, 44, 55, PCPV
A11	34, 64, 73

1.5 The role of high risk HPVs in cervical disease.

To date over 100 different HPV types have been identified in *homo sapiens* of which about 40 have been shown to infect the mucosal epithelium. Depending on their association with cervical cancer, the mucosal HPVs have been categorized as either high risk or low risk (Table III).

Table III. List of high risk and low risk mucosal HPV types categorized according to their association with cervical disease. * HPV 54 and HPV 66 have been classified as both high risk and low risk.

Category	HPV Types
Low Risk	**6, 11, 34, 40, 42, 43, 44, 53, 54*, 57, 66*, MM8**
High Risk	**16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 54*, 55, 56, 58, 59, 66*, 68, 69, MM4, MM7, MM9**

The list of low risk and high risk mucosal HPV depicted in Table III changes as causal relationships are established between the HPV types and cervical cancer. For example, HPV 68 was previously designated as low risk but is now considered a high risk type. Other discrepancies exist in the classification of high risk and low risk HPV types. Some researchers classify HPV types 54 and 66 as low risk (Gravitt *et al.*, 1998) whereas others classify them as high risk (Lorincz *et al.*, 1992; Tawheed *et al.*, 1991; zur Hausen, 1996). The low risk mucosal HPV 6 and HPV 11 are associated with benign proliferations such as those seen in condyloma acuminata whereas high risk mucosal HPV types are associated with cervical disease which manifest as flat warts on the cervix and are visible as flat white lesions when 3%-5% acetic acid is applied to the cervix.

Cervical abnormalities are graded according to the Bethesda system (Lundberg, 1989) (Table IV) as the Papanicolaou system is unacceptable for diagnostic cytopathology in modern practice in that it does not communicate clinically relevant information in a reliable manner.

Both high risk and low risk HPV types are associated with low grade cervical lesions (Feoli-Fonseca *et al.*, 2001). Persistent infection with HPV has been shown to be a risk factor for the development of cervical neoplasia and it has been shown that infection with

Table IV. The Bethesda system for grading cytological abnormalities of cervical squamous epithelium. (Adapted from Lundberg, 1989)

Terminology	Cytological Abnormality
ASCUS	Atypical cells of unknown significance
LSIL*	Cellular changes associated with HPV infection
	Mild/slight dysplasia/cervical intraepithelial neoplasia grade 1 (CIN1)
HSIL*	Moderate dysplasia/cervical intraepithelial neoplasia grade 2 (CIN2)
	Sever dysplasia/cervical intraepithelial neoplasia grade 3 (CIN3)
	Carcinoma in situ/CIN3
Squamous cell carcinoma	Invasive carcinoma

*LSIL Low grade squamous intraepithelial lesion
*HSIL High grade squamous intraepithelial lesion

high risk HPV types is more likely to persist (Hildesheim *et al.*, 1994; Franco *et al.*, 1999), whereas infections with low risk types are more readily resolved. This is reflected in the distribution of HPV types through increasing grades of cervical neoplasia with high risk HPV types more significantly associated with high grade lesions than low risk HPV types (Lombard *et al.*, 1998; Remmink *et al.*, 2000; Feoli-Fonesca *et al.*, 2001). However the mechanisms of persistence by high risk types are still unknown.

During 1995 the International Agency for Research on Cancer (IARC) conducted a series of multi-centre case-control studies on cervical cancer in various populations (IARC, 1995). Based on the results of these studies HPV 16 and HPV 18 were classified as human carcinogens of group one. Further analysis on this cohort by the IARC (Munoz, 2000) assessing the risk for the development of carcinoma associated with various HPV types, showed that HPV types 31, 33, 35, 45, 51, 52, 58 and 59, can now also be considered human carcinogens of group one.

1.6 Molecular mechanisms differentiating high risk HPVs from low risk HPVs

HPV DNA is found episomally in low grade CIN lesions, whereas cervical cancer biopsies may contain integrated and episomal HPV DNA (Howley, 1996). Low risk HPV types are more likely to remain in an episomal form than high risk HPV types. High risk HPV 18 is found in an integrated form in stable cervical cell lines such as HeLa cells (Howley, 1996) and is able to immortalize human keratinocytes whereas low risk types have failed to do so (Pirisi *et al.*, 1987; Durst *et al.*, 1991). The mechanisms of the integration events by high risk HPV types are not yet characterised.

During the integration of high risk HPV types the E2 gene is disrupted. The E2 gene products regulate the production of E6 and E7 and as the synthesis of proteins encoded by E2 is halted, E6 and E7 are over-expressed (Stoler *et al.*, 1992). The E6 and E7 ORF's are consistently retained and expressed in the transformed cells (Cone *et al.*, 1992). Unlike the high risk HPV types, the E6 and E7 genes of the low risk HPV types are expressed from two independent promoters which permit E2 to regulate E6 and E7 separately (Ponten and Guo, 1998).

In the host cell, tumour suppressor gene products halt cell growth. The E6 and E7 genes bind to p53 and pRb respectively resulting in uncontrolled cell growth. The E6 and E7 oncogenes of high risk HPV types bind strongly to p53 and pRB, whereas the binding affinity of low risk HPV's E7 for pRB is about 10 fold lower than that of the high risk HPVs (zur Hausen, 1996).

It should be noted however, that the vast majority of high risk HPV infections do not result in malignant progression (IARC, 1995) indicating that other co-factors are most likely involved in malignant progression.

1.7 The evolution of HPV types

The PVs are ancient viruses and it is thought that they co-evolved over the millenia with their respective hosts (Chan *et al.*, 1992). The HPVs are thought to have emerged with *homo sapiens*, or its immediate ancestors, from Africa some 200 000 to 1 million years ago (Cavelli-Sforza *et al.*, 1993).

Individual HPV types are stable species and there is no evidence that new types arise by recombination of existing types (Ho *et al.*, 1993). The HPVs are slowly evolving viruses and based on a study on HPV 18 by Ong *et al.* (1993) it is postulated that a point mutation within a HPV genome takes 12 000 years to become fixed within a population. Ancient populations migrated from East Asia across Beringia into Alaska approximately 12 000 years ago. Ong *et al.* (1993) showed that an HPV 18 isolate from native American Indians differed by only 1 point mutation in the hypervariable LCR, from that of East Asian HPV 18 isolates. Given that the American Indians were infected at the beginning of this migration this postulate could hold true. The stepwise accumulation of these point mutations would give rise to the different HPV types.

Genomic variants that are intermediates exist between HPV 18 and HPV 45 (Ong *et al.* 1993). However the same does not hold true for HPV 16 and its two closest relatives HPV 31 and HPV 35. Therefore based on the theory of viral and host co-evolution, HPV 18 and

HPV 45 would have evolved more recently from a common ancestor than HPV 16 with HPV 31 and HPV 35.

However, the taxonomic groupings and the phylogenetic studies of the PVs have still not clarified how the different PVs evolved. The range of host specific PVs and epithelium specific PVs supports a role for selection. But this does not explain why different PV types exist within an ecological niche eg. in humans approximately 40 HPV types have been found to infect mucosal epithelium of the genital tract.

Another anomaly in the typing of HPVs is the apparent lack of sub types. Only 5 subtypes have been reported (Stewart *et al.*, 1996) whereas many variants (<2% difference within the L1 gene) of HPV types have been described. If the HPV types arose from an accumulation of point mutations one would expect to observe some evolutionary evidence linking the different types. According to Chan *et al.* (1992) this could be due to the current operational definition of HPV types. However this still does not adequately explain the lack of HPV sub types showing a 3-10% variance from each other within the L1 gene.

The HPVs have been well studied and to date 110 HPV DNA genomes have been fully sequenced. Even though novel genital HPVs are still being identified (Kino *et al.*, 2000) there appears to be a slowing down of new genital HPVs detected (Chan *et al.*, 1995). This would indicate that the major HPV types associated with cervical disease have been identified. However, novel HPV types are still being detected in immunocompromised patients such as transplant patients (Longuet *et al.*, 1996), HIV positive patients (Volter *et al.*, 1996; Terai and Burk, 2001) and sex workers (Chow and Leong, 1999)

1.8 Transmission of HPV

Cervical HPV infections are thought to be the most common sexually transmitted disease in adults (Richardson *et al.*, 2000) with young sexually active women at highest risk of acquiring an HPV infection. A study by Kjaer *et al.* (2001) on 100 virgins who were all HPV DNA negative on enrollment, showed that only those women who initiated sexual activity became HPV DNA positive. Monogamous Thai women whose male partners had unprotected sex with commercial sex workers were shown to be at higher risk for the development of cervical cancer (Thomas *et al.*, 2001). The commercial sex workers had a higher prevalence of oncogenic HPV types and these viruses were being transmitted from the sex workers by the male partners to their monogamous partners.

One of the predominant risk factors for acquiring genital HPV infection in young women is the number of lifetime and recent sexual partners (Bauer *et al.*, 1993; Wheeler *et al.*, 1993; Burk *et al.*, 1996; Peyton *et al.*, 2001). Burk and co-workers (1996) investigated young female college students in which 76% of the participants were under the age of 23 and it was shown that the predominant risk factor for the acquisition of genital HPV was the number of different sexual partners in the preceding 6 months. It was also shown that the number of lifetime sex partners of the participants' male partners was a risk factor for the acquisition of HPV infection. By using multivariate analysis in which high risk HPV types were considered separately to low risk HPV types, several studies have shown that this does not hold true for low risk HPV types (Franco *et al.*, 1995; Silins *et al.*, 2000 and Richardson *et al.*, 2000). Richardson (2000) showed that lifetime number of sexual partners and oral sex was only associated with infection with high risk HPV types and that low risk HPV types were invariant with respect to sexual activity. This would suggest a difference in the transmission of low risk HPVs as compared to high risk HPVs.

HPV DNA has been isolated from semen (Chan *et al.*, 1994; Lai *et al.*, 1997 and Olatunbosun *et al.*, 2001) although the role of the male reproductive tract as a reservoir for HPV has not been widely studied. In all three studies, sperm cells were separated by Percoll gradient centrifugation and washed with sterile phosphate buffered saline to ensure that the HPV detected, was localized to the sperm cells. However, the mechanism by which the sperm cells take up exogenous HPV DNA is not known. A further study by Rintala *et al.* (2002) on vasectomised men indicated that HPV was harboured in the vas deferens of men with no clinical symptoms of HPV infection. Despite the reports of HPV infecting the male reproductive tract, it has been shown that the concordance of different HPV types between sexual partners is low (Hipplainen *et al.*, 1996).

Although it is accepted that high risk HPVs are transmitted sexually there is evidence that HPV can be transmitted by non-sexual routes. Studies have also shown that vertical transmission of HPV DNA from mother to infant is possible (Cason *et al.*, 1995; Kaye *et al.*, 1996; Puranen *et al.*, 1997). Each study proved by means of gene analysis that the HPV types detected in the infant were identical to those infecting the mother. However, it should be noted that in none of the aforementioned studies was there 100% concordance between the HPV types detected in the mother and the infant and that infants were infected with HPV types not detected in the mother. This would indicate that the infants acquired HPV infection via another unknown route, in addition to the vertical transmission.

Further evidence of non sexual transmission of HPV was published by Giaquinto *et al.* (2000) who reported the development of a vulvar carcinoma in a 12 year old girl infected with human immunodeficiency virus (HIV) which was shown to harbor HPV 16 DNA sequences. The child had acquired HIV via vertical transmission and there was no evidence

of sexual abuse as was established by counselors at the hospital clinic. As the child had never had sexual intercourse, the authors concluded that the HPV infection was acquired by a non-sexual route.

Despite the evidence that mucosal HPV infections could be spread by non-sexual routes, there are conflicting reports in the literature as to whether this is possible. In a study on genital, anal and oral samples collected from infants at birth, 6, 12, 18, 24, and 36 months, Watts *et al.* (1998) showed that only 1,5% of genital samples contained HPV DNA, 1,2% of anal samples contained HPV DNA and none of the oral samples contained HPV DNA. These results had all been preceded or followed by a negative HPV DNA result and the infants were born to mothers who had shown either the presence or the lack of HPV DNA at 34 and 36 weeks' gestation. The authors therefore concluded that there was a low risk of vertical transmission or the results could be due to contamination and that no compelling evidence of transmission from mother to child was seen in the study. A study by Koch *et al.* (1997) on pre-school children showed only 1 of 392 oral samples and 4 of 249 anal samples to contain HPV DNA. However the authors note that the results should be interpreted with caution as low copy numbers of HPV DNA are present in the oral cavity.

All of the above studies used PCR methods for HPV DNA detection. There were however, methodological differences. One of the studies that showed vertical transmission used nested PCR, the second study used 2 sets of PCR primers that targeted either the L1 gene or the E7 gene and the third study re-amplified samples showing a discordant negative result between mother and child. The studies that did not show vertical transmission all used consensus primers targeting the L1 gene and no nested PCR reactions were performed. The discrepancies between the studies could therefore be due to the PCR methodologies

employed. This highlights the importance of using sensitive techniques when trying to detect HPV DNA in samples that may contain low copy numbers of virus.

1.9 Geographical distribution of high risk HPV types

Internationally HPV 16 accounts for approximately 50% of cervical cancers and HPV 18 for 20% (Bosch *et al.*, 1995) but even though certain high risk HPV types have been found to be commonly associated with high grade cervical lesions, there are geographical differences in the types of high risk HPVs circulating in different populations. In a study on Japanese women from Okinawa with cervical cancer (Nagai *et al.*, 2001), it was noted that HPV 16 accounted for only 29.6% of cervical cancers. This is considerably lower than the international incidence of 50%. The PCR method used in this study could detect HPV types 16, 18, 31, 33, 35 and 58. These HPV types are most commonly associated with cervical cancer internationally, yet they only accounted for 52% of the HPV positive cancers in the study. This indicated that less common high risk HPVs made up 48% of the HPVs in this study or alternately that HPV types considered as low risk could be associated with cervical cancer within the group studied.

A study on Chinese women from Hong Kong (Chan *et al.*, 1999) showed a prevalence rate of 23.8% for the less common high risk HPV 58 in women with cervical cancer or precursor lesions. Infection with HPV 58 within this group also showed a significant association with CIN/carcinoma with a significant trend of increase in prevalence with increasing severity of cervical lesion.

In Mozambique, it was shown that the most common HPV type associated with cervical neoplasia is HPV 35 (Castellsague *et al.*, 2000). The four most common HPV types in this

cohort of women were HPV 35 (30%), HPV 58 (17%), HPV 16 (13%) and HPV 33 (13%). Internnationally, HPV types 16, 18, 45 and 31 have been shown to be the four most common HPV types associated with cervical neoplasia (Bosch, 1995). The results from the Mozambique study therefore vary considerably from international results.

These geographical differences have major implications for vaccine design as well as the formulation of public health policies. As cancer of the cervix is a multi-step process preceded by precursor lesions which increase in severity over time, a prophylactic vaccine developed to immunize women against HPV infection would need to include those HPV types associated with the precursor lesions. It is therefore important to establish which HPV types are prevalent within a given population and in particular, which HPV types are associated with cervical precursor lesions.

1.10 Typing of HPV

1.10.1 Introduction

HPV is a poor immunogen as it is a non-lytic virus and productive infections are confined to the terminally differentiating epithelium. Therefore there is no systemic phase which would allow for antigen presentation (Frazer, 1998). As a result there are no serological assays available for the identification of HPV types. The difficulty of typing HPVs is exacerbated by the fact that there are no *in vitro* cultivation systems because HPV requires terminally differentiating cells. As terminally differentiated cells no longer divide, cell cultures cannot successfully be maintained. These limitations have therefore hampered the study of HPV infections. The diagnosis of HPV infections can be broadly divided into three methods of detection; clinical observation, cytological methods and DNA detection methods

1.10.2 Clinical observations

The exophytic anogenital warts such as condyloma acuminata (Figure 4a) caused by HPV types 6 and 11 and the keratotic genital warts caused by HPV types 1 and 2 are easily diagnosed by their gross histological features and the tendency to be positioned on the external genitalia whereas the HPV lesions found on the cervix tend to be flat. Visualisation of these cervical lesions can be accomplished by colposcopic examination after staining with a 3-5% acetic acid application. HPV infections appear as flat, shiny, white lesions (Figure 4b).

A disadavantage of this method is that determination of HPV type is not possible. It is also considered to be insensitive, non-specific and an inadequate guide for therapy (Barasso, 1992). This might be applicable in developed countries where efficient health care systems are in place. In developing countries, however, there is a role for colposcopy and acetowhitening as a diagnostic tool for HPV infection. The method is inexpensive and requires only a single visit as the patient can be treated immediately. A study done by Goldie *et al.* (2001) on black South African women indicated that the benefits of this method outweighed the disadvantages of false positive diagnoses leading to unnecessary cryotherapy.

1.10.3 Cytological methods

HPV infected cells undergo cytological changes with koilocytosis being a feature of HPV infection. These cells have a characteristic enlarged, dark nucleus with an irregular border encircled by a clear cytoplasmic ring. Koilocytic cells can be detected by means of the Papanicolaou (Pap) smear (Figure 5) and are considered part of the spectrum of the

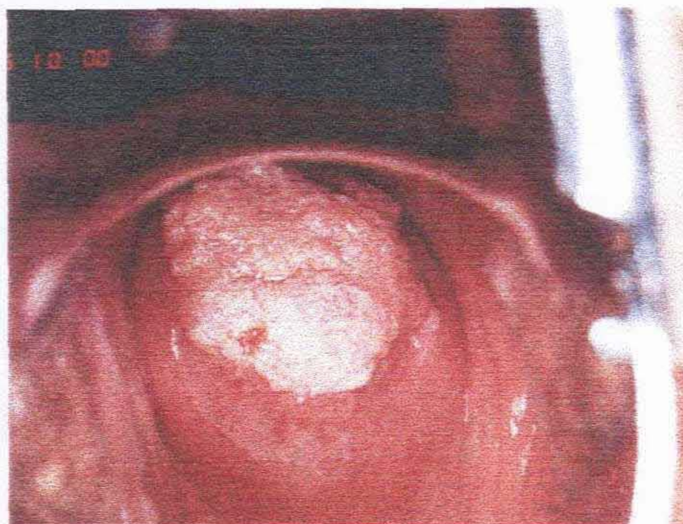

Figure 4a. Photo image of a condyloma acuminata lesion of the cervix. Cauliflower like lesion caused by infection with HPV 6/11(Clinical Virology, 1997. Churchill Livingstone Inc.)

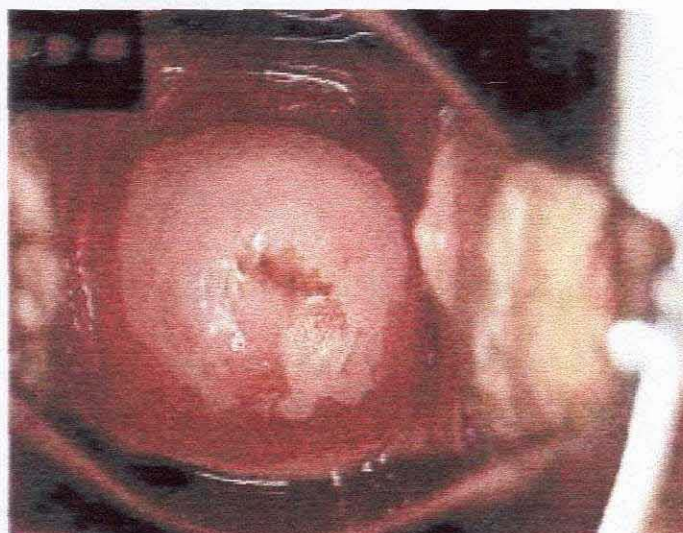

Figure 4b. Photo image of an HPV infected cervix. Aceto whitening of the tissue after application of 3%-5% acetic acid. (Clinical Virology, 1997. Churchill Livingstone Inc.)

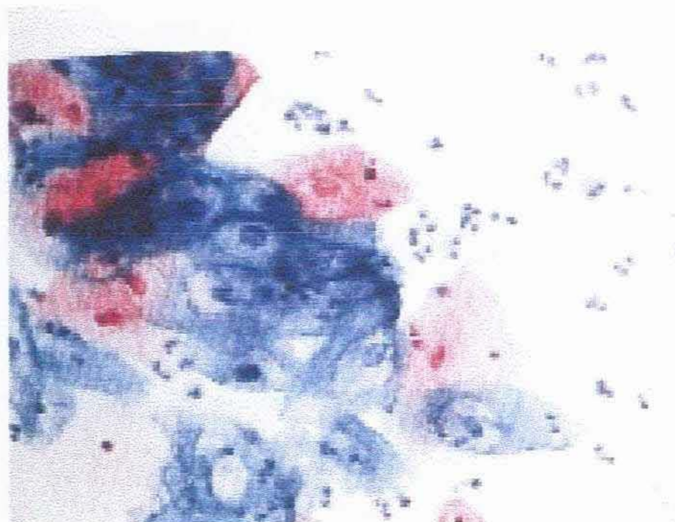

Figure 5. Pap smear of a low grade squamous intraepithelial lesion showing the cytological pattern of HPV. This includes intermediate and superficial squamous epithelial cells with enlarged atypical nuclei, hyperchromatic, with coarse chromatin. There is perinuclear clearing surrounded by a dense zone of cytoplasm.(Shingleton and Orr, 1995).

lowest grade of CIN. However latent HPV infections do not display these cytological changes and the sensitivity of the Pap smear is poor (Reid *et al.*, 1991). To date no comprehensive cervical cancer screening program has yet been implemented in South Africa (Michelow *et al.*, 1999). Obstacles that would have to be overcome before a screening program could be implemented would include educating the target population, training nursing staff and increasing the number of trained cytologists to screen the smears.

1.10.4 HPV DNA detection methods

After extraction of DNA from sample tissue, various methods can be employed to detect and type HPV DNA. Various non amplification methods such as Southern blot hybridization, dot blot hybridization and *in situ* hybridization utilize labeled probes for HPV detection. The advantage of these methods is that the type of HPV DNA detected can be determined by specific probes and a method such as *in situ* hybridisation can establish

the location of the HPV DNA within the cell. The disadvantage of these methods is that repeated rounds of probing need to be performed to establish which HPV types are present in the tissue and the sensitivity is less than that of amplification methods such as PCR (polymerase chain reaction). Although detection systems can utilize non radioactive means, another disadvantage lies in the use of radioactive materials for labeling probes. Designated areas and equipment are required, the disposal of radioactive contaminated materials needs to be strictly monitored and personnel handling the radioactive material need to be trained and monitored.

The hybrid capture™ assay is more sensitive than the methods described above as it uses a signal amplification system and ninety samples can be processed simultaneously making it is suitable for high through put applications. However, the assay only differentiates between high risk and low risk HPV types and does not identify a specific type within each sample. Therefore if a patient has a positive result on more than one occasion, the method is unable to establish if the patient is consistently infected with the same HPV type. Another disadvantage is that the method is expensive and therefore not suitable for large screening programs in developing countries.

PCR is a widely used technique for the detection of HPV DNA. The method is specific and is able to detect small quantities of DNA. It has been shown to be the most sensitive method for the detection of HPV DNA in clinical samples (Guerrero *et al.*, 1992; Kuypers *et al.*, 1993; Morris *et al.*, 1990; Schiffman *et al.*, 1991).

PCR primers that are used to detect HPV DNA can either be type specific or consensus. Type specific primers target a specific DNA sequence within a particular HPV type and can be designed to target any of the HPV genes depending on the aims of the study. The

advantage of using type specific PCR lies in the fact that the primers will only bind to the DNA sequence of a specific HPV type. Therefore, when the PCR product is run on an agarose gel, the presence of a band of the expected size will indicate the presence of a particular HPV type and no further testing is required. The disadvantage of this method is that only 1 HPV type can be detected and it is therefore not suitable for epidemiological studies designed to detect a wide range of HPV types. A second disadvantage is that the method is unable to amplify novel HPV types.

Consensus PCR primers are able to detect a wide range of HPV types including novel HPV types. Consensus primers are group specific eg. they will target only mucosal HPV types and they target the L1 gene as it is the most conserved of the HPV genes. The most commonly used PCR primers for the detection of HPV DNA are the My09/MY11 primers (Manos *et al.*, 1989). The advantage of using consensus primers lies in their ability to detect a wide range of HPV types as well as novel types and they are therefore highly suitable for epidemiological studies requiring the identification of all HPV types within a population. The disadvantage, however, is that the method does not distinguish between the different HPV types within a group. Further laboratory manipulations are therefore required to identify which HPV types have been detected. This can be done by various hybridization techniques involving probing the PCR product with known probes or by sequencing the PCR product.

Alternately HPV types can be identified by restriction fragment length polymorphism (RFLP). After analysis of the DNA sequence of the PCR fragment, suitable restriction endonucleases are chosen. The amplified PCR product is digested with the selected restriction endonucleases and the digested product electrophoresed on an agarose gel. The various sized fragments are separated by the gel resulting in different RFLP patterns which

are able to differentiate between the different HPV types. Unknown or novel HPV types are indicated by an unknown pattern or an undigested fragment and can be identified by sequencing of the PCR product.

The main advantage of using RFLP for HPV typing is the identification of novel types and variants, whereas hybridization techniques employ specific probes and can only identify the known HPV types for which the probes were designed.

The reverse line blot assay utilizes both PCR and hybridization. The advantage of this method is the sensitivity achieved by DNA amplification followed by hybridization. The disadvantages are the cost of the PCR which uses an expensive *Taq* polymerase and the method only identifies certain known HPV types. If a specimen contains a dual infection of which one is a novel HPV type or an HPV type other than those immobilized on the strip, the method will not indicate the presence of the unknown HPV type.

1.11 HPV typing studies in South Africa

The development of a prophylactic or therapeutic vaccine against HPV infection would play a large role in the prevention of cervical cancer. Due to geographical differences in the prevalence of HPV types a vaccine would need to be developed specifically for the HPV types circulating within the target community.

A literature search reveals that few studies have been performed in South Africa on the types of HPV prevalent in women with cervical intraepithelial neoplasia (CIN) or cancer of the cervix. A study by Williamson *et al.* (1989) used Southern blot hybridisation to detect HPV types 6, 11, 16, 18, 31, 33 and 35 in women with CIN but this technique was not as

sensitive as PCR and only 66 of the 98 specimens tested positive for HPV DNA. In addition the study could only identify a limited number of HPV types as only 7 different type specific probes were used. A second study by Williamson *et al.* (1994) on cervical carcinomas employed consensus PCR as a means of detecting HPV. The sensitivity of this study was therefore an improvement over the previous study. However, HPV typing was performed by hybridisation of PCR products with type specific and group specific oligonucleotide probes, thereby limiting the number of different HPV types that could be identified. A further study by Cooper *et al.* (1991) presented evidence of integration of HPV types 16, 18 and 33 in cervical squamous cell cancers in South African and British women. To date, in South Africa, there have been no published studies employing techniques capable of detecting a wide range of known and novel HPV types in women with CIN lesions or cancer of the cervix.

Due to the lack of published data, it is not yet known which HPV types need to be targeted by a prophylactic vaccine for South African women.

1.12 Project motivation

Various prophylactic vaccines are being developed to prevent HPV infection in women. Internationally it has been shown that HPV 16, 18, 45 and 31 (IARC 1996) are the most prevalent HPV types associated with cervical cancer. However, there are geographical differences in the distribution of HPV types amongst populations. For a prophylactic vaccine to be effective the 4 most prevalent types need to be included in the vaccine to ensure herd immunity. A humoral response with neutralizing antibodies is desired as part of a prophylactic vaccine and as there is no cross reactivity between HPV types it is

essential that epidemiological studies are performed to establish which HPV types are associated with disease within the various populations.

As has been noted, few studies have been performed in South Africa on women with cervical cancer or CIN. These studies are dated and the methods employed were not very sensitive nor able detect a wide range of high risk mucosal HPV types. The first aim of this study therefore, was to establish the prevalence of HPV types in Western Cape women with cervical neoplasias using a sensitive nested PCR that employs degenerate consensus primers. These primers are designed to detect a wide range of mucosal HPV types as well as novel HPV types. The second aim of the study was to develop a RFLP method that is able to identify the 10 mucosal HPV types declared as human carcinogens by the IARC, as well as HPV 11 which is commonly detected in oral specimens.

The significance of the presence of anogenital HPVs in the oral cavity has yet to be established, as is shown by the conflicting results of various studies. It has also not yet been fully established if the oral cavity acts as a reservoir for mucosal HPVs or if the oral cavity plays a role in the transmission of mucosal HPVs. A review of the published data reveals that few studies have been performed on the HPV types infecting the oral mucosa of women with cervical intraepithelial neoplasia (CIN) and there are no published data on the anogenital HPV types infecting the oral mucosa of women with CIN in South Africa. The third aim of this study was to establish the prevalence of mucosal HPVs in the oral mucosa of women with CIN and fourthly to establish whether the same HPV types infect the oral cavity and the cervix within the same woman.

Chapter 2

Methods and Materials

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2. Materials and Methods

2.1 Study population

The patient study population consisted of 163 women attending the colposcopy clinic or receiving in-patient care at Groote Schuur Hospital, Cape Town, between April 1993 and November 1997, who were diagnosed on colposcopy with cervical intraepithelial neoplasia (CIN) 1, 2 or 3. Signed consent was obtained from the participants once the nature of the study was explained to them by trained medical staff.

Approval for this study was obtained from the Ethics and Research committee of the University of Cape Town.

2.2 Specimens and clones

2.2.1 Biopsy samples.

Cervical punch biopsies were collected from all 163 participants by gynaecologists in attendance at the clinics and placed in a sterile plastic container and stored at –20°C shortly after excision.

2.2.2 Buccal samples

As the buccal study was performed retrospectively, samples were only available from the last 31 participants of the cervical study. Buccal samples were collected from all 31 patients by twirling a Cervi-brush (Pharmaceutical Enterprises, Cape Town) against the mucous membrane of the inner cheek. Each Cervi-brush was placed in a sterile tube, sealed and stored at -20°C.

2.2.3 HPV clones.

Plasmids containing known HPV types were obtained from Dr. Matsukuru of the National Institute of Infectious Diseases, Japan; the Digene Corporation, USA and Dr E-M de Villiers, Referenzzentrum fur Humanpathogene Papillomviren, Germany. The following HPV types were available for use in this study: HPV 11, 16, 18, 31, 33, 45, 58 and 59.

2.3 DNA extraction.

2.3.1 Tissue digestion using Proteinase K.

Proteinase K (Boehringer Mannheim) was used to cleave the peptide bonds of amino acids, thereby breaking down the proteins of the cell and enabling the DNA to be released from the cell. It was used in conjunction with a detergent to lyse the cells and denature proteins enveloping nucleic acids and therefore it was not necessary to phenolize the cell lysate. Excessive sample handling was reduced, minimising the probabliity of cross-contamination between samples. As the *Taq* polymerase used in the PCR reaction was susceptible to proteolytic degradation, the proteinase K was inactivated by heat treatment after digestion of the tissue sample.

The frozen cervical biopsies were thawed on ice and finely minced using a sterile disposable scalpel in a sterile petri dish. To prevent cross contamination of specimens, a new pair of gloves, sterile petri dish and disposable scalpel were used for handling each specimen and an extraction control consisting of lysis buffer only, was placed after every tenth sample. All work was performed in a laminar flow hood (BioFlow-11) in a dedicated room free from plasmid DNA. The minced tissue was transferred to a sterile microfuge tube containing 300 µl proteinase K lysis buffer (Appendix A) and incubated at 56°C for at

least 2 hours or until there were no visible pieces of tissue. The samples were incubated for 10 minutes at 95°C to inactivate the proteinase K and then stored at 4°C until required.

2.3.2 DNA extraction using the Qiamp DNA mini kit.

DNA was extracted from the patient buccal samples using a Qiamp mini kit (Qiagen). This method employs a spin column in which digested cells are applied to a column containing a silica-gel membrane. The presence of chaotropic salts in the buffer supplied by the manufacturer modifies the structure of water and facilitates the binding of DNA to the silica-gel membrane. The bound DNA is washed with buffers supplied by the manufacturer. The salt and pH conditions of the buffered lysate ensure that any protein or other contaminants are not retained on the membrane. The DNA is eluted from the silica-gel membrane with ultra pure water or the elution buffer supplied.

Buccal cells were removed directly from the Cervi-brushes by adding 200 μl phosphate buffered saline (PBS) (Appendix A) to the sterile tube containing the brush. After the tubes were pulse vortexed for 1 minute, 20 μl Proteinase K (as supplied by Qiagen) was added to 200 μl aliquots of phosphate buffered saline containing the buccal cells. A further 200 μl buffer solution (as supplied by Qiagen) was added to each specimen and the samples pulse vortexed for 15 seconds to ensure adequate mixing. The samples were incubated at 56°C for 10 minutes to lyse the cells. 200 μl ethanol was added to each sample and pulse vortexed for 15 seconds. The buffered lysate was applied to a spin column placed in a collection tube and centrifuged at 6000 x g for 1 minute. The collection tube containing the lysate was discarded and the spin column placed in a fresh collection tube. The bound DNA was washed by adding 500 μl buffered ethanol (as supplied by Qiagen) to the spin column and centrifuged for 1 minute at 6000 x g. The collection tube was discarded and

the spin column placed in a new collection tube. A second washing step was performed by adding 500 µl buffered ethanol (as supplied by Qiagen) to the spin column and centrifuged at 6000 x g for 1 minute. The collection tube was discarded and the spin column placed in a fresh tube. DNA was eluted by adding 200 µl ultra-pure water (BDH Ltd.) to each spin column and standing at room temperature for 1 minute. DNA was recovered from the membrane by centrifugation at 6000 x g. The extracted DNA was stored at 4°C until required for PCR.

All DNA extractions were performed in a dedicated area free of plasmid DNA and amplified DNA products. All equipment in this area was used for DNA extractions only.

2.4 Polymerase chain reaction

PCR is a sensitive and rapid in vitro method for the amplification of a targeted sequence of DNA (Mullis *et al.*, 1987). After heat denaturation of the DNA, two oligonucleotide primers of approximately 17 – 30 nucleotides in length are hybridised to complementary sequences flanking the DNA sequence to be amplified. Primer annealing is facilitated by the presence of salts such as potassium chloride (KCl) in a buffered solution. A heat stable DNA polymerase (*Taq*) derived from the bacterium *Thermus aquaticus* synthesizes a complementary DNA strand by incorporating deoxynucleoside triphosphates (dNTPs) in the presence of free magnesium chloride ($MgCl_2$). Repeated cycles of denaturation, annealing and elongation allow for the exponential increase of target fragments.

2.4.1 Optimisation of PCR reactions

Optimisation titrations were performed on both single step PCR (for the detection of human DNA) and nested PCR (for the detection of HPV DNA). The primers, reagent

concentrations and cycling conditions for both the single step PCR and the nested PCR have been previously described by Michael *et al.* (1997) and Williamson & Rybicki, (1991) respectively. To ensure optimal PCR conditions both primer titrations and $MgCl_2$ titrations were performed for the single step PCR and the nested PCR as the commercial reagents and thermocycler used in this study were different to the published protocols.

Thermocycling was performed in a Perkin Elmer (USA) GeneAmp 6700 thermocycler. The primers were manufactured by the Department of Molecular and Cellular Biology, University of Cape Town and diluted to a stock concentration of 50 pmol/µl. Commercial PCR reagents were supplied at the following concentrations: Supertherm *Taq* (J.M.R.Holdings) at 5 units/µl; $MgCl_2$ (J.M.R.Holdings) at a concentration of 25 mM, dNTP mix (Abgene) containing 200 µM of each dNTP and ultrapure water (BDH).

Human DNA extracted from a cervical biopsy was used as the template in the $MgCl_2$ and primer titrations for the single step PCR and plasmid containing the HPV 18 genome was used in the nested PCR titrations.

2.4.1.1 Magnesium chloride titration

Mg^{2+} in the form of $MgCl_2$ influences the enzyme activity of *Taq* polymerase, forms soluble complexes with dNTPs to produce the substrate that the *Taq* polymerase recognizes and increases the melting temperature (T_m) of double stranded DNA. The optimal amount of $MgCl_2$ concentration ranges from 0.5 mM to 5 mM. An excess of $MgCl_2$ results in the production of non specific PCR product due to a decrease in the specificity of the reaction and increases the incidence of primer dimers. A lower concentration of $MgCl_2$ increases the specificity of the reaction but results in a lower yield of PCR product.

$MgCl_2$ titrations were performed by adding $MgCl_2$ concentrations as shown in Table V, to a PCR reaction mix containing 10 X PCR buffer, 50 pmol each primer, 2 units of *Taq* polymerase, 200 μM each dNTP, template DNA and ultra pure water to give a final volume of 50 μl.

Table V. Volumes and concentrations of $MgCl_2$ used in the $MgCl_2$ titration for single step and nested PCR. Figures in parentheses indicate the final concentration of $MgCl_2$ used in a 50μl PCR reaction mix.

Reaction tube number	MgCl2 25 mM
1	2 μl (1 mM0
2	3 μl (1.5 mM)
3	4 μl (2 mM)
4	5 μl (2.5 mM)
5	6 μl (3 mM)
6	7 μl (3.5 mM)
7	8 μl (4 mM)

2.4.1.2 Primer titration

Primer concentrations of between 10 pmols and 50 pmols are generally optimal. High primer concentrations can lead to mispriming and the accumulation of nonspecific products. Insufficient primer concentrations can result in the exhaustion of primers before the reaction is complete, thereby resulting in low yields of PCR product. A primer titration was of particular importance in the optimisation of the nested PCR as degenerate primers were used, which results in a decrease in concentration of the individual primers within the reaction.

Primer titrations were performed by adding primer concentrations as shown in Table VI, to a PCR reaction mix containing $1/10^{th}$ volume (5μl) PCR buffer, 200 μM each dNTP, 4 mM $MgCl_2$, 2 units of *Taq* polymerase, template DNA and ultra pure water to give a final volume of 50 μl.

Table VI. Volumes and concentrations of oligonucleotide primers used in the primer titration for single step and nested PCR. Figures in parentheses indicate the final concentration of primer in a 50µl PCR reaction mix.

Reaction Tube Number	Primer 1: 50pmols/ul	Primer 2: 50pmols/ul
1	0.2 µl 10 pmols	0.2 µl 10 pmols
2	0.4 µl 20 pmols	0.4 µl 20 pmols
3	0.6 µl 30 pmols	0.6 µl 30 pmols
4	0.8 µl 40 pmols	0.8 µl 40 pmols
5	1 µl 50 pmols	1 µl 50 pmols

2.4.2 Single step PCR

To ensure the presence of amplifiable DNA that no PCR inhibitors were present, single step PCR using primers (Appendix B) for the human CCR-5 gene (Michael *et al.* 1997) was performed on both the biopsy and buccal samples. After optimisation of the PCR reaction, single step PCR was performed by adding 5 µl of sample DNA to 45 µl of PCR master mix.

Thermocycling conditions included an initial denaturation step of 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and primer extension at 72°C for 1 minute. A final primer extension step at 72°C for 7 minutes was performed after the 30 cycles of DNA amplification.

Preparation of PCR master mixes was performed with dedicated equipment in a separate area free of amplified DNA products. Only plugged tips were used in the preparation of master mixes. Sample DNA was added to the master mix in a separate area using dedicated pipettes and plugged tips.

2.4.3 Nested PCR

Nested PCR using degenerate consensus primers (Williamson & Rybicki, 1991) was used for the amplification of HPV DNA in both the buccal samples and the biopsy samples as well as amplifying known clones of HPV. The primers amplify a section of the L1 gene which is the most conserved of the HPV genes (Baker, 1987.) Degenerate primers are a mixture of primers with similar sequence but with nucleotide variations at one or more positions and can be employed to search for novel members of a known family (Wilks, 1989). Consensus primers are able to amplify different types of organisms from one genomic locus with a single primer pair.

The degenerate consensus primers were used in a nested reaction to increase the sensitivity of the PCR. A nested PCR reaction is one in which a pair of outer primers is used to amplify a fragment of DNA and a second pair of inner primers is used to amplify a smaller DNA fragment within the original amplicon. A nested PCR reaction is therefore more sensitive than a single step PCR reaction as the amplicon generated by the outer primers is the target DNA for the inner primers resulting in the amplification of a pre-amplified product.

As nested PCR provides an extremely sensitive means of amplifying small amounts of target DNA (Rolfs *et al.* 1992) the method is prone to PCR contamination due to the

presence of amplicons from the first round of amplification with the outer primers. Strict control measures were therefore employed to minimise the risk of contamination.

Both outer and inner PCR master mixes were made up and dispensed into the required number of tubes in a dedicated area with dedicated equipment and pipettes. To control for contamination a water control consisting of PCR master mix and ultra-pure water in lieu of sample DNA was placed after every fifth sample. The sample DNA was then loaded into the outer reaction and water was added to the water controls in a separate dedicated area with a pipette used for this purpose only. Loading of template for the nested reaction took place in a designated area after the area had been decontaminated with 10% v/v sodium hypochlorite for 5 minutes. Plugged tips were used in all the PCR steps and gloves and a protective gown were worn in each dedicated area. All work was performed in bio-safety cabinets allocated to the different areas and a strict one way flow of work traffic was adhered to. After handling of amplified product the PCR master mix area and the DNA loading area were not re-entered on the same day.

After optimisation of the PCR reaction, 4 µl of sample DNA containing an unknown quantity of DNA was added to 46 µl of the master mix containing the outer primers. A positive control consisting of an known HPV positive biopsy and a negative control consisting of a known HPV negative biopsy were included in the assay. After an initial denaturation step of 94°C for 5 minutes, the samples were subjected to 30 reaction cycles of denaturation at 94°C for 30 seconds, primer- template annealing at 45°C for 30 seconds and primer extension at 72°C for 1 minute. At the end of the 30 reaction cycles a final extension step was performed at 72° C for 7 minutes.

After completion of the outer reaction, 1 µl of amplified product was added to 49 µl of master mix containing the inner primers. The inner reaction was performed under the same cycling conditions as the outer reaction.

2.4.4 Colony PCR

PCR products from patients suspected of harbouring multiple HPV infections as determined by RFLP (section 2.6) were cloned as described in section 2.7. Colony PCR was performed on the transformed colonies followed by RFLP to confirm the presence of multiple HPV types.

Li, *et al.* (1988) described a method for the release of DNA from cells by direct lysis which was adapted by Atlas and Bej (1990) for the release of DNA from bacterial cells. However, in our experience the initial denaturation step of the thermocycling process of 94°C for 5 minutes, allows sufficient lysis of the bacterial cell membrane and the release of adequate amounts of DNA for PCR purposes.

Due to the large copy numbers of HPV DNA in a single transformed bacterial colony it was not necessary to perform a nested reaction and a single step PCR was performed using the inner HPV primers only.

After transformation of competent *E.Coli* cells with HPV from the patient samples, five white colonies from each sample were selected for colony PCR. A single bacterial colony was emulsified in 50 µl of ultra pure water (BDH) and 1 µl of the emulsion was added to

49 μl of PCR master mix containing the HPV inner primers of the nested reaction. Thermocycling conditions were the same as described in 2.4.3.

2.5 Purification of PCR products

The Qiaquick PCR purification kit method employs a spin column containing a silica-gel membrane to bind the PCR amplified DNA. A high salt buffer facilitates the adsorption of PCR products ranging in size from 100 bp – 10 kb to the silica-gel membrane, but allows the removal of primers of up to 40 bp in length. The silica-gel bound PCR product is washed with buffer (supplied by the manufacturer) to remove any contaminants and then eluted in ultra-pure water or buffer.

All samples showing amplified PCR product on gel electrophoresis were purified using the Qiaquick PCR purification kit (Qiagen) in preparation for RFLP and sequencing. The protocol was followed as per manufacturers instructions with the exception of the elution step, in which ultra-pure water was used instead of the buffer supplied by the manufacturer. This was done as selected PCR products were to be sequenced and the manufacturers of the ABI automated sequencer used in this study recommend that samples are eluted in water as certain buffers interfere with the sequencing reaction.

Prior to cleaning up the PCR products with the Qiaquick PCR clean up kit, the concentration of PCR product for each sample was established by visually comparing 4 μl of PCR product to marker VI on an ethidium bromide stained 1.8% agarose gel.

To clean up the PCR samples, 5 volumes of buffer (supplied by the manufacturer) was added to 1 volume of PCR reaction and mixed by inverting the tube. A spin column was

placed in a collection tube and the PCR/buffer mixture applied to the spin column. The collection tube with spin column was centrifuged at 6000 x g for 1 minute. The flow through was discarded and the spin column replaced in the collection tube. 750 µl of wash buffer (supplied by the manufacturer) was applied to the spin column and centrifuged at 6000 x g for 1 minute. The collection tube was discarded and the spin column placed into a fresh Eppendorf tube. Depending on the concentration of PCR product from each sample, varying quantities of ultra pure water were added to the spin column to obtain an approximate final DNA concentration of 500 ng-1000 ng. The spin column was allowed to stand for 1 minute and then spun at 6000 x g for 1 minute to collect the eluted DNA. Samples were stored at –20°C until required.

2.6 Restriction fragment length polymorphism

2.6.1 Gene analysis using computer software

One of the primary aims of this study was to develop a means of typing the mucosal HPVs associated with cervical disease. The RFLP was developed to identify HPV types 16, 18, 31, 33, 35, 45, 51, 52, 58, and 59 as they have been declared human carcinogens of group 1 by the IARC and therefore contribute significantly to the disease burden of cervical neoplasias. The RFLP was also designed to identify HPV 11 as this HPV type is commonly detected in the oral cavity.

The DNA sequences of the following mucosal HPV types were downloaded from the NCBI Genbank data base (http://www.ncbi.nlm.nih.gov/): 6a, 6b, 7, 11, 13, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 57b, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 82, and 83. These mucosal HPVs represent the full list of mucosal HPV types lodged with the Genbank at the time of developing the RFLP and were

all analysed to ensure that the restriction patterns used in the RFLP would be unique and accurately identify the different types.

The outer and inner primers (Appendix B) were mapped to the L1 gene to ensure sufficient homology for annealing as mismatched nucleotides at the 3' end of the primers do not allow for efficient primer annealing and primer extension. Restriction sites were identified within the region amplified by the inner primers using the DNAman software program. The RFLP was designed to be performed in 2 stages viz. a screening digest and a confirmatory digest. The screening digest consisted of a single digest of *BstE*11 and a double digest of *Pst*1 and *Bgl*1. The confirmatory digest consisted of selected restriction endonucleases where required (Table VII).

Table VII. List of restriction endonucleases required for the RFLP confirmatory digests of mucosal HPV types

HPV type	Restriction endonuclease
11	*Dra* 111
18	*Pvu*11
31	*Nco* 1
33	*ApaL* 1
45	*Eco* 47111
59	*Bgl* 11

2.6.2 RFLP methodology

Restriction endonucleases are enzymes produced by bacteria to destroy foreign DNA by cleaving the internal phosphodiester bonds of the foreign DNA. They are classified as types 1, 11 and 111 (Old & Primrose, 1994). Type 11 restriction endonucleases are of particular importance to molecular techniques as they recognise a specific DNA sequence, known as the recognition sequence, and can cleave DNA at a specific site within or adjacent to a

specific DNA sequence (Sambrook *et al.* 1989). The recognition sequences are pallindromic ie. the 5'-3' sequence of the forward DNA strand reads the same as the 5'-3' of the reverse DNA strand (Figure 6).

Cleavage of DNA with restriction endonucleases in conserved genes of a species can detect base sequence variations, resulting in different lengths of DNA fragments. The HPV primers used in this study were consensus primers designed to amplify a highly conserved

Endonuclease	**Recognition Sequence**	**Bacterial Source**
*Pst*1	C T G C A ▼ G G ▲ A C G T C	*Providencia stuartii*
*Bgl*11	A ▼ G A T C T T C T A G ▲ A	*Bacillus globigii*

Figure 6. Examples of pallindromic DNA sequences recognised by type II restriction endonucleases. Arrows indicate the site of DNA cleavage by the restriction endonuclease.

region of the L1 gene of different mucosal HPV types. By digesting this region with selected restriction endonucleases, the various HPV types could be identified with the different restriction maps created by the fragment length polymorphisms.

5 µl containing approximately 500 ng of DNA of the inner nested PCR product from cervical biopsy and buccal samples was added to a 20 µl reaction mix containing reaction buffer, 2 units of enzyme and ultrapure water. The reaction mixes were incubated for 1 hour at 37°C in an incubator except for the *BstE*11 digests which were incubated at 60° C for 1 hour. RFLP was performed on cloned HPV DNA using the same method to confirm RFLP patterns.

2.7 Cloning

This is achieved by ligation of the DNA of interest into a vector and subsequent transformation of competent *E.Coli* cells with the chimeric molecule to obtain multiple copies of the clone. Each copy of the vector ligates with only one copy of the insert therefore each colony of the transformed *E.Coli* cell would contain multiple clones of the DNA fragment of interest. Cloning was performed to verify RFLP results that indicated the presence of more than one HPV type in a patient sample. The HPV PCR used in this study used consensus primers, therefore samples containing more than one HPV had all the HPV types amplified simultaneously. Cloning provided a means of separating out the different HPV types.

2.7.1 Subcloning into pGem®-T easy vector

PCR amplification using a thermostable *Taq* polymerase that does not have proofreading activity can result in the addition of deoxyadenosine to the 3' end of the amplified PCR product. This is done in a template independent fashion. As the *Taq* used in this study did not have proofreading activity, pGem®-T easy vector (Appendix D) was the vector of choice. pGem®-T easy vector has 3' terminal thymidine overhangs which have been added to both ends of the multiple cloning site (MCS) and are complementary to the deoxyadenosine of the PCR product, thereby enabling more efficient ligation of the PCR product into the vector. These 3' overhangs also help prevent the recircularisation of the vector.

The MCS found in the vector enables the release of the insert by digestion with the appropriate restriction endonucleases. However for this study, PCR was performed directly on bacterial colonies containing the vector and insert, thereby releasing the insert of interest.

2.7.1.1 Preparation of vector and insert DNA

The molar ratio of vector to insert is important for efficient ligation. If the concentration of insert is too low, recircularization of the vector is favoured due to the reduced frequency of intermolecular reactions resulting in a lower number of recombinants. When using the pGem®-T easy vector system a ratio of 3:1 to 1:3 of vector to insert DNA is recommended.

The concentration of purified PCR products was determined by spectrophotometry and the appropriate amount of PCR product for inclusion in the ligation reaction was calculated as follows:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

2.7.1.2 Ligation

During ligation, bacteriophage T4 DNA ligase catalyzes the formation of a phosphodiester bond between the 5' phosphate group and the 3'hydroxyl group of adjacent nucleotides. This enables the DNA fragment of interest to ligate into the vector.

Ligation mixes were made up by adding the appropriate amount of PCR product (as calculated in 2.8.1.1) to 1 µl (50 ng) pGem®-T Easy vector, 5 µl of 2 x ligation buffer and 1 µl of T4 ligase (at a concentration of 3 Weiss units per µl). The volume was made up to 10 µl with deionized water and the ligation mixes were incubated overnight at 4°C .

A positive control with a known insert and a background control with no insert were run with each batch of ligation reactions.

2.7.1.3 Preparation of competent *Escherichia coli* cells

100 µl of a glycerol stock of DH5α *E.Coli* cells (Stratagene) was added to 10ml 2X yeast tryptone (YT) broth (Appendix A) and grown overnight at 37°C. An 0.5 ml inoculum of overnight culture was added to 50 ml of warm (37°C) 2 x YT broth in a 500 ml flask. The culture was incubated at 37°C with vigorous shaking until an $O.D_{600}$ of 0,5-0,6 was reached. The culture was cooled on ice and spun at 4°C for 5 minutes at 4000 x g. The supernatent was poured off and the pellet gently resuspended in 60 mM $CaCl_2$, 10 mM Hepes (pH 7,2). The suspension was kept on ice for 20 minutes and spun at 4°C for 5 minutes at 4000 x g. The supernatent was poured off and the tubes drained well. The cells were resuspended in 60 mM $CaCl_2$, 10 mM Hepes and 15% glycerol. Aliquots of 100 µl were snap frozen in a mixture of ethanol and dry ice and stored at -70°C until required.

2.7.1.4 Transformation of competent *E.Coli* cells

E.Coli favours glucose as an energy source. However if glucose is not present, the cell can use lactose as an energy source by hydrolysis to galactose and glucose. The lactose utilization genes, made up of lac Y, lac Z and lac A form an operon ie each gene is transcribed on the same mRNA delineated by a single promoter upstream of lac Z and a single terminator downstream of lac A (Murray *et al.* 1996). The products of the lac I gene, which is situated just upstream of the operon, regulate the expression of the operon. The gene product of Lac I is named the lactose repressor. In the absence of lactose the promoter of the operon is blocked by the lactose repressor. In the presence of lactose or an inducer such as Isopropyl-β-D-thio-galactopyranoside (IPTG), the repressor is bound to the lactose resulting in a conformational change. The repressor – inducer complex dissociates from the DNA molecule and transcription can take place.

The MCS of the pGem®-TEasy vector is inserted within the α peptide coding region of the lac Z (β-galactosidase) gene without disrupting the reading frame. A functional enzyme molecule is generated when an *E.Coli* cell containing a mutant β-galactosidase (β-gal) protein lacking the N-terminus combines with the α-peptide. This is known as α-complementation (Ullman *et al.* 1967). In the presence of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) the colonies turn blue. However, if DNA is inserted into the MCS the synthesis of the α-peptide is prevented and α-complementation does not take place. Therefore no accumulation of enzymatically active β-gal takes place and colonies remain white in the presence of X-gal.

Competent DH5α *E.Coli* cells were thawed on ice. 2 μl of ligation mix, as described in 2.8.1.2, was transferred to a 1,5 ml microfuge tube and kept on ice. 100 μl of competent DH5α *E.Coli* cells was transferred to the ligation mix and kept on ice for 10 minutes. The ligation/competent cells were heat shocked for 2 minutes in a 42°C water bath. 900 μl of room temperature 2 x YT broth was added to the ligation/competent cells and incubated for 45 minutes at 37°C. 100 μl of each transformed culture as well as a positive control and background control were plated out on separate 2 x YT agar plates containing ampicillin at a concentration of 100 μg/ml agar; IPTG at a concentration of 1 mg/ml agar and X-gal at a concentration of 0.1 g/ml agar. Plates were incubated overnight at 37°C.

2.7.1.5 Screening for recombinants

PGem®-TEasy vector contains an ampicillin resistance gene, therefore only *E.Coli* colonies containing the insert plus vector, or vector only, will grow on agar plates containing

ampicillin. Any contaminating *E.Coli* colonies would be unable to grow in the presence of ampicillin. Recombinants can be differentiated from untransformed cells by the colour of the colonies (as described in 2.7.3).

After overnight incubation at 37°C the competent cells were screened for recombinants. Five white *E.coli* colonies were selected for each sample and colony PCR performed as described in 2.4.4.

2.8 DNA electrophoresis.

2.8.1 Agarose gels

An agarose gel is a complex network of polymeric molecules of varying pore size, depending on the concentration of agarose and the buffer composition used. DNA fragments of different molecular weights are separated as the DNA migrates through the matrix under the influence of an electrical charge. DNA fragments of an unknown size can be electrophoresed with a DNA marker of known sizes allowing for the accurate determination of the unknown fragment.

Agarose was mixed with 1x Tris-borate with ethylenediaminetetra-acetic acid (TBE) buffer and brought to boiling point in a microwave to ensure complete dissolving of the agarose. After cooling to approximately 50°C, gels of 3 mm – 4 mm were poured and allowed to set at room temperature. A 3% agarose gel was used to separate the smaller products of restriction endonuclease digestion to allow for better separation of product and a 1,8% agarose gel was used to separate PCR products. 20 µl of each reaction was loaded into the agarose wells with loading buffer (Appendix A) and run against Marker V111 (Boehringer Mannheim, Germany) for the RFLP products and against Marker V1

(Boehringer Mannheim, Germany) for PCR products. (Appendix C). The gels were electrophoresed at 100 volts for 1 hour.

2.8.2 Buffer conditions

As DNA has a net negative charge, a buffering system is required for electrophoresis. Water is electrolysed during electrophoresis, generating protons at the anode and hydroxyl ions at the cathode. This results in the anodal end of the chamber becoming basic and the cathodal end becoming acidic. The net charge of the DNA is negative in the presence of the basic buffer resulting in migration of the DNA to the anode. The interaction of TBE with agarose results in a smaller pore size reducing the broadening of DNA bands due to dispersion and diffusion. TBE (Appendix A) was the buffer of choice as it enables better resolution of DNA fragments smaller than 1 kb. All the expected PCR products and RFLP fragments in this study ranged in size from 37 bp – 340 bp.

TBE buffer was made up as detailed in appendix A and stored at room temperature until required. When electrophoresing an agarose gel, sufficient TBE buffer was added to the gel electrophretic system to cover the gel to a depth of 3-5 mm.

2.8.3 Ethidium bromide staining

To enable visualisation of the separated DNA fragments, gels were stained with ethidium bromide which is a fluorescent dye that intercalates between the DNA bases. DNA stained with ethidium bromide can be detected by exposure to ultra-violet radiation.

After electrophoresis, the gels were stained in 200 ml fresh TBE containing 20 µl ethidium bromide (10 mg/ml.) (Appendix A), for 20 minutes with gentle agitation. The gels were

destained for 20 minutes with gentle agitation in 500 ml distilled water to reduce background staining and exposed to ultra violet (UV) light to view the bands.

2.9 Sequencing

Sanger *et al.* (1977) described a chain-terminator sequencing procedure which capitilised on the ability of DNA polymerase to synthesize a complementary strand of DNA from a single stranded DNA template and to use the analogue, 2',3'- dideoxynucleoside triphosphates (ddNTP) as a substrate. Chain elongation occurs at the 3' end of the DNA strand by the formation of phosphodiester bonds between the 3'-hydroxyl group and the 5'-phosphate group of the incoming dNTP. The 3' end of the analogue lacks a hydroxyl group and therefore no longer acts as a substrate for the growing DNA strand. Once the analogue is incorporated into the growing point of the DNA strand, the growing DNA strand is terminated.

The automated sequencer used in this project is a cycle sequencer that employs a dye-labelled terminator method for sequencing of DNA products. Cycle sequencing is successive rounds of denaturation, annealing and extension in a thermal cycler which results in the linear amplification of DNA products . The 4 dideoxy terminators are each

labelled with a different fluorescent dye. The growing chain is simultaneously terminated and labelled with the dye that corresponds to that base. The dye-labelled DNA fragments migrate through the polyacrylamide gel and separate according to size. At the lower portion of the gel a laser beam scans across the gel. The laser excites the fluorescent dyes which then emit light at specific wavelengths for each dye and the results are interpreted by sequence analysis software.

2.9.1 Preparation of specimens for sequencing

After completion of HPV typing of the biopsy and buccal samples by RFLP, 52 randomly chosen samples were sequenced to confirm the RFLP typing results. Where possible, two samples of each known RFLP pattern were sequenced. Samples showing an unknown RFLP pattern or an undigested band were also sequenced.

An aliqot of the same PCR product used for RFLP was diluted to give an approximate concentration of 3-10 ng DNA. This was achieved by visually comparing 4 µl of the PCR product to the known concentrations of DNA molecular weight marker VI (Appendix C) on an ethidium bromide stained 1.8% agarose gel.

Sequence reaction mixes were made up with 3.2 pmol of the inner forward primer of the HPV nested primer pair, 4 µl terminator mix enzyme (AB Applied biosystems), 4 µl 2.5 x buffer (ABI systems) 10-50 ng PCR product and ultra-pure water to make up a total volume of 20 µl. Sequencing reactions underwent thermocycling in a GeneAmp 6700 (Perkin Elmer, USA) at 96°C for 1 minute followed by 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Sequencing reactions were then directly sequenced using an ABI 377 (Perkin Elmer, USA) automated sequencer.

2.10 Reverse line blot assay

2.10.1 Specimen collection

Gravitt *et al.* (1998) published an HPV detection and typing method using consensus PCR and reverse line blot assay. To assess the validity of the nested HPV PCR and the RFLP, results were compared to results obtained by the reverse line blot assay. The comparison between the two assays could not be performed on the original biopsies used in the

epidemiological study and development of RFLP as these studies had been performed retrospectively. The extracted DNA had been stored at 4°C for over a period of 2 years resulting in the partial degradation of DNA. The samples were able to be amplified by the nested PCR but were not amplifiable using the PCR protocol of the reverse line blot assay. However, during the original study, biopsies that were very large had been cut in two and the second piece of biopsy stored at -20°C. A total of 92 frozen biopsies were available for the comparison study. The biopsies were drawn from the same Groote Schuur hospital cohort and included specimens from patients with CIN 1, CIN 2, CIN 3 and cancer of the cervix. Frozen biopsies were thawed on ice and DNA extracted using the Quiagen extraction method as described in 2.3.2.

2.10.2. Hot start PCR

Target DNA was amplified by means of "hot start" PCR. The *Taq* polymerase used in the protocol has an attached antibody which renders the *Taq* inactive. This prevents non specific amplification at temperatures below the annealing temperature. Once the PCR mix has been heated to 95°C for 10 minutes, the antibody dissociates from the *Taq* polymerase rendering it active.

The primers (PGMY09/PGMY11) used in the PCR protocol are 5' biotin labelled consensus primers which amplify a 441 bp fragment of the L1 gene. Included in the PCR protocol are primers (GH20 and PC04) for the human ß-globin gene which act as an internal control.

Each amplification was performed in a 100 µl reaction containing 10 x PCR buffer (as supplied by manufacturer), 6 mM $MgCl_2$, 200 µmol each dATP, dCTP, dGTP and 600

µmol dUTP, 50 pmol each 5' biotinylated HPV L1 consensus primer (PGMY09 and PGMY11), 2,5 pmol each biotinylated ß-globin primer (GH20 and PCO4) and 7,5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). PCR reactions were denatured for 9 minutes at 95°C followed by 40 cycles of denaturation for 30 seconds at 95°C, primer annealing for 1 minute at 50°C and primer extension for 1minute at 72°C. This was followed by a final extension step of 72°C for 5 minutes.

2.10.3. Single-hybridisation reverse line blot detection

The hybridisation protocol employs a plastic backed nylon membrane strip (as supplied by Roche) containing 27 BSA-conjugated immobilised probe lines which allow for the identification of 18 high risk HPV types and 9 low risk HPV types. To ensure that amplifiable DNA is present, the strip has 2 probes for the human ß-globin gene which act as a strong positive control and a weak positive control. Single stranded PCR products that are complementary to the bound probe lines will hybridise whereas non-specifically hybridised product are removed by means of a stringency wash at a higher temperature. The correct stringency temperature is calculated on the melting temperature (T_M) of the probe-target hybrid. The T_M is the temperature at which the probe and the target are 50% dissociated. After the stringency washes any positive hybridisations are detected by streptavidin-horseradish peroxidase colour mediated precipitation at the probe site. Avidin is a protein that binds strongly to biotin. Unbound conjugate is rinsed off with a wash buffer and colour development is activated by incubating with hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine in dimethylformamide. Results are interpreted by means of a plastic overlay marked with lines corresponding to the position of the different probes in relation to the reference line. A schematic diagram of the assay is shown in Figure 7.

PCR products were denatured to single stranded DNA by adding 100 µl Amplicor™ denaturation solution to the PCR product at room temperature. Hybridisation solution (Appendix A) was warmed to 53°C and 3 ml added to each genotyping strip. 75µl of the denatured PCR reaction was added to its corresponding genotyping strip and incubated in a shaking water bath at 53°C for 30 minutes. The hybridisation solution was aspirated and each strip was washed in 3 ml room temperature washing buffer (Appendix A) and immediately aspirated to remove any residual hybridisation buffer. A second wash at 53°C was performed for 15 minutes in a shaking water bath. The wash buffer was aspirated and 3 ml streptavidin-horseradish peroxidase (Appendix A) added to each strip. The strips were incubated at room temperature for 30 minutes with shaking. The streptavidin-horseradish peroxidase was aspirated and the strips washed in room temperature wash buffer. The wash buffer was aspirated and then 3 ml room temperature wash buffer added to each strip and incubated at room temperature for 10 minutes with shaking. The wash buffer was aspirated and the 10 minute washing step repeated. The wash buffer was aspirated and 3 ml sodium citrate solution added to each strip and allowed to stand at room temperature for 5 minutes.

After aspirating the sodium citrate solution, 3 ml of colour development solution (as supplied by Roche) was added to each strip and shaken at room temperature for 5 minutes. The colour development solution was aspirated and the strips were rinsed thoroughly in distilled water. A positive HPV result was indicated by the presence of one or more a solid blue lines. The HPV types were identified by aligning a marked plastic overlay (as supplied by Roche) with the reference line on each strip.

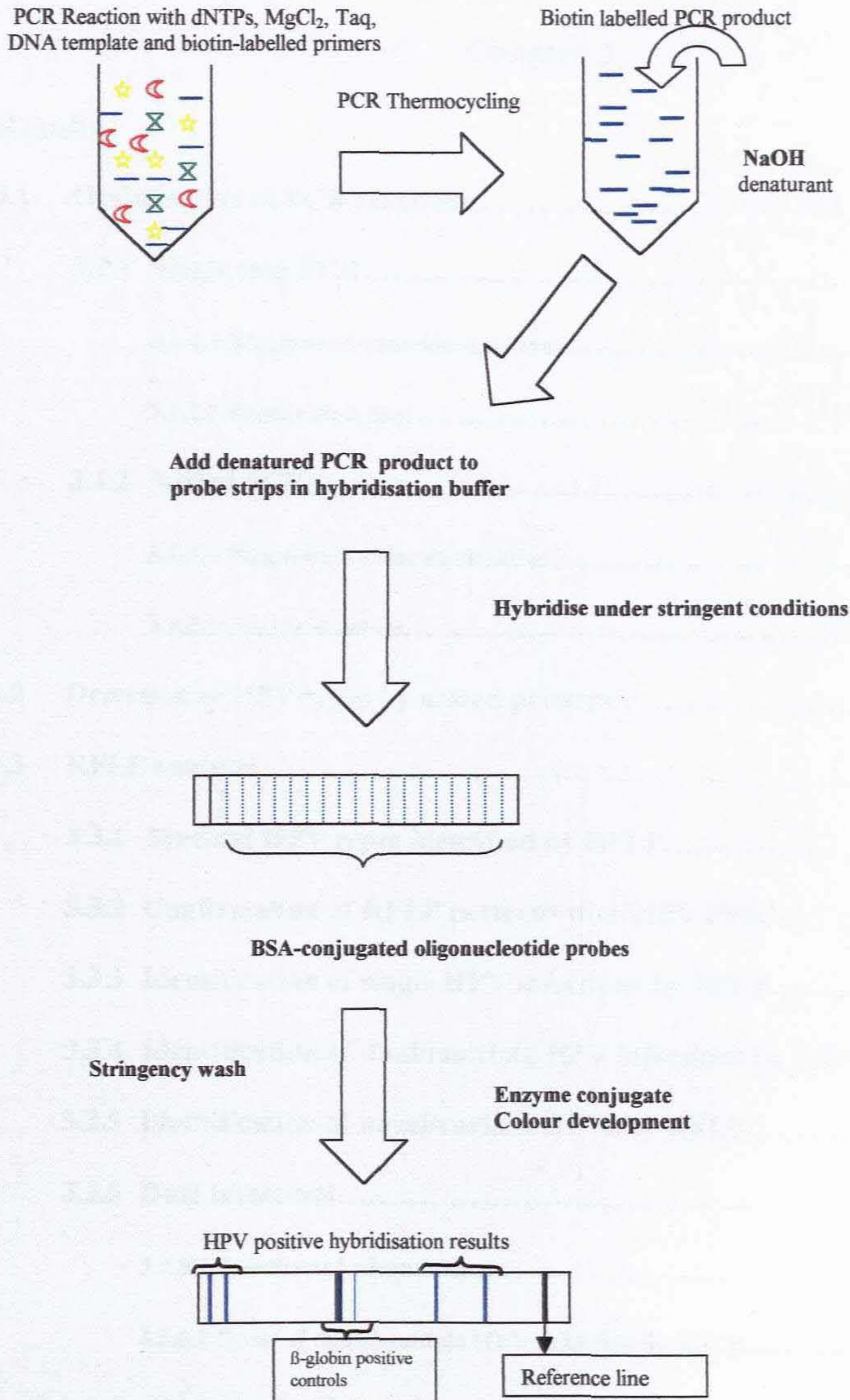

Figure 7. Schematic diagram of HPV genotyping of PCR product by reverse line blot assay. The reference line on the hybridized strip is aligned to a plastic overlay labelled with 27 different HPV types so as to establish which HPV types are present in the unknown sample.

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3. Results

3.1 Optimisation of PCR reactions

3.1.1 Single step PCR

3.1.1.1 Magnesium chloride titration

Differences in copy numbers of target DNA do not always make it possible to simultaneously optimise for sensitivity, specificity and reproducibility when performing PCR on clinical samples. After performing the magnesium chloride titration with the CCR5 primers, the correct size fragment of 225 bp was visualized on an ethidium bromide stained 2% agarose gel. High concentrations of $MgCl_2$ can result in non specific products due to a decrease in specificity, but it was noted even at the higher $MgCl_2$ concentrations that there were no non specific bands present (Figure 8). A concentration of 1,5mM $MgCl_2$ was therefore chosen as higher concentrations could facilitate primer dimer formation in some of the clinical samples. Figure 8 shows the results of the $MgCl_2$ titration to the right of molecular weight marker V1 (Appendix C).

3.1.1.2 Primer titration

An excess of primer concentration in a PCR reaction can result in mispriming, whereas too low a concentration results in insufficient amplification product. The primer titration using the CCR5 primers in the single step PCR indicated that 10 pmol yielded too little product and concentrations of 20 pmol and above yielded the same amount of product. Therefore 20 pmol was the concentration of choice as higher concentrations could promote mispriming. Figure 8 shows the results of the primer titration to the left of molecular weight marker V1 (Appendix C).

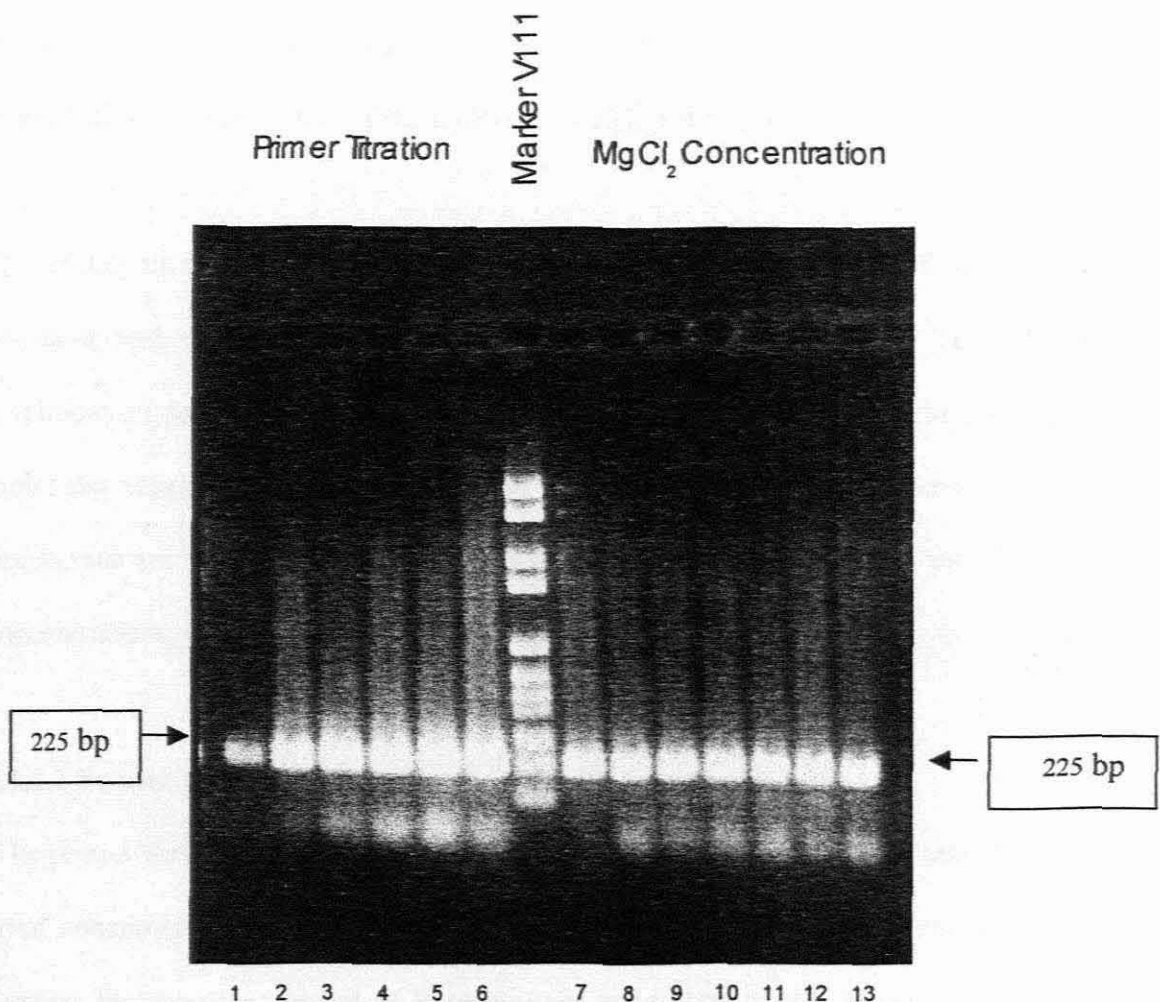

Figure 8. Primer titration and $MgCl_2$ titration for the single step PCR. Lanes 1-6: Primer titration of 10 pmol, 20 pmol, 25 pmol, 30 pmol, 40 pmol and 50 pmol.using CCR5 primers in single step PCR.Lanes 7-13: $MgCl_2$ titration of 1mM, 1,5mM, 2mM, 2,5mM, 3mM, 3,5mM and 4mM using CCR5 primers in single step PCR Sizes of the molecular weight marker V1 in base pairs are as follows: 2176,1766,1230,1033,653,517,453,394,298,234/220 and 154

3.1.2 Nested PCR

3.1.2.1 Magnesium chloride titration

The $MgCl_2$ optimisation indicated that there was insufficient amplification of the outer product of the PCR nested reaction with concentrations of 1 mM and 1,5 mM $MgCl_2$. Non specific amplification products of about 145 bp and +/- 70 bp were observed with

concentrations of ∃1,5 mM $MgCl_2$. Figure 9 shows the correct size amplification outer product of 441 bp to the left of the molecular weight marker V1.

The $MgCl_2$ titration yielded no difference in amplification products of the inner nested fragment and no non specific amplification products were present. The correct size amplification product of 320 bp of the inner nested fragment is shown to the right of the molecular weight marker V1 in Figure 9. As the primers used in the nested reaction are degenerate and no non specific amplification products were present at the higher $MgCl_2$ concentrations, a 2 mM $MgCl_2$ was the concentration of choice.

3.1.2.2 Primer titration

The primer titration on the outer reaction of the nested PCR yielded the least product at 10 pmol concentration and the most product at 50 pmol primer concentration. The primer titration for the outer nested PCR reaction is positioned to the left of molecular weight marker V1 in Figure 10.

The primer titration of the inner nested reaction gave the same results as those of the outer PCR reaction (lanes 7 –12 of figure 10). It was noted that there were some non specific amplification products of about 700 bp at the primer concentration of 20pmol. As the nested primers are consensus primers and are therefore a mixture of primers, higher concentrations *of primer are advisable to ensure adequate concentrations of each primer. A 50 pmol primer* concentration was therefore chosen for both the outer and the inner nested reactions.

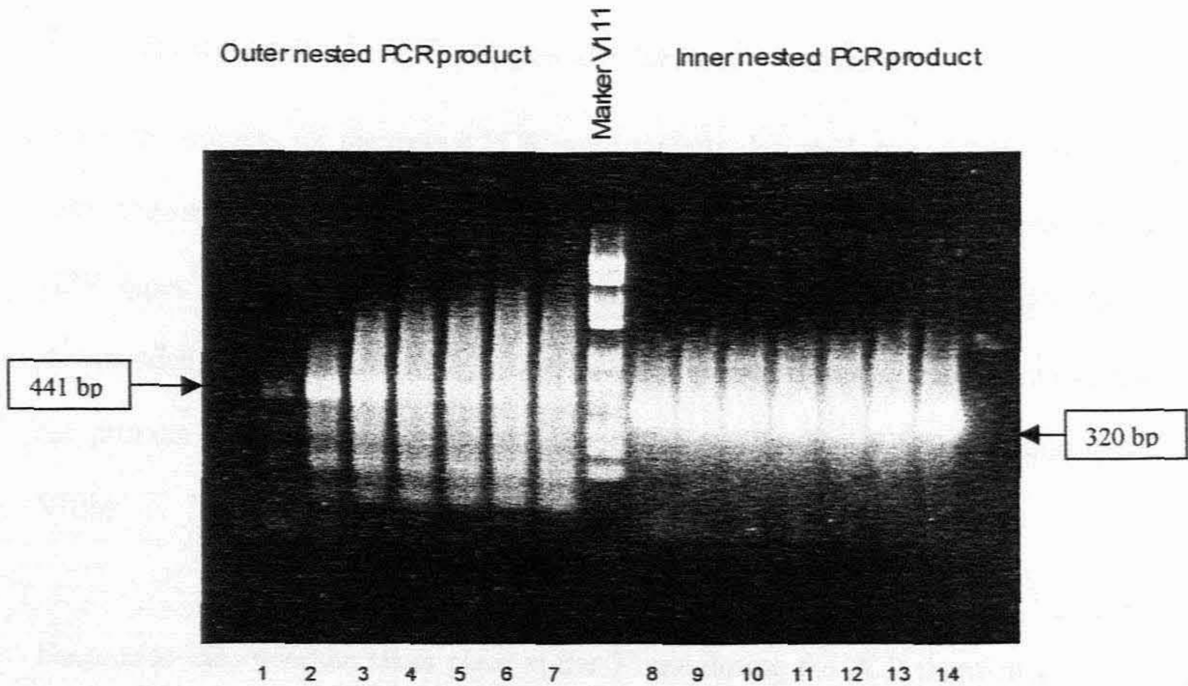

Figure 9. $MgCl_2$ titrations for the nested PCR. Lanes 1-7: $MgCl_2$ titration of 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM and 4 mM for the outer nested PCR reaction. Lanes 8-14: $MgCl_2$ titration of 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM and 4 mM for the inner nested PCR reaction.
Sizes of molecular weight marker V1 in base pairs are; 2176,1766,1230,1033,653,517,543,394,298,234/220 and 154

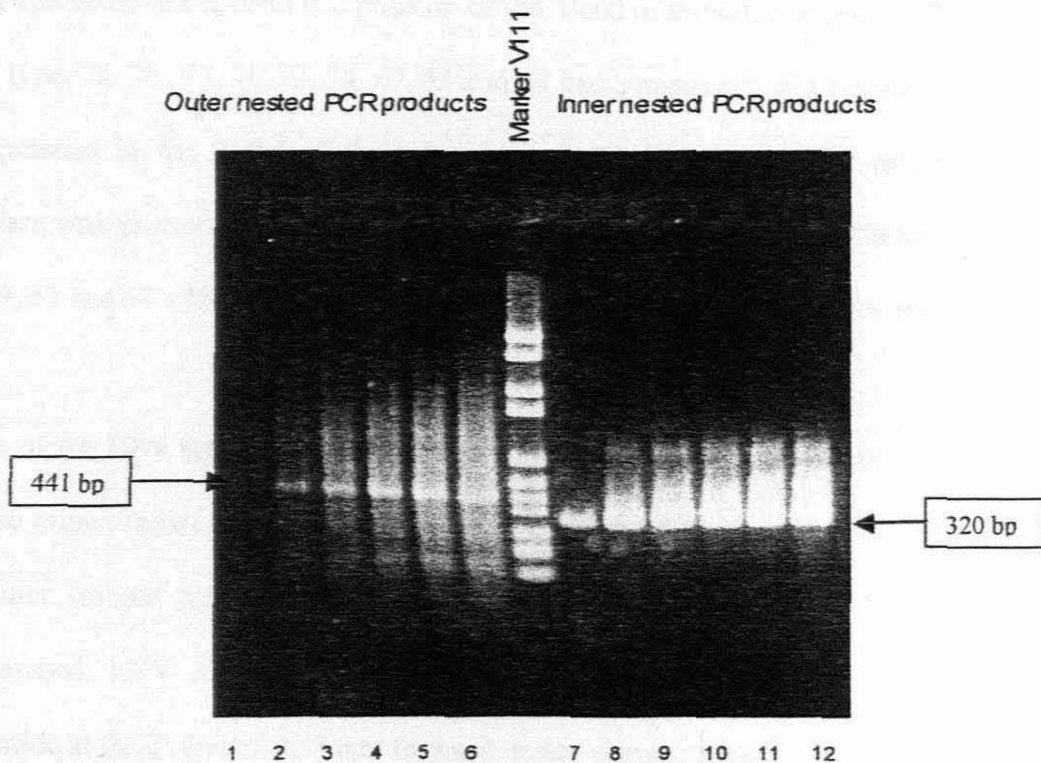

Figure 10. Primer titrations for the nested PCR. Lanes 1-6: Primer titration of 10 pmol, 20 pmol, 25 pmol, 30 pmol, 40 pmol and 50 pmol for the outer nested PCR reaction. Lanes 7-12: Primer titration of 10 pmol, 20 pmol, 25 pmol, 30 pmol, 40 pmol and 50 pmol for the inner nested PCR reaction.
Sizes of molecular weight marker VI in base pairs are; 2176,1766,1230,1033,653,517,543,394,298,234/220 and 154

3.2 Detection of HPV types by nested primers

When the primers for the nested PCR were initially designed, only 6 HPV genomes had been sequenced (Williamson and Rybicki, 1991). The primers were therefore based on HPV types 6b, 11, 16, 18, 31 and 33. Alignment of the primers to HPV sequences downloaded from Genbank as described in 2.6.1 of materials and methods, indicated that the primers would be able to detect a broad range of mucosal HPVs (Table VIIIa and VIIIb).

Nucleotide incorporation takes place at the 3' end during the PCR thermocycling process therefore it is important that there are no mismatches of the target DNA at the 3'end of the oligonucleotide PCR primers. HPV types 26, 39, 51, 56, 67, 69, 70 and 73 had a mismatch of an adenine at the second last position of the 3'end of the outer forward HPV primer and HPV types 26, 39, 42, 51, 53, 54, 69, 83 and 84 had a mismatch of a cytosine at the second last position at the 3'end of the outer reverse HPV primer. These mismatches could interfere with primer extension in particular with regards to the outer PCR reaction of HPV 26, 39, 51 and 59 which had mismatches at both the outer forward and reverse primers.

Three of the HPV types (HPV 35, 57 and 82) showed nucleotide inserts within the outer reverse primer region (Table IX) and HPV types 35 and 82 had mismatches at the 3' end of the outer reverse primer with three of the four nucleotides at the 3'end of HPV 35 mismatched. HPV 35 and 57 also had a mismatch of a cytosine at the second last nucleotide at the 3' end of the inner forward nested primer. Despite the mismatches and the nucleotide inserts the primers were able to amplify cloned HPV 35 and detect HPV 35 in cervical

Table VIIIa. Alignment of the outer forward and reverse HPV oligonucleotide primers to mucosal HPV types.

	Outer Forward Primer: 5'-CAGGATGGvGAwATGGT - 3'	Outer Reverse Primer:* 3' -CxCGCAGTACyAAwATG -5'
HPV 6a:	CAGGATGGCGATATGGT	CACGCAGTACCAACATG
HPV 6b:	CAGGATGGCGATATGGT	CACGCAGTACCAACATG
HPV 11:	CAGGATGGGGACATGGT	CACGCAGTACAAATATG
HPV 16:	CAGGATGGTGATATGGT	CACGCAGTACAAATATG
HPV 18:	GAAGATGGTGATATGGT	CTCCCAGTACCAATTTA
HPV 26:	GAGGATGGCGATATGAT	CCCGCAGTACTAACCTT
HPV 31:	CAAGATGGGGATATGGT	CACGTAGTACCAATATG
HPV 33:	GAGGATGGTGATATGGT	CTCGCAGTACTAATATG
HPV 34:	CAGGATGGTGATATGAT	CTAGAAGCACAAACTTT
HPV 39:	GAGGATGGTGATATGAT	CCCGTAGTACCAACTTT
HPV 40:	CAGGATGGCGACATGGT	CTCGTAGCACTAATTTA
HPV 42:	CAGGATGGGGATATGGT	CCCGTAGTACTAACATG
HPV 45:	GAGGATGGTGATATGGT	CCCGCAGTACTAATTTA
HPV 51:	CAGGATGGCGATATGAT	CCAGAAGTACAAATTTA
HPV 52:	CAGGATGGGGACATGGT	CTCGTAGCACTAACATG
HPV 53:	GAGGATGGAGACATGGT	CCAGGAATACAAACATG
HPV 54:	CAGGATGGTGATATGGT	CCCGTAGTACTAACCTA
HPV 55:	GAAGATGGTGATATGGT	CACGTAGTACAAACATG
HPV 56:	GAGGATGGGGACATGAT	CTAGAAGTACTAACATG
HPV 58:	CAGGATGGTGACATGGT	CTCGTAGCACTAATATG
HPV 59:	GAAGATGGTGATATGGT	CTCGCAGCACCAATCTT
HPV 67:	GAGGATGGGGACATGAT	CACGTAGTACCAACATG
HPV 69:	GAGGATGGTGATATGAT	ACCGCAGTACCAACCTC
HPV 70:	GAGGATGGCGATATGAT	CACGTAGTACTAATTTT
HPV 71:	CAGGATGGTGATATGGT	CACGTATTACAAATATG
HPV 72:	CAGGATGGTGACATGGT	CTCGCAGTACTAATGTA
HPV 73:	CAGGATGGTGATATGAT	CTAGAAGCACTAATTTT
HPV 83:	CAGGATGGCGACATGGT	CCCGCAGTACCAATATT
HPV 84:	GAGGATGGTGATATGGT	CCCGCAGCACCAATTTT

Nucleotides homologous to the primers are in black script and bold red underlined script indicates a mismatch. The reverse primer is shown as the inverse complement. Ambiguity codes are as follows: v = T,G,C; w = T,C; x = A,T; y = A,T,C; z = G,C.

Table VIIIb Alignment of the inner forward and reverse oligonucleotide primers to mucosal HPV types

	Inner Forward Primer: 5' - TGwAAATATCCxGATTATxT - 3'	**Inner Reverse Primer:** 3' - GATACCACxCzCAGTAC - 5'
HPV 6a:	TGTAAATATCCAGATTATTT	GATACCACACGCAGTAC
HPV 6b:	TGTAAATATCCAGATTATTT	GATACCACACGCAGTAC
HPV 11:	TGCAAATATCCTGATTATTT	GATACCACACGCAGTAC
HPV 16:	TGCAAATATCCAGATTATAT	GATACTACACGCAGTAC
HPV 18:	TGTAAATATCCTGATTATTT	GATACCACTCCCAGTAC
HPV 26:	TGTAAATATCCTGATTATCT	GATACCACCCGCAGTAC
HPV 31:	TGTAAATATCCAGATTATCT	GATACCACACGTAGTAC
HPV 33:	TGCAAATATCCAGATTATTT	GATACCACTCGCAGTAC
HPV 34:	TGTAAATATCCAGATTATCT	GATACTACTAGAAGCAC
HPV 39:	TGTAAATATCCTGATTATTT	GACACTACCCGTAGTAC
HPV 40:	AGTAAATATCCAGATTATTT	GACACCACTCGTAGCAC
HPV 42:	ACTAAATATCCTGATTACTT	GATACTACCCGTAGTAC
HPV 45:	TGTAAATATCCAGATTATTT	GACACTACCCGCAGTAC
HPV 51:	TGTAAATATCCTGATTATTT	GATACTACCAGAAGTAC
HPV 52:	TGTAAGTATCCTGATTATTT	GATACCACACGCAGTAC
HPV 53:	TGTAAATATCCCGATTATTT	GATACCACCAGGAATAC
HPV 54:	TGTAAATATCCTGATTACCT	GATACCACCCGTAGTAC
HPV 55:	TGCAAATATCCTGACTATTT	GATACTACACGTAGTAC
HPV 56:	TGTAAATATCCTGACTATTT	GATACTACTAGAAGTAC
HPV 58:	TGCAAATATCCAGATTATTT	GATACCACTCGTAGCAC
HPV 59:	TGTAAATATCCTGATTATTT	GATACTACTCGCAGCAC
HPV 67:	TGTAAATATCCTGATTATCT	GACACTACACGTAGTAC
HPV 69:	TGCAAATATCCAGATTACCT	GATACTACCCGCAGTAC
HPV 70:	TGTAAATATCCTGATTATTT	GACACTACACGTAGTAC
HPV 71:	TGTAAATAT*CAGATTATTT	GACACATCACGTAGTAC
HPV 72:	TGCAAATATCCTGACTATTT	GATACTACTCGCAGTAC
HPV 73:	TGTAAATACCCAGATTATTT	GATACTACTAGAAGCAC
HPV 83:	TGTAAATATCCTGATTATTT	GATACTACCCGCAGTAC
HPV 84:	TGTAAATATCCTGATTATTT	GATACCACCCGCAGCAC

Nucleotides homologous to the primers are in black script and bold red underlined script indicates a mismatch. An asterisk denotes a nucleotide deletion. The reverse primer is shown as the inverse complement. Ambiguity codes are as follows: v = T,G,C; w = T,C; x = A,T; y = A,T,C; z = G,C.

Table IX. Alignment of HPV types 35, 57 and 82 to oligonucleotide primers.

	Outer Forward Primer:	**Outer Reverse Primer:**
	5'- CAGGATGGvGAwATGGT – 3'	3'- CxCGCAGTACyAAwATG – 5'
HPV 35:	CAAGACGGGGACATGGT	GTGACAGTAC AAAAATG
		⇧ ATAT
HPV 57:	GAAGATGGGGATATGGT	CACGC AGCACAAATGTC
		⇧ CG
HPV 82:	GAGGATGGCGATATGGT	TAAGCAGTACA TA CATG
		⇧ T ⇧ AGG
	Inner Forward Primer:	**Inner Reverse Primer:**
	5' - TGwAAATATCCxGATTATxT – 3'	3'-GATACCACxCzCAGTAC – 5'
HPV 35:	TGCAAATATCCTGATTATCT	GATACAACCCGTAGTAC
HPV 57:	TGTAAATATCCAGACTATCT	GACACCACGCGCAGCAC
HPV 82:	TGTAAATACCCTGATTACTT	GACACTACTAAAAGTAC

Arrows indicate the position of nucleotide inserts in the region of the outer reverse primer. The outer and inner reverse primers are shown as the inverse complement. Bold red underlined letters indicate a mismatch. Ambiguity codes are as follows: v = T,G,C; w = T,C; x = A,T; y = A,T,C; z = G,C.

biopsy samples. This is probably due to the low annealing temperature which allows for maximum amplification efficiency and for mismatches between the primers and template DNA. No plasmids of HPV 57 and 82 were available and no biopsy samples appeared to contain these two HPV types when typed by nested PCR and RFLP, as well as the reverse line blot assay. Therefore it could not be verified as to whether the HPV nested primers would efficiently amplify HPV 57 and 82.

3.3 RFLP Analysis

3.3.1 Mucosal HPV types identified by RFLP

To ensure that the RFLP patterns would differentiate between the mucosal HPV types and that the RFLP pattern would be unique to each mucosal HPV type, the RFLP was designed to be performed in 2 stages viz. a screening restriction endonuclease digest and a confirmatory restriction endonuclease digest where required. The screening digest was performed with *BstE*11 and a double digest of *Pst*1 and *Bgl*1 and depending on the pattern of the screening digest, selected restriction enzymes where then used for a confirmatory digest.

HPV types 16, 18, 31, 33, 35, 45, 51, 52, 58 and 59 were identified by digesting the PCR fragments with the restriction endonucleases as shown in Table X. In addition the low risk HPV 11, which is commonly found in the oral mucosa, was also identified. The RFLP was able to identify a wider range of mucosal HPV types if additional restriction endonucleases were used, but the assay was limited to the eleven HPV types listed above due to their clinical significance.

When viewing the restriction endonuclease digests on an agarose gel, the fragment sizes of the screening digests could be established by comparison to the known fragments of the molecular weight marker electrophoresed on the same gel. After establishing the sizes of the fragments it was possible to identify the HPVs present by comparing the fragments to those listed in Table X to establish which HPV types were present in the sample. HPV types 16, 52, 35 and 58 were identified by the screening digest only. The other seven HPV types required a confirmatory digest with the restriction endonucleases listed in Table X.

Table X. RFLP patterns generated by the restriction endonucleases for the screening and confirmatory digests

Screening Digests			**Confirmatory Digests**					
	Pst1/Bgl1	*BstE11*	*Pvu11*	*Nco1*	*ApaL1*	*Eco47111*	*Bgl11*	*Dra111*
HPV31	—	—		126+209				
HPV45	—	—				160+182		
HPV59	—	—					103+232	
HPV16	—	43+79+213						
HPV52	—	109+110+119						
HPV18	33+302	—	179+156					
HPV33	86+249	—			75+260			
HPV35	135+194	122+207						
HPV58	81+86+168	—						
HPV51*	33+86+219	—						
HPV11	14+302	—						133+183

Fragment sizes generated by the restriction endonucleases are given in base pairs.
— indicates no digestion with the restriction endonuclease.
*HPV 51 needs to be confirmed by sequencing as the pattern generated is the same as HPV 57

Sequence analysis by DNAman software showed that HPV 18 contains a unique *Pvu*11 site resulting in 2 fragments of 156 and 179 bp and that HPV 16 generates a unique pattern of 43, 79 and 213 bp when digested with *BstE*11.

3.3.2 Confirmation of RFLP patterns with HPV clones

RFLP analysis of plasmids containing inserts of the full genomes of HPV types 16, 18, 31, 33, 35, 45, 58 and 59 was performed to confirm the predicted RFLP patterns. The RFLP patterns of all the clones were the same as predicted by DNAman analysis. Figure 11 shows the RFLP patterns of clones of HPV types 35, 31, 33, 45 and 52 compared against molecular weight marker VIII (Boehringer Mannheim) (Appendix C).

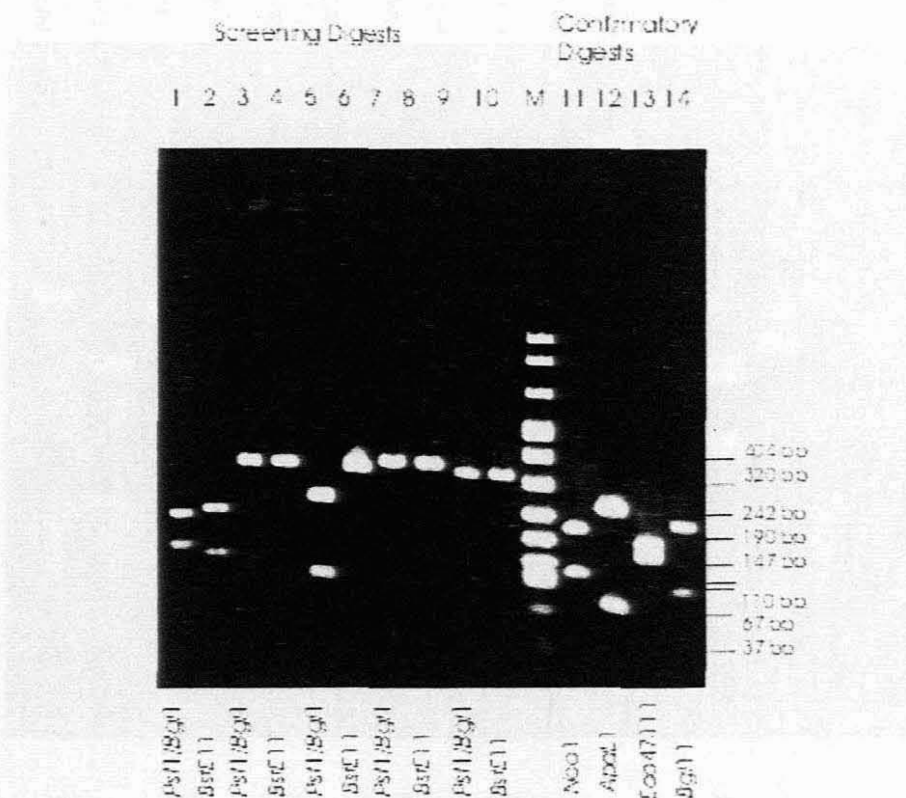

Figure 11. Screening and confirmatory digests of HPV clones 35, 31, 33, 45 and 52. The screening digests (*Pst*1/*Bgl*1 and *BstE*11 for each clone) are to the left of the molecular weight marker V111. The confirmatory digests (where required) are to the right of the molecular weight marker V111. Lanes 1 and 2 are the RFLP fragments for HPV 35; lanes 3,4 and 11, HPV31; lanes 5,6 and 12, HPV33; lanes 7,8 and 13, HPV45 and lanes 9,10 and 14, HPV 52. The base pair sizes of the molecular weight marker are as follows: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 and 37/34/26.

3.3.3 Identification of single HPV infections by RFLP

Single HPV infections were indicated by complete digestion of the PCR fragment with the restriction endonucleases in the screening and confirmatory digests. The following known HPV types were identified in the buccal and biopsy samples by RFLP: 11, 16, 18, 31, 33, 35, 52 and 59. Figure 12 shows the screening and confirmatory digests of HPV types 16, 18, 31, 33 and 52 which were detected in biopsy samples.

Figure 12. Screening and confirmatory digests of single infections of HPV 16, 18, 31, 33 and 52 in biopsy samples. All HPV types were detected in biopsies. The screening digests are to the left of the molecular weight marker VIII and confirmatory digests are to the right of the molecular weight marker VIII. The base pair sizes of the molecular weight marker are as follows: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 and 37/34/26.

3.3.4 Identification of dual/multiple HPV infections by RFLP

The RFLP was able to detect the presence of dual/multiple infections in the biopsy and buccal samples. A dual/multiple infection was indicated by the presence of an undigested band in the screening and confirmatory digests. Figure 13 shows a dual infection of HPV 11 and 18 and a triple infection of HPV 11, 16 and 18 that were detected in two of the buccal samples. As can be noted in table X, digestion with the *Pst*1/*Bgl*1screening enzymes results

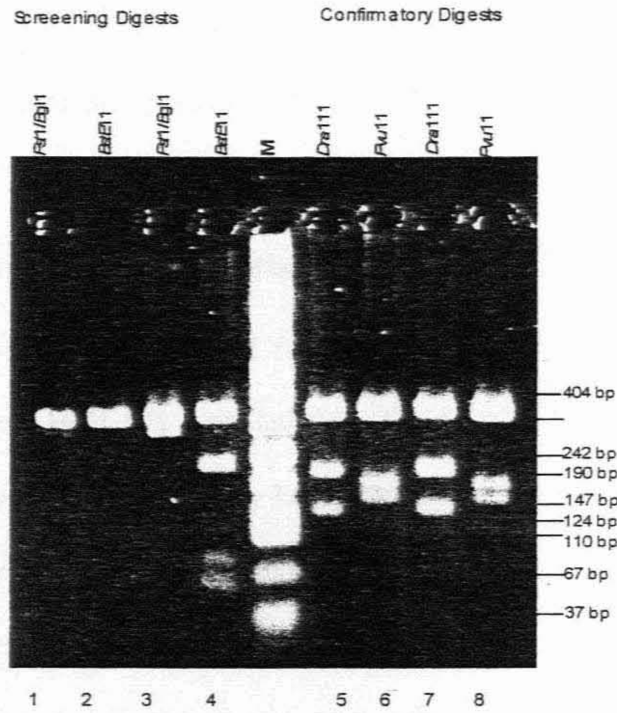

Figure 13. Screening and confirmatory digests of dual/multiple infections identified in buccal samples. Lanes 1 (*Pst*1/*Bgl*1) and 2 (*Bst*EII) , are screening digests and lanes 5 (*Dra*III) and 6 (*Pvu*II) the confirmatory digests for a dual infection of HPV 11 and 18. Lanes 3 (*Pst*1/*Bgl*1), 4 (*Bst*EII), are the screening digests and lanes 7 (*Dra*III) and 8 (*Pvu*II) the confirmatory digests for a triple infection of HPV 11, 16 and 18. The sizes of the molecular weight marker VIII in base pairs are as follows: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 and 37/34/26.

in two fragments of 14 and 302 base pairs for HPV 11 and 33 and 302 base pairs for HPV 18. Due to the size of the smaller fragments it was noted that they were often not visible on an agarose gel as can be seen in lane 1 of figure 13. However, the small digested fragments along with the 302 base pair fragment are visible in the *Pst*1/*Bgl*1 digest in lane 3 of figure 15. Neither HPV 11 nor HPV 18 digest with *Bst*E11, therefore no digested products were viewed for the screening digest for the dual infection of HPV 11 and 18. Confirmatory digests (as laid out in table X) were performed to establish which HPV types were present. Digestion with *Dra*III yielded two fragment sizes of +/-133 and 183 base pairs and digestion with *Pvu*II yielded two fragments of +/-179 and 156 base pairs indicating the presence of both HPV 11 and 18. The triple infection indicated exactly the same digestion

results with the exception of the *Bst*EII digest which yielded three bands of +/- 43, 79 and 213 base pairs indicating the presence of HPV 16.

3.3.5 Identification of novel/variant HPVs by RFLP

No novel HPV types were detected in this study, however a newly described HPV, HAN2294 (candidate HPV 87), was detected. The HAN2294 DNA sequence was lodged directly with the Genbank database by Menzo *et al.* (2001) and is still being subjected to final NCBI review as it is not yet published. HAN2294 was detected in a biopsy from a patient with CIN 2. The RFLP pattern showed the presence of HPV 16 and an undigested fragment. The undigested fragment was gel purified and then sequenced as described in 2.9.1 of materials and methods. The product was sequenced off the forward inner primer. Figure 14 shows the alignment of the nested inner product of the unknown HPV to HAN2294. The HPV DNA amplified from the biopsy showed a 99.4% homology to HAN2294 with the only difference in the sequenced region being a thymine at nucleotide position 193.

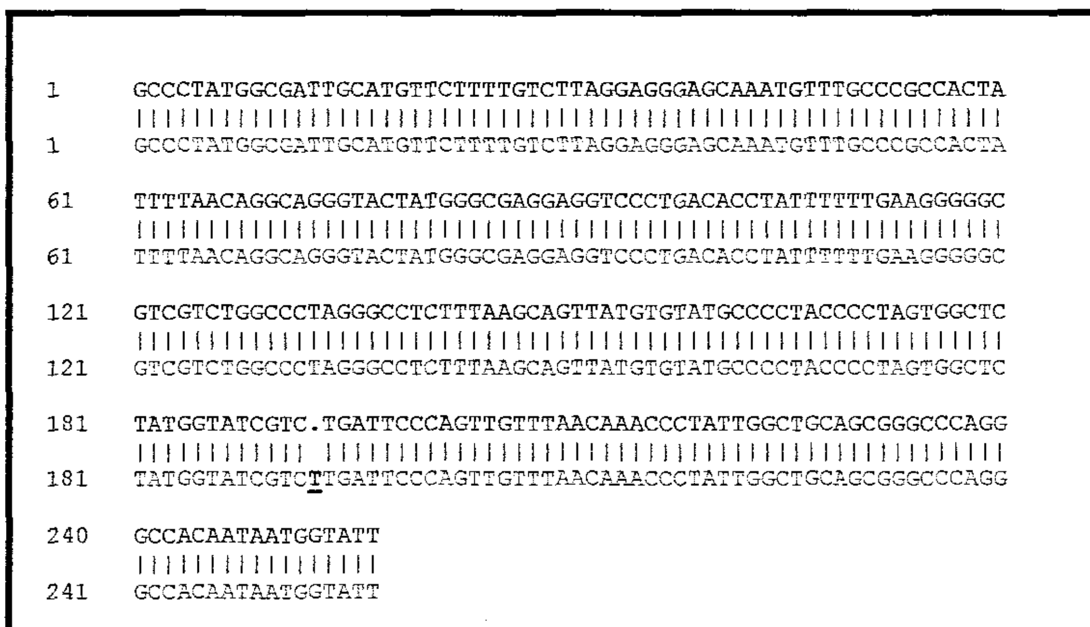

Figure 14. Alignment of HAN2294 to an unknown HPV detected in a biopsy sample. Script in black denotes the prototype sequence lodged with Genbank by Menzo *et al.* Script in red denotes the sequence of the HPV type detected in the biopsy sample.

An HPV amplified from a CIN 3 biopsy showed the *Pst*1/*Bgl*1 and *ApaL*1 RFLP pattern of HPV 33, as well as the *Nco*1 cut site of HPV 31. These results were confirmed by sequencing of the inner product of the nested PCR. The electropherogram of the sequence, however, had a high background indicating the possibility of a dual infection although the endonuclease restriction digest had shown complete digestion of the fragment. To ensure that the RFLP pattern represented a single infection, cloning was performed as described in 2.8. of materials and methods. Two clones (A and B) are shown in figure 15 confirming that the HPV had both a *Nco*1 cut site of HPV 31 and a *ApaL1* cut site of HPV 33 and therefore both sites were on the same fragment and not the result of a dual infection. Sequencing of the genome amplified by the inner primers revealed a 98% homology to HPV 33 indicating that this could possibly be a variant. The full length L1 gene would need to be sequenced to confirm this result.

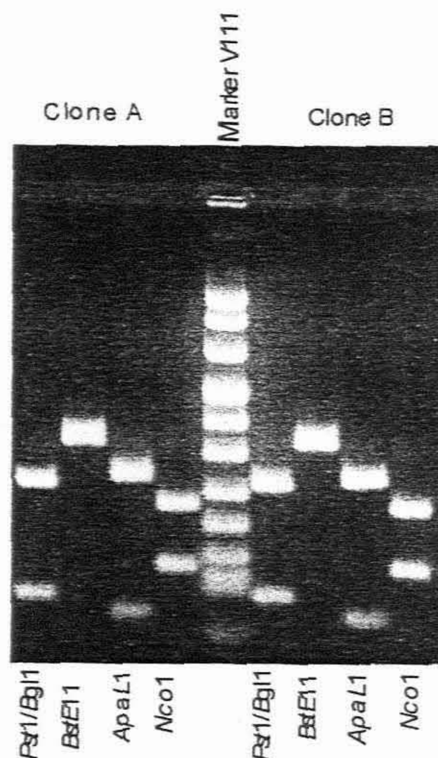

Figure 15. Screening and confirmatory digests of clones showing the *Nco*1 site of HPV 31 and *ApaL*1 site of HPV 33 within the same virus. Clone A is positioned to the left of the marker and clone B to the right of the marker. The sizes of the molecular weight marker VIII in base pairs are as follows: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 and 37/34/26.

Five of the biopsies showed the same *Pst*1/*Bgl*1 cut sites as HPV 58. However, unlike HPV 58, they also digested with *BstE*11 yielding 2 fragments of 126bp and 209bp (lane 4 of figure 16).

All of the above samples were sequenced and shown to be a variant of HPV 58. This sequence had previously been lodged with the NCBI Genbank by this laboratory in 1991 as CIN 46 (accession number M72146.1). The *BstE*11 cut site is the result of a nucleotide change at position 6539 of an adenine in the wild type HPV 58, to guanine in the local

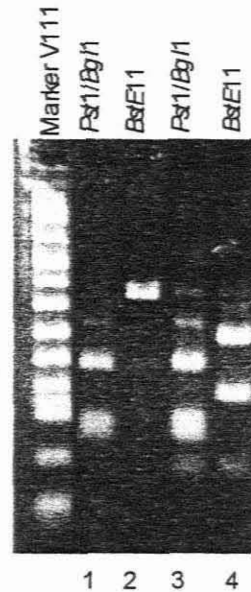

Figure 16. Screening and confirmatory digests of a variant of HPV 58. Lanes 1 and 2 are the *Pst*1/*Bgl*1 and *BstE*11digests of the wild type HPV 58 and lanes 3 and 4 the digests of the local variant of HPV 58. Wild type HPV 58 does not digest with *BstE*11 whereas the local variant yields 2 fragments of 126bp and 209bpThe sizes of the molecular weight marker VIII in base pairs are as follows: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 and 37/34/26.

variant (highlighted in red in Figure 17). This translates into an amino acid change of isoleucine to methionine. (Figure 18). Despite nucleotide changes at other positions in some of the sequences of the HPV 58 variant, none of these point mutations translated into an amino acid change.

Studies have shown that neutralizing antibody responses against the L1 protein of HPV are sufficient to protect against papillomavirus infection in animals immunized with virus-like particles (VLPs) composed of the L1 major capsid protein (Breitburd *et al.*, 1995). It would therefore be desirable for a prophylactic vaccine to induce a neutralizing antibody response. However, HPV L1 neutralizing epitopes are conformation dependant and changes in amino

```
AGTTTGTTCTTTTTTCTTAGACGTGAGCAGATGTTTGTTAGACACTTTTTTAATAGGGCT      HPV 58
                             G           G                 C      Biopsy C39
                             G           G                 C      Biopsy C54
                             A           A                 T      Biopsy D17
                             G           G                 C      Biopsy D77
                             G           G                 C      Biopsy D36
***************************** *********** *****************

GGAAAACTTGGCGAGGCTGTCCCGGATGACCTTTATATTAAAGGGTCCGGTAATACTGCA      HPV 58
                       G                                          Biopsy C39
                       G                                          Biopsy C54
                       T                                          Biopsy D17
                       G                                          Biopsy D77
                       G                                          Biopsy D36
*********************** ************************************

GTTATCCAAAGTAGTGCATTTTTTCCAACTCCTAGTGGCTCTATAGTTACCTCAGAATCA      HPV 58
 T   C                                      G                     Biopsy C39
 T   C                                      G                     Biopsy C54
 C   T                                      G                     Biopsy D17
 T   C                                      G                     Biopsy D77
 T   C                                      G                     Biopsy D36
* *** ************************************** ***************

CAATTATTTAATAAGCCTTATTGGCTACAGCGTGCACAAGGTCATAACAATGGCATTTGC



************************************************************

TGGGGCAATC                                                        HPV 58
                                                                  Biopsy C39
                                                                  Biopsy C54
                                                                  Biopsy D17
                                                                  Biopsy D77
                                                                  Biopsy D36
**********
```

Figure 17. Sequence alignment of the inner PCR product of HPV 58 wild type with the 5 local variants of HPV 58. An asterisk indicates sequence homology between the wild type and all 5 variants. Bold, black, underlined nucleotides indicate a discrepancy when compared with the wild type sequence. The nucleotide change at position 6539 of an adenine in the wild type HPV 58 to guanine in the local variant is highlighted in red script.

HPV 58 Wild Type:	KMASEPYGDSLFFFLRREQMVRHFFNRAGKLGEAVPDDLYIKGSGNTAV
HPV 58 Variant:	KMASEPYGDSLFFFLRREQMVRHFFNRAGKLGEAVPDDLYIKGSGNTAV
HPV 58 Wild Type:	IQSSAFFPTPSGSITSESQLFNKPYWLQRAQGHNNGICWGNQLFVTVVDT
HPV 58 Variant:	IQSSAFFPTPSGSMTSESQLFNKPYWLQRAQGHNNGICWGNQLFVTVVDT

Figure 18. Amino acid alignment of the inner PCR product of the wild type HPV58 with the local variant of HPV 58. The amino acid change is highlighted in red script.

acids within this region could result in a different conformation. If HPV 58 were to be included in a prophylactic vaccine for South African women, further studies would need to be performed to establish if the amino acid change in the local HPV 58 variant results in a conformational change, because a vaccine incorporating the wild type HPV 58 might not raise neutralizing antibodies.

3.3.6 Dual infections

3.3.6.1 Selection of recombinants

The presence of dual/multiple infections were indicated by an undigested fragment on an ethidium bromide stained agarose gel after restriction endonuclease digestion. To present proof of dual/multiple HPV infections by RFLP, the inner nested PCR product was cloned into pGem®-T easy vector (chapter 2.8.2), transformed into competent DH5α *E.Coli* cells (chapter 2.8.4) and plated onto 2 x YT agar with IPTG, X-gal and ampicillin. After overnight incubation 5 white colonies were selected and colony PCR performed as previously described in 2.4.5 of methods and materials.

3.3.6.2 Proof of dual/multiple HPV infection by RFLP

The PCR products from the 5 colonies were purified using a Qiagen PCR clean up kit and RFLP was performed on the inner amplified product of the colony PCR as described in 2.6 of methods and materials. If all 5 clones showed the same RFLP pattern a further 5 white colonies were selected and the process repeated until clones with different RFLP patterns were selected.

An example of a dual infection is shown in Figure 19 with the original RFLP patterns and the RFLP patterns after cloning. The original RFLP pattern indicated the presence of both HPV 16 and HPV 31 by RFLP as the screening *BstE*11 pattern showed the unique pattern of HPV 16 and an undigested band indicated the presence of another HPV type. A confirmatory digest with *Nco*1 yielded fragment sizes consistent with HPV 31 and an undigested band. Clone A proved to be HPV 31 as it did not digest with either of the screening enzymes, but did digest with *Nco*1 giving fragment sizes consistent with HPV 31. Clone B proved to be HPV 16 as indicated by the unique pattern generated by digestion with *BstE*11. Both clones digested completely with their respective enzymes indicating that only 1 HPV type was present in each clone.

3.4 Sequencing results

HPV types 13, 26, 58 (variant), 68 and HAN2294 were not identified by the RFLP and were therefore typed by sequencing as described in 2.9.1 of methods and materials. A further 30 randomly chosen samples were sequenced to confirm the RFLP results and the

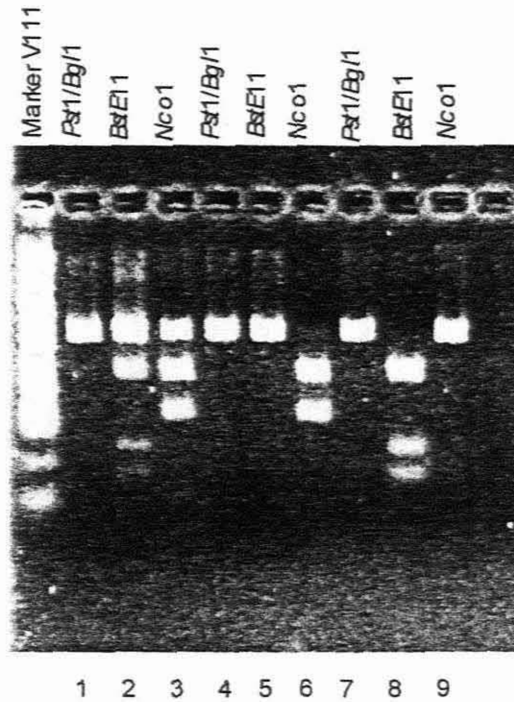

Figure 19. Screening and confirmatory digests showing the original RFLP patterns of a dual infection and the RFLP patterns of 2 of the clones. Lanes 1, 2 and 3 show the original RFLP pattern (HPV 16 and 31). Lanes 4, 5 and 6 show the RFLP pattern of clone A (HPV 31). Lanes 7, 8 and 9 show the RFLP pattern of clone B (HPV 16).

presence of dual infections. Sequencing of these samples identified only single HPV infections (figure 20a) or the predominant type of a dual infection (figure 20b). The presence of a dual HPV infection was visible by the presence of smaller peaks underlying the peaks of the predominant HPV type in the electropherogram. But as the peaks in the electropherogram reflect the signal intensity of each fluorescent dye labelled nucleotide, only the nucleotides of the HPV type with the higher signal intensity are recorded by the sequencer software.

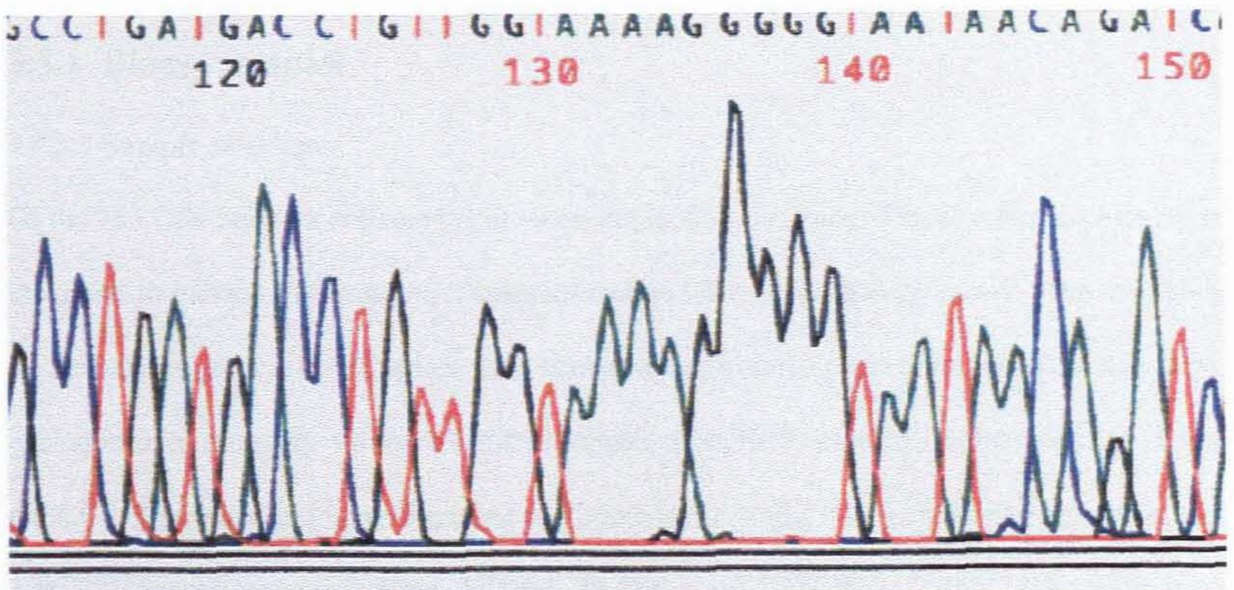

Figure 20a. Electropherogram of a sequence of a single HPV infection. No smaller underlying peaks are present indicating that only one HPV type is present in the sample.

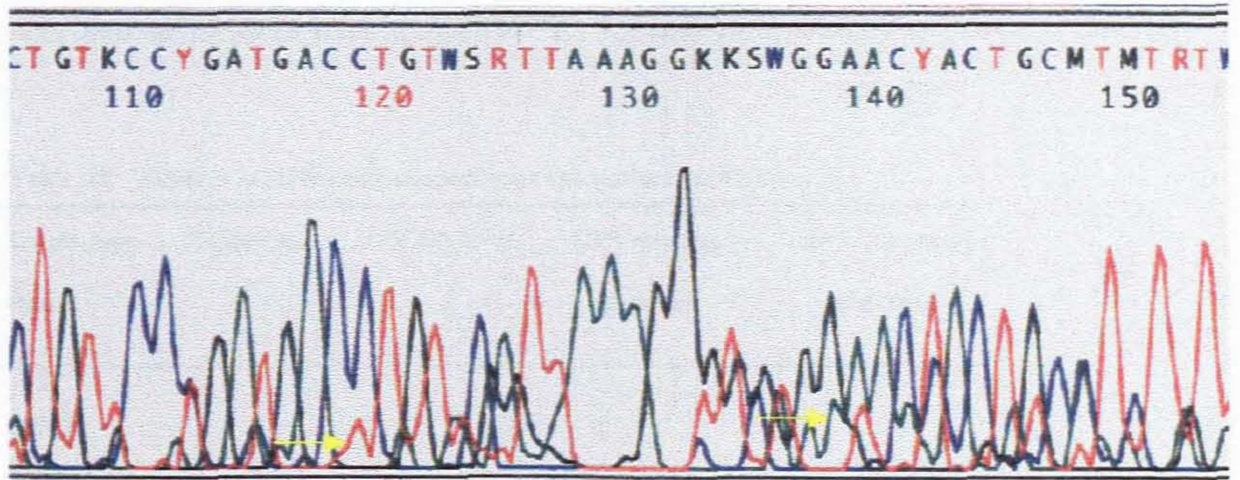

Figure 20b. Electropherogram of a sequence of a dual HPV infection. The second infection is indicated by the underlying smaller peaks. The yellow arrows highlight two of the peaks of the underlying dual infection.

3.5 Epidemiological data

3.5.1 Biopsy samples

3.5.1.1 Sample selection

Of the 163 CIN biopsies collected, 150 were included in the study. Three of the biopsies were too small to include in the study; 3 biopsies had no CIN; no pathology results were available on 3 of the biopsies and 4 biopsies were shown to be invasive cancer and therefore had to be excluded from the study. Of the 150 CIN biopsies, 139 (92%) showed amplifiable DNA using the CCR 5 primers and were therefore included in the study. The colposcopy results on the 139 CIN biopsies were as follows: 4 CIN 1, 26 CIN 2 and 109 CIN 3.

3.5.1.2 HPV prevalence rates in biopsy samples using nested PCR

Of the 139 biopsies showing amplifiable DNA, 115 (83%) were positive for HPV DNA using the nested PCR protocol. Two of the 4 CCR 5 positive CIN1 lesions, 16/26 of the CIN 2 lesions and 97/109 of the CIN 3 lesions were positive for HPV DNA. There were 16 dual infections, all of which were detected in CIN 3 biopsies and 99 single infections. (Table XI)

Table XI. Number of HPV positive specimens categorized by lesion grade

Colposcopy Result	CCR5+ve	HPV DNA+ve	HPV+ve dual infections	HPV+ve single infections
CIN 1	4	2	0	2
CIN 2	26	16	0	16
CIN 3	109	97	16	81
Total	139	115	16	99

The CIN 3 tumours showed a higher prevalence of high risk HPV DNA (Figure 21) and analysis of these results using Epi Info version 5 software (Centre for Diseases Control Epidemiology Program Office, Atlanta, Georgia) indicated that CIN 3 lesions were statistically associated with HPV infection when compared to the CIN 1 and CIN 2 lesions (*P* value=0.0002 and odds ratio 5.39 [1.90<OR<15.44] and relative risk 1.69 [1.12<RR<2.54]).

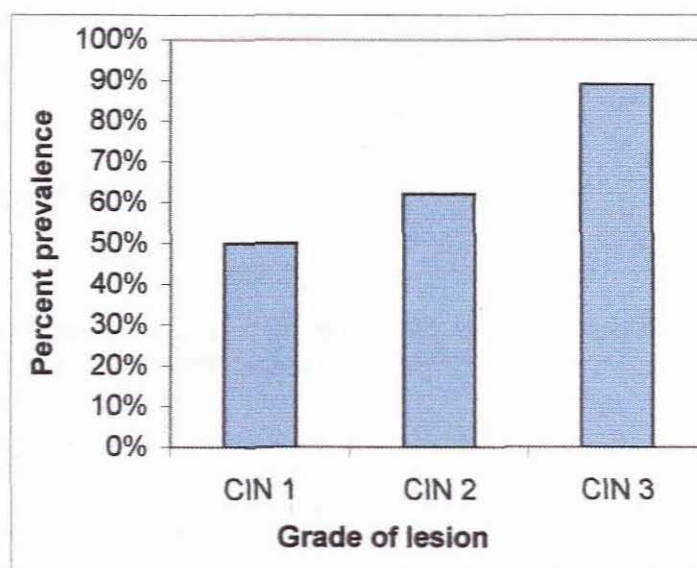

Figure 21. Prevalence rate of high risk HPV in CIN 1, 2 and 3 cervical lesions

3.5.1.3 HPV types detected in biopsy samples using RFLP

Twelve different HPV types were detected across all grades of lesions (Table XII). It has been shown that low grade cervical lesions are more likely to harbour low risk HPV types and high grade lesions are more likely to harbour high risk types (Lombard *et al.* 1998). However, due to the small number of CIN 1 and CIN 2 lesions in this study, no statistical inferences could be drawn on the HPV types detected in the different grades of lesions. Although when single

infections were compared to dual HPV 16 was statistically associated with dual infections (*P* value=0.003).

Table XII. Number of HPV types detected in the HPV DNA+ve CIN lesions

Colposcopy Result	HPV types											
	16	**33**	**31**	**58**	**35**	**18**	**26**	**52**	**6**	**11**	**68**	**HAN2294**
CIN 1(n=2)	1	1										
CIN 2(n=16)	6	3	1	2	3		1					
CIN 3(n=97)*	72	16	13	3	1	2	1	2	1	1	1	1
Total	**79**	**20**	**14**	**5**	**4**	**2**	**2**	**2**	**1**	**1**	**1**	**1**

n= number of HPV positive specimens. As all the dual infections were detected in CIN 3 lesions the total number of HPVs detected is greater than 97

The four most prevalent HPV types were HPV 16 (67%), HPV 33 (16%), HPV 31 (11%) and the local variant of HPV 58 (4%) and would therefore need to be included in a prophylactic vaccine developed for this cohort. An international study by Bosch *et al.* (1995) showed HPV 16 to be the predominant HPV type in CIN lesions and in cervical cancer biopsies. As the predominant HPV type detected in this study was also HPV 16 it would appear that the results are in keeping with international results.

HPV 35 was detected in three biopsies of which two were CIN 2 lesions and one a CIN 3 lesion. There were five HPV 58 positive biopsies of which two were CIN 2 lesions and three were CIN 3 lesions. Both HPV 35 and 58 belong to the A9 group of HPVs which includes HPV 16, 31, 33, 35, 52, 58 and 67. Of the sixteen HPV DNA positive CIN 2 lesions, fifteen

contained HPV types of group A9. Only one CIN 2 lesion contained an HPV from another group ie. HPV 26 which belongs to the A5 group. This is in contrast to a study by Matsukura and Sugase (2001) in which they found group A9 to be predominant in CIN 3 lesions and groups A5, A6 and A7 to be predominant in CIN 2 lesions.

The HPV types identified in the CIN 3 lesions were also predominantly from the A9 group with 95/97 HPV positive CIN 3 lesions containing HPVs of group 9. All sixteen dual infections detected in the CIN 3 lesions contained group 9 HPV types. Both HPV types were from group 9 in 14/16 dual infections and 2/16 dual infections contained an HPV type from group 9. This high prevalence of group 9 HPV types in CIN 3 lesions is consistent with the results in the study by Matsukura and Sugase (2001).

3.5.2 Buccal samples

3.5.2.1 Sample selection

All thirty one buccal samples showed amplifiable DNA by PCR using the CCR5 primers and could therefore be included for HPV typing.

3.5.2.2 HPV prevalence rates in buccal samples using nested PCR

HPV DNA was detected in 23 of the 31 buccal samples giving a prevalence rate of 74%. In contrast studies have shown that the prevalence rate of HPV DNA at the cervix in sexually active women with normal cervical pathology ranges from 20%-40% (Bosch, *et al.* 1995). The prevalence rate of HPV DNA in the buccal samples with normal pathology in this study therefore appears to be high when compared to the HPV DNA prevalence rate in normal cervical samples. However, HPV prevalence rates in other studies on normal oral pathology varies from 1% to 81.1% (Kelloski *et al.*, 1992; Ostwald *et al.*, 1994; Badaracco *et al.*, 1998

and Terai *et al.*, 1999) indicating that the results obtained in this study are in keeping with the published literature.

3.5.2.3 HPV types detected in buccal samples using RFLP

Two of the samples positive for HPV DNA had insufficient DNA for HPV typing, therefore only 21 samples were typed. Thirteen of the samples (62%) were single HPV infections, 5 (24%) were dual infections and 3 (14%) were triple infections.

HPV types 11, 13, 16, 18 and 33 were detected in the buccal samples (Figure 22). The prevalence rates of the different HPV types were as follows: HPV 11 (18/23), HPV 18 (8/23), HPV 16 (3/23), HPV 13 (2/23) and HPV 33 (1/23).

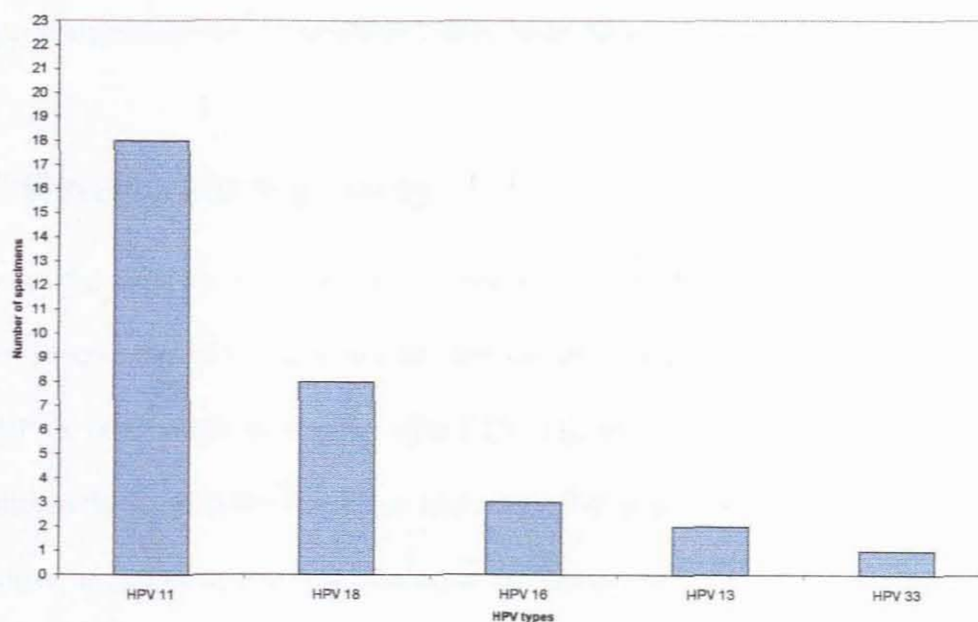

Figure 22. Prevalence of HPV types detected in buccal specimens

3.6 Correlation of HPV types between buccal and biopsy samples

Two of the patients did not have amplifiable DNA at the cervix and were therefore not able to be included in the correlation study and fifteen patients were HPV positive at both sites. Only two patients had the same HPV type at both sites. HPV 11 was detected in the oral cavity and the cervix in one patient and a second patient had HPV 16 at the cervix and HPV 16, 11 and 18 in the oral cavity. As the genomes were not sequenced it cannot be stated that the same HPV genome was infecting both sites. Despite this, it was concluded that there was no correlation between the HPV types found in the oral cavity and those found in the cervix of the same patient as only two of the patients had the same HPV type at both sites (Table XIII). If, however, a larger proportion of the patients had the same HPV types at both sites, the RFLP would not have been an appropriate method of establishing a correlation between the HPV genomes infecting both sites. PCR, followed by cloning of the PCR product and sequencing would have generated more reliable data.

3.7 Reverse line blot assay

Two of the aims of this study were to develop an RFLP method based on the amplicons of a sensitive nested PCR and to use this method for establishing the prevalence rate and types of HPVs in a cohort of women with CIN. The method was validated by comparing the results to the published reverse line blot assay. The epidemiological results for the subset of biopsies using the reverse line blot assay are summarised in Table XIV. PCR amplification of the human B globin gene was performed to ensure that there were no PCR inhibitors present and is used on the strip of immobilised probe lines as an internal control. HPV X indicates a positive PCR results with the consensus HPV PGMY09/PGMY11 primers but the HPV type was not identified by the reverse line blot assay.

Table XIII. Correlation of HPV types between the oral cavity and cervix.

	Biopsy Samples			Buccal Samples		
	CCR5 PCR	HPV PCR	HPV Type	HPV Type	HPV PCR	CCR5 PCR
Sample No.						
1	+	+	16	11+18	+	+
2*	+	+	11	11	+	+
3	+	+	35	11+16+18	+	+
4	+	+	58		-	+
5	+	+	16+33		-	+
6	+	+	16	11	+	+
7	+	+	16	11	+	+
8	-	-		13	+	+
9	+	+	16		-	+
10	+	-		13	+	+
11	+	+	16		-	+
12*	+	+	16	11+16+33	+	+
13	+	-		11	+	+
14	+	+	31	11+16+18	+	+
15	+	+	31	11	+	+
16	+	+	6		-	+
17	+	-		11+18	+	+
18	-	-		11	+	+
19	+	+	16		-	+
20	+	-		11	+	+
21	+	+	16		-	+
22	+	+	16	11+18	+	+
23	+	+	16	11	+	+
24	+	+	16	11+18	+	+
25*	+	-			-	+
26	+	-		11+18	+	+
27	+	+	31+33	18	+	+
28	+	+	16	11	+	+
29	+	+	52	11	+	+
30	+	+	16+33	ins DNA+	+	+
31	+	-		ins DNA+	+	+

The results in the table are depicted as mirror images of each other for easier comparison between the HPV types detected in the oral cavity and those detected at the cervix. Samples marked with an asterisk indicate that the same HPV type was detected at both anatomical sites.

Table XIV. Prevalence rates of HPV types in a subset of biopsy samples using the reverse line blot assay

B-Globin positive biopsies	92
HPV positive biopsies	64
Dual/multiple infections	12
Single infections	52
HPV types:	
HPV 16	33
HPV 45	14
HPV 52	9
HPV 18	5
HPV 33	5
HPV 31	4
HPV 35	3
HPV 51	3
HPV 58	3
HPV 66	3
HPV X	3
HPV 26	1
HPV 39	1
HPV 53	1
HPV 56	1
HPV 59	1
HPV 68	1

The HPV results of the reverse line blot assay were compared to the results obtained by nested PCR and RFLP. Figure 23 shows a comparison of the HPV types as detected by the two assays. Only the eleven HPV types detected by the RFLP were used in this comparison.

Of the twenty eight disparate results (Table XV), nine were the result of either one of the assays not detecting all the HPV types within a dual/multiple infection. Thirteen of the results were disparate in that one of the assays gave a negative HPV result while the other had a positive result. This would be due to PCR sensitivity and not a reflection of the typing assays. Six of the biopsies had completely disparate results between the two assays

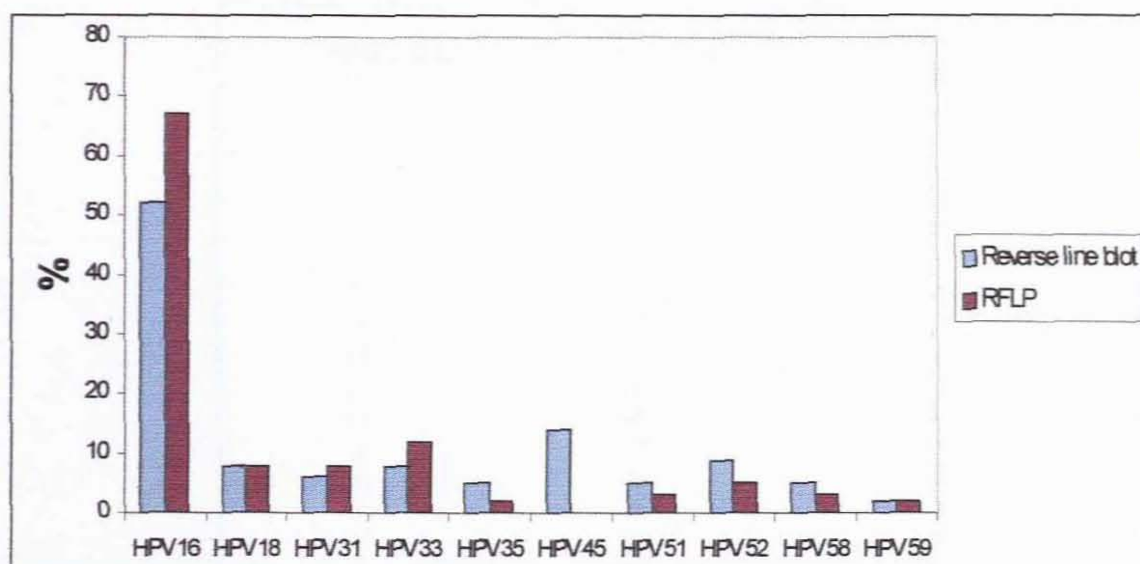

Figure 23. HPV types detected by the reverse line blot assay as compared to the RFLP. Only the eleven HPV types identified by the RFLP were included in the comparison

ie. the HPV type/types detected by the one assay was completely different to those of the second assay. The reason for these six discrepancies could be due to non specific hybridisation on behalf of the reverse line blot assay or a reflection of nucleotide changes within the amplified target DNA resulting in the RFLP mistakenly identifying an HPV type as another. However, it is more likely that these six biopsies are dual/infections and the disparate results are the result of differences in PCR amplification efficiencies due to low viral copy number or primer annealing.

The most significant difference was the apparent inability of the nested PCR to detect HPV 45. None of the nine biopsies showing an HPV 45 infection by reverse line blot assay, were positive using the nested PCR and RFLP. Where HPV 45 was present as a single infection, there was no amplification with the nested PCR. When the HPV 45 was present as part of a dual or multiple infection the nested PCR only amplified the other HPV types present.

Table XV. List of biopsies giving disparate results between the reverse line blot assay with the nested PCR and RFLP.

Biopsy number	Reverse line blot	RFLP
1	Neg	16
2	Neg	16
3	Neg	16
4	Neg	16
5	Neg	16
6	Neg	16
7	Neg	X
8	Neg	16, 33
9	45	Neg
10	45	Neg
11	45	Neg
12	45	Neg
13	45	Neg
14	45	33
15	X	16
16	X	16
17	X	16
18	31	16,31
19	16, 52	33
20	16, 58	16
21	16, 35	16
22	51, 52, 68	31
23	33, 35, 53	33
24	16, 33, 52, 66	33
25	56, 66	33
26	18, 45	16, 18
27	16, 39, 45, 59	16, 59
28	18, 45, 58, 51	18, 51, 58

X denotes a positive HPV result by PCR but not identified by the typing method.

When the HPV 45 sequence was analysed for mismatches with the nested primers it was found that the outer forward primer of the nested primer pair only had one mismatch at the 5'end and the inner forward primer had no mismatches. The outer reverse primer had a mismatch of a cytosine at the second last nucleotide position of the 3'end as well as three mismatches in the inner nested reverse primer. However, the HPV nested primers were able to amplify plasmid containing HPV 45 (figure 11). It is unlikely that the HPV 45 circulating in the cohort differed sufficiently enough from the wild type to prevent annealing of the nested HPV primers. The conditions governing efficient amplification in

PCR are multifactorial therefore it remains unclear as to why the HPV nested primers were unable to amplify HPV 45 in clinical samples.

Conversely the reverse line blot assay missed HPV 16 in twelve biopsies and of these the reverse line blot assay gave a negative HPV result in seven of the biopsies. This is significant for individual patient care as internationally HPV 16 has been shown to be the most common HPV type associated with CIN lesions and cervical cancer and a woman with a persistent HPV 16 infection needs to be clinically monitored at regular intervals.

A prophylactic HPV vaccine would include the four most prevalent HPV types circulating in a community. The epidemiological results obtained from the two assays gave slightly different results. Results of the reverse line blot assay indicated that HPV 16 was the most prevalent HPV type followed by HPV 45, 52, and 33/18 (Table XVI). Results of the RFLP indicate that HPV 16 was the most prevalent HPV type followed by HPV 33, 18/31 and 52.

Table XVI. The four most prevalent HPV types identified by reverse line blot assay and RFLP.

	Reverse line blot		RFLP	
HPV 16	52%	(1)	67%	(1)
HPV 45	14%	(2)	0%	
HPV 33	8%	(4)	12%	(2)
HPV 18	8%	(4)	8%	(3)
HPV 31	6%	(5)	8%	(3)
HPV 52	9%	(3)	5%	(4)

Figures in parentheses indicate the hierachy in prevalence of that particular HPV type as established by each assay. The figures given as a percentage indicate the prevalence rate of the HPV type as established by each assay.

Chapter 4

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Chapter 4

Discussion

4.1 Sensitivity and detection of HPV types using nested PCR

Various primers amplifying DNA fragments from different regions of the HPV genome have been developed to detect HPV in clinical samples. Due to the highly conserved nature of the L1 gene, consensus primers targeting the L1 region have become the most widely used PCR primers in epidemiological and clinical studies. The My09/My11 primer set (Manos *et al.,* 1989) has been predominantly used in studies conducted in North and South America and Asía and the Gp5+/GP6+ primer set (Snijders *et al.*, 1990) has been predominantly used in Europe (Qu *et al.*, 1997). Both sets of primers are group specific and are designed to only detect mucosal HPVs, but each primer pair utilizes a different PCR strategy to achieve optimal template amplification. The My09/My11 primers are synthesized with several degenerate nucleotides within each primer and are therefore a mixture of 25 primers. This allows for the detection of a broad range of mucosal HPVs. In contrast, GP5+/GP6+ primers have a fixed nucleotide sequence but employ a low annealing temperature during the thermocycling process. This effectively lowers the stringency of the assay allowing for the detection of a broad range of mucosal HPVs.

It has been shown that a nested PCR reaction is more sensitive than a single step PCR reaction. In a study by Husnjak *et al.* (2000) 250 cervical samples that had tested PCR negative using the My09/My11 primers were re-tested using a nested reaction, with the MY09/My11 primers amplifying a ~ 450 bp fragment of the L1 gene and the GP5+/GP6+ primers amplifying a 140-150 bp fragment within the region amplified by the My09/My11 primers. Of the 250 previously PCR negative cervical biopsy samples, 38.8% were found

to be positive for HPV DNA using the nested reaction. Nested reactions are therefore of value when dealing with samples that contain low copy numbers of HPV DNA, as large amounts of genomic DNA in proportion to the target DNA could inhibit the ability of the primers to find their target DNA (Tucker *et al.*, 1993). This results in a reduction of the efficiency of the PCR reaction.

The HPV nested primers used in this study takes advantage of all three strategies ie primer degeneracy and a low annealing temperature in a nested reaction. The primers should therefore be efficient at amplifying a broad range of mucosal HPV types as was shown in the amplification of the newly described HAN2294. The HPV nested primers described in this study would therefore be of value in studies aimed at detecting novel HPV types as well as studies designed to detect a wide range of known mucosal HPV types.

An intermethod comparison by Qu *et al.* (1997) highlighted differences in the ability of the My09/My11 and GP5+/GP6+ primers to detect different HPV types. The My09/My11 primers failed to detect HPV types 31, 35 and 59 in some samples whereas the GP5+/GP6+ primers failed to detect HPV 53 and 61 in samples. Not all mucosal HPV types will have 100% homology to the consensus primers and might therefore not be efficiently amplified. Qu *et al.* (1997) showed that there was an inverse relationship between the number of mismatched base pairs and the ability of the primer pair to amplify the HPV type. However, de Roda Husman *et al.* (1995) demonstrated that the GP5+/GP6+ primers showed no decrease in sensitivity with 3 mismatches within the primer pair. With four or more mismatches the sensitivity decreased by 1 000-fold.

Sequence analysis indicated that there were several mismatches between some HPV types and the nested HPV primers used in this study (Tables VIII a and VIII b). Fourteen of the

nineteen HPV types had a thymine at the sixth nucleotide position of the inner nested reverse primer (Table VIII b). By including a degeneracy of an adenine and a guanine at this position, all of the mucosal HPVs examined (with the exception of HPV 72) would be homologous to the primers at this position. HPV types 26, 31, 34, 54, 67 and 69 had a mismatch of a cytosine at the second last nucleotide at the 3' end of the inner forward nested primer. This could be rectified by incorporating a cytosine in the degeneracy of adenine and thymine at this position. As the mismatches described above appear to be relatively common, it is likely that they could be present in novel HPV types. Therefore, the ability of the HPV nested primers to detect novel HPV types could be improved by re-designing the oligonucleotide primers. But, as there are many variables determining the efficiency of PCR amplification theoretical predictions might not hold true. The amplification efficiency of a primer set can only be determined through empirical studies.

The lack of HPV 45 detected in this study appears to be due to the inability of the nested HPV primers to amplify HPV 45 in clinical samples as the results of the reverse line blot assay showed that HPV 45 was present in nine of the subset of biopsies. However, as can be seen in Figure 11 of the results, the nested PCR is able to amplify plasmid containing wild type HPV 45 and the RFLP is able to identify wild type HPV 45. A sequence analysis of the HPV 45 genome does not reveal any other areas of primer homology within the genome that could potentially interfere with efficient amplification of the required fragment. The homology of the primers to the HPV 45 genome is not 100%, with a total of seven mismatches. This could lead to inefficient binding of the primers. However, the primers have less homology to the HPV 35 genome with ten mismatches and a four nucleotide insert and it was shown that there was efficient amplification of both biopsy samples and plasmid DNA. It is therefore unclear as to why the primers did not amplify HPV 45 in the patient samples but were able to amplify plasmid DNA.

Both Qu *et al.* (1997) and Tucker *et al.* (1993) have shown that amplification of HPV DNA in samples containing multiple HPV types can produce inaccurate results. Qu *et al.* found that the GP5+/GP6+ primers failed to detect multiple HPV types in almost 50% of samples in which the MY09/My11 primers had detected multiple HPV types. This indicated that some consensus primers are more efficient at amplifying HPV DNA from samples containing more than one HPV type. Tucker *et al.* (1993) performed an experiment in which varying quantities of cloned HPV 11 and 16 were mixed together. PCR using primers specific for HPV 11 were used to amplify the mixture. The final product showed equal quantities of HPV 11 and 16 even though the primers had six mismatches with HPV 16 but were 100% homologous to HPV 11. The same results were achieved when the input ratios of HPV DNA were varied 9/1 and 1/9.

The HPV nested primers used in this study detected a total of twenty one dual infections and three triple infections in the biopsy and buccal specimens. This might not be an accurate reflection of the true prevalence rates of multiple HPV infections within this cohort, as no empirical study was performed to establish how efficient the primers are at amplifying HPV DNA from samples containing multiple types. However, it was noted that the nested PCR did not detect dual/multiple infections in seven of the biopsies showing dual./multiple infections with reverse line blot assay. This could be due to inefficient amplification by the nested primers or non specific DNA hybridisation to the line probe of the reverse line blot assay.

Both Karlsen *et al.* (1996) and Smits *et al.* (1995) have shown that to gather the most accurate data on HPV types and prevalence rates in a study, more than one PCR method should be used. However, most studies employ only one PCR method for their detection

and typing of HPV. The integrity of the various PCR assays must ensure that there is no significant difference in the HPV prevalence rates of an HPV study and the predominant HPV types should also be detected with equal frequency. Each PCR method will show variations in sensitivity, their ability to efficiently amplify certain HPV types and their ability to amplify all HPV types in samples containing multiple HPV genomes. It is therefore important to take into account any limitations of an assay when interpreting data from HPV studies employing consensus primers.

Other than the ability to detect HPV 45 in clinical specimens the nested HPV primers used in this study were able to detect a broad range of mucosal HPV types in both single and dual/multiple infections. The most prevalent HPV types (with the exception of HPV 45) were the same when the nested PCR results were compared to the reverse line blot assay.

4.2 Typing of mucosal HPVs using RFLP

Various methods are used to type HPVs after PCR amplification using consensus primers (1.10.4.6 literature review). RFLP analysis can be used to type HPVs (Contorni and Leoncini, 1993; Lungu *et al.*, 1992; Chen *et al.*, 1994; Bernard *et al.*, 1994) or detect variants of an HPV type (Bible *et al.*, 2000) and novel HPV types (Peyton *et al.*, 1994; Manos *et al.*, 1994; Peyton and Wheeler, 1994). One of the aims of this study was to establish the prevalence rates and HPV types infecting women with CIN lesions. Even though it has been shown by various studies that the HPV types detected in women with CIN lesions vary between geographical regions, it has been established that mainly high risk or oncogenic types are associated with these lesions. The RFLP was therefore designed to only primarily detect these types as it was likely that the 10 HPV types declared as

human carcinogens by the IARC would be the most prevalent HPV types harbored in the cohort used in this study.

The RFLP identified HPV types 16, 18, 31, 33, 35, 52 and 58 in the cervical biopsies and buccal samples. As these HPV types are potential human carcinogens of group one the RFLP would be useful as a typing method for studies designed to detect the presence of high risk mucosal HPV types, especially in situations where automated sequencing facilities are not available . An added advantage is that the RFLP is based on the amplicons of a sensitive nested PCR reaction and could be used as an epidemiological tool for studies investigating the role of high risk mucosal HPVs at other anatomical sites where the HPV copy number is low eg. the oral cavity.

It has been shown internationally that HPV 16 is the most common HPV type associated with cervical cancer (Munoz, 2000). It is also postulated that women with HPV 18 associated cervical cancer have a poorer prognosis (Lombard *et al.*, 1998; Schwartz *et al*,, 2001) and a more rapid disease progression (Shyu *et al*,, 2001). The RFLP described in this study could provide a quick and inexpensive means of screening samples for HPV 16 and HPV 18 as they have unique patterns requiring no confirmatory digest. Only two restriction endonucleases would be required ie. *BstE*11 for HPV 16 and *Pvu*11 for HPV 18.

Mounting evidence indicates that HPV variants play a role in the pathogenesis of cervical neoplasias due to differences in oncogenic potential (Xi *et al.*,1997; Villa *et al.*, 2000) as well the ability of the virus to persist (Xi *et al.*, 1995). Three of the HPV 58 variants detected in this study were found in CIN 3 lesions and two were detected in CIN 2 lesions with the variant being the fourth most prevalent HPV type detected in biopsy samples in this study. The significance of the HPV 58 variant detected in this study has not yet been

established. The RFLP developed in this study could also play a valuable role in screening samples for the variant of HPV 58. By performing a *Pst*1 digest and a *BstE*11 digest, the wild type is differentiated from the variant.

A study by Stewart *et al.* (1996) on intratypic variation of less prevalent mucosal HPV types, found that the majority of nucleotide changes were synonymous ie the nucleotide change does not translate into an amino acid change. However, the nucleotide change of the HPV 58 detected in this study was non synonymous. This change has taken place in the L1 gene which codes for the major capsid protein and could potentially interfere with the antigenic properties of the virus. Neutralizing antibodies to HPV are directed to conformational epitopes situated on the L1 capsid protein, therefore a prophylactic vaccine directed against HPV 58 would need to elicit a conformationally correct antibody response to be effective. It is therefore important to establish if the amino acid change of the L1 capsid protein in the HPV 58 variant leads to a change in the protein folding.

Even though the RFLP was effective in typing the HPVs detected in the cohort, the method had drawbacks as it was time consuming when trying to establish if an undigested band was the result of a multiple infection, a novel HPV type, an HPV type not identified by the method, or if the band was the result of incomplete restriction endonuclease digestion. However, this drawback is counterbalanced by the ability of the RFLP to detect novel HPV types and variants. Direct sequencing would also provide a means of identifying novel HPV types and variants especially if the samples only contain single HPV infections. But as the PCR primers are consensus primers, direct sequencing of PCR products from samples containing multiple HPV infections would also be time consuming. Each sample

would need to be cloned and multiple clones of each sample screened by means of sequencing to establish the presence of novel HPV types or variants.

4.3 Epidemiological data

4.3.1 HPV prevalence rates in CIN lesions within the international context

The causal relationship between HPV with cancer and its precursor lesions is now well established. However, only 25% of all squamous intraepithelial lesions (SIL) will progress to a higher grade SIL lesion and of these 10% will progress to carcinoma *in situ* and 1% to invasive cancer. One third of all grades of SIL will regress and 41% will persist (NIH consensus statement, 1996). It is the HPV types that are associated with cervical lesions that persist or progress that are of clinical significance. If a patient presents with cervical neoplasia and an HPV positive test result, it is important that the patient be clinically followed up to establish if the same HPV type is persisting and whether the lesion is regressing. A prophylactic or therapeutic vaccine designed to prevent or treat cervical cancer, will not be aimed at the cancer *per se*, but at the prevention or treatment of the HPV type associated with the lesions. It is therefore necessary to establish which HPV types are associated with cervical neoplasias within a population, as epidemiological data have highlighted the existence of geographical differences.

In a worldwide survey on invasive cervical cancer, Bosch *et al.* (1995) showed that HPV types found in group A9 (HPV 16, 31, 33, 35, 52 and 58) were detected in 66,5% of all cervical cancers internationally and the HPV types found in group A7 (HPV 18, 39, 45, 59 and 68) were detected in 27,3% of the CaCx samples. Even though this study was

performed on CIN biopsies and not cervical cancer biopsies as in the Bosch study, 97/116 (84%) of the biopsies were from CIN 3 grade lesions and are therefore likely to reflect the HPV group that would be found in patients with cervical cancer. In this study 97.6% of CIN biopsy samples contained HPVs from the A9 group which is higher than the Bosch study.

The international study by Bosch (1995) showed the following HPV prevalence rates in cervical cancer samples: HPV 16 (50%), HPV 18 (14%), HPV 45 (8%) and HPV 31 (5%). The predominant HPV type detected in this study was HPV 16, which is in keeping with international trends. However, the lack of HPV 18 and the presence of a variant of HPV 58 as the fourth most common HPV type, indicated geographical differences when compared to international trends.

A study in Senegal by Chabaud *et al.* (1996) showed an increase in HPV 18 prevalence through increasing grades of cervical lesion. The study also revealed that HPV 18 was the second most prevalent type after HPV 16. Rattray *et al.* (1996) also showed that HPV 18 was uncommon in patients with no abnormal cytology or low grade cervical lesions, however the prevalence increased in high grade and cervical cancer. In contrast, Matsukura and Sugase, (2001) presented evidence of only five HPV 18 infections in three hundred and fifty four cases of CIN lesions in Japanese women.

In this study, HPV 18 was detected in only 2/116 HPV positive samples. A possible explanation for the low prevalence of HPV 18 could lie in the collection of biopsy samples. Anatomically, the cervix consists of the ectocervix which is lined with squamous epithelium and the endocervix which is lined with cylindrical mucus producing cells. The junction between the two types of epithelium is known as the transformation zone. HPV

18 is associated with adenocarcinoma (Bosch *et al.*, 1995) which would be more likely to arise from the mucus producing cells of the endocervix. The samples collected in this study were punch biopsies of lesions detected on colposcopy. The endocervix is not easily visible on colposcopy and therefore most lesions observed and sampled in this study would be of squamous cell origin of the ectocervix. The Matsukura and Sugase study (2001) collected only punch biopsies from lesions visible by colposcopy. Therefore, the cervical cells were collected from a focal point and the study showed a low prevalence rate of HPV 18. In contrast, the Chabaud study (1996) used an Ayre spatula and the Rattray study (1996) an endocervical brush to collect cervical samples. Both of these sample collection methods would allow for the collection of cells from a wide area including the transformation zone and both studies found a increasing prevalence of HPV 18 through CIN grades. The low prevalence of HPV 18 detected in this study as well as the Japanese study could therefore be partially explained by sample bias.

It is of concern to note that HPV 45 was the second most common HPV type detected in CIN biopsies when using the reverse line blot assay (Table XIV of results) to type the HPVs present in the biopsies. Based on these results HPV 45 would have to be included in a prophylactic vaccine. As noted earlier, the nested HPV primer pair was unable to amplify HPV 45 from clinical samples. This highlights the importance of the integrity of assays used in epidemiological studies and suggests that more accurate results could be obtained by using more than one PCR primer set when performing epidemiological studies.

4.3.2 HPV prevalence rates in CIN lesions within the South African context

Only three articles have been published on the prevalence rates of HPV in CIN lesions within a South African population. Markowitz (1986) detected HPV in 99% of CIN 3

lesions. This study was based on histological evidence of HPV infection only and did not employ any methods to type the HPVs present. A second study by Williamson *et al.* (1989) on CIN 3 biopsies showed a prevalence rate of HPV 16 (16%), HPV 33 (8%), HPV 31 (2%) and HPV 18(1%). The HPV types detected in HPV positive CIN 3 biopsies in this study using nested PCR and RFLP were HPV 16 (66%), HPV 33 (15%), HPV 31 (12%) and HPV 58 (3%). Even though the three most prevalent types were the same for each study, the Williamson study showed a lower prevalence rate of each type which would be due to sensitivity differences of the protocols employed in the studies. Williamson did not use a DNA amplification technique such as PCR before performing Southern blot hybridization. In contrast, the nested primers used in this study are able to detect low copy numbers of HPV DNA and are therefore able to amplify concentrations of HPV DNA that are below the detection limit of Southern blots. The fourth most prevalent types were HPV 18 in the Williamson study and HPV 58 in this study. Once again the difference is due to the protocols employed, as the Williamson study used a limited number of type specific probes for Southern blots and the protocol did not include probes for HPV 58. Therefore any HPV 58 DNA present in the samples would have gone undetected.

The third study on HPV prevalence in CIN 2 and CIN 3 lesions was done by Cooper *et al.* (1991) using non-isotopic in situ hybridisation (NISH). The four most prevalent types in this study were HPV 16 (50%), HPV 33 (25%), HPV 18 (22%) and HPV 35 (16%). The two most prevalent HPV types in the Cooper study were the same as the nested PCR with RFLP results of this study, ie HPV 16 followed by HPV 33. It should be noted that the sample population of the Cooper study were drawn from Durban patients and therefore any differences in the HPV prevalence rates between the two studies could be a reflection of geographical differences. Despite the differences in methodology and population HPV 16 and HPV 33 were the two most prevalent HPV types in the Cooper study, the Williamson

study and this study. This could putatively indicate that these are the two most common HPV types found in CIN patients in South Africa.

The lack of published studies on South African women using sensitive techniques able to detect a wide range of HPV types needs to be addressed. The disease burden of cancer of the cervix is extremely high, with black South African women having a 1 in 21 lifetime risk of developing cancer of the cervix (Sitas *et al.*, 1996). The NIH consensus statement states that with the causal relationship between HPV infection and cervical neoplasia having being established, prophylactic and therapeutic vaccine strategies should be given the highest priority. However, these vaccine strategies can only be based on the HPV types circulating within a given population and to date, in South Africa, there are no comprehensive epidemiological studies providing this information.

4.3.3 Significance of HPV prevalence rates in buccal samples

As discussed in 1.2 of the literature review, there are widely discrepant results between studies on the prevalence rates of HPV in the oral cavity in both normal and neoplastic tissue. This could be due to the low copy numbers of HPV DNA found in the oral cavity resulting from high levels of degradative enzymes compared to the cervix (Maitland *et al.*, 1987).

In the light of these discrepant results, one of the aims of this study was to establish the prevalence of mucosal HPVs in the oral cavity of women with CIN, using a sensitive detection technique and secondly to establish any correlation between the types found in the oral cavity and those found in the cervix. Badaracco *et al.* (1998) found an HPV prevalence rate of 18% in the oral cavity using PCR employing the My 09/11 primers

followed by hybridisation for HPV typing. A study by Kellokoski *et al.* (1992) showed an HPV prevalence rate of 16,5% using HPV type specific primers for HPV types 6, 11, 16 and 18. This prevalence rate was increased to 29,4% when the PCR products were probed using dot blot hybridisation. The results of this study reveal a far higher prevalence rate compared to the above two studies, with 74% of the buccal samples showing the presence of HPV DNA. This is probably due to the use of nested PCR as a detection means. A study by Terai *et al.* (1999), however, also showed a high prevalence rate of HPV in normal oral mucosa with 81,1% of samples showing the presence of HPV DNA.

This study showed a relatively high rate of multiple infections with 38% of the buccal samples showing the presence of more than one HPV type. Similarly, both Terai (1999) and Badaracco (1998) showed a high number of multiple infections with 56,7% and 35% respectively of samples showing the presence of more than one HPV type. In a study of oral squamous cell carcinomas Woods *et al.* (1993) detected dual/multiple HPV infections in nine of fourteen oral biopsies. This would indicate that multiple HPV infections are common in both normal oral mucosa and oral squamous cell carcinomas.

The most prevalent HPV type in the oral samples in this study was HPV 11 (78%) followed by HPV 18 (35%) and HPV 16 (13%). The presence of high risk mucosal HPV types in the normal oral mucosa is not yet fully understood. Terai *et al.* (1999) detected HPV 18 DNA in 86,7% of normal oral tissue. Kelloski *et al.* (1992) detected high risk HPV 16 or HPV 18 in seven of twenty five biopsies of normal oral mucosa. Badaracco *et al.* (1998) detected HPV 16 in five of sixteen normal oral samples that were positive for HPV DNA. The presence of mucosal high risk HPV types in normal oral mucosa without any accompanying pathology could be explained by the evidence that the viral genome is rarely, if ever, integrated in human oral tissue (Maitland, *et al.* 1987). However, this

appears to be an unlikely explanation as it has been shown that the viral genome is also not integrated in oral neoplasms (Maitland *et al.* 1987) indicating that co-factors are required for the development of oral neoplasias.

The results of this study indicated that there was no correlation between the HPV types found in the oral cavity and those of the cervix of the same patient (Table XIII of results) with only one patient having the same HPV type at both sites. A literature search reveals only two other studies which have looked at concurrent HPV infection in oral and genital mucosa. (Badaracco *et al.*, [1998] Kelloski *et al.*, [1992]). Neither the Kelloski nor Badaracco studies showed concordance between the oral and genital HPV types. Only 2/25 patients had the same HPV type at both sites in the Kelloski study and 3/29 patients had the same HPV type in the Badaracco study. It should be noted, however, that neither of the above two studies nor this study performed genome sequence analysis to verify that the HPV genomes that appeared to be concordant were in fact so. It cannot therefore be stated *de facto*, that the identical HPV was infecting both sites.

The significance of detecting HPV in the oral cavity still needs to be established as there appears to be no evidence of auto inoculation between the oral cavity and the cervix as shown by the difference in HPV types at each site. It has been suggested (Broker *et al.*, 2001) that HPV is part of the microflora that colonizes humans and that it should be considered as a commensal. This is indicated by the ubiquitous nature of both cutaneous and mucosal HPVs indicating that mucosal HPVs are not only acquired sexually but also by other means. The oral results obtained in this study could therefore be a reflection of this hypothesis.

Chapter 5

Conclusions

The primary aims of this study were to establish the HPV prevalence rate by means of a sensitive nested PCR and to develop a novel RFLP method to type the high risk mucosal HPV types infecting these women. The secondary aim of this study was to establish the prevalence rate and HPV types infecting the oral mucosa of women with CIN lesions as well as to establish if there is any correlation between the HPV types infecting the oral mucosa and those infecting the cervix.

The HPV nested PCR used in this study was sensitive and able to detect a wide range of HPV types. It would therefore provide a valuable tool for epidemiological studies involving samples containing low copy numbers of HPV. However, the inability of the nested HPV primers to amplify HPV 45 in clinical specimens is of concern and needs to be taken into consideration when using these PCR primers for epidemiological studies. This could be overcome by re-designing the primers or clinical samples could be screened using the nested PCR primers and then samples re-screened using HPV 45 type specific PCR primers. The nested PCR would also provide a valuable tool for the detection of novel or unusual HPV types. This is of particular importance for the South African scenario which faces an AIDS epidemic, as it has been shown that novel and unusual HPV types are often harbored in immunocompromised patients.

The integrity of the typing results of the RFLP was confirmed by sequencing and the reverse line blot assay. The RFLP developed in this study was able to identify the 10 high risk mucosal HPV types considered as human carcinogens of group 1 by the IARC and

would therefore be valuable in screening clinical samples for these high risk HPV types and in particular the rapid screening of samples for HPV 16 and HPV 18 DNA. Although the RFLP is unable to identify the presence of novel HPV types or variants conclusively, it could prove valuable by indicating the presence of variants or novel HPV types by an unknown RFLP pattern. This was highlighted in this study by the difference in RFLP pattern of the wild type HPV 58 and the local variant.

The epidemiological results of this study show that HPV 16 is the predominant HPV type associated with CIN lesions in women from the Western Cape when using both the RFLP method and the reverse line blot assay. This confirmed the results of other studies done on South African women and an HPV vaccine would therefore need to include HPV 16. However, it still needs to be established conclusively as to which HPV types are the second, third and fourth most prevalent types associated with CIN lesions due to the discordant results of the two typing assays employed in this study. Epidemiological typing studies would also have to be performed in the rest of South Africa as a prophylactic or therapeutic vaccine would be aimed at the whole female population and the results from the Western Cape do not necessarily reflect the prevalence rates of the different HPV types circulating in the rest of the country.

This study showed a high prevalence rate of mucosal HPVs in the oral cavity, of which the significance is not understood. Although the number of buccal samples in this study was small, there was no correlation between the HPV types found in the oral cavity and those found in the cervix. This result was in accordance with other studies. It would appear therefore, that HPV is not transmitted by autoinoculation from the mouth to the cervix or *vice versa* and these results could reflect the hypothesis that HPV is a commensal.

Directions for future studies

The inability of the nested primers to amplify HPV 45 from clinical samples needs to be investigated. This would include designing new primers or re-designing the nested primers, analyzing the DNA sequences of the clinical samples and comparing the sequences to the cloned HPV 45 to establish if secondary structures are interfering with primer annealing or if a HPV 45 variant is circulating within the population.

Further epidemiological studies need to be performed to establish the HPV prevalence rates in women with cervical neoplasias from other parts of South Africa. The cohort used in this study are relatively isolated from the rest of the population when compared to the social dynamics occurring elsewhere in the country.

Only a small number of buccal samples were collected in this study and the role of HPV infections in the oral cavity needs to be investigated further. Studies on both normal and neoplastic oral samples need to be performed to establish if HPV is transmitted via the oral route and if infection with HPV is necessary for the development of oral neoplasias.

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Appendix A

Proteinase K lysis buffer.

KCl 1mM	5 ml
Tris HCl pH 8,3	1 ml
$MgCl_2$ (2,5mM)	250 µg
NP 40 (0,5%)	500 µl
Triton X100 (0,5%)	500 µl
Proteinase K (120µg/ml)	12 mg

Make up to 100 ml with distilled water and store aliquots at –20°C until required.

Phosphate Buffered Saline

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Dissolve in 800 ml distilled water. Adjust pH to 7.4 with HCl and make up to 1 liter with distilled water. Autoclave. Store at room temperature until required.

Ethidium Bromide Stock Solution 10 mg/ml.

Ethidium bromide	1 g
Distilled water	100 ml

Stir on a magnetic stirrer for 1 - 2 hours. Transfer to a dark bottle or cover bottle with tin foil and store at room temperature.

1 X Tris-Borate with EDTA

Tris	1.08 g
Boric Acid	5.5 g
EDTA	7.45 g

Dissolve in 800 ml. distilled water and make up to 1 liter with distilled water. Store at room temperature.

2 X Yeast Tryptone Broth/Agar

Tryptone	16 g
Yeast Extract	10 g
Sodium chloride	5 g

For broth: dissolve in 1000 ml distilled water and dispense into appropriate aliquots. Autoclave. For agar: prepare as for broth and add 15 g agar.

Reverse line blot hybridisation solutions

1X hybridisation solution (4XSSPE, 0,5%SDS)

20X SSPE	200 ml
20% SDS	25 ml

Make up to 1liter with distilled water and store at room temperature until required

1X wash solution (1X SSPE,0,1%SDS)

20X SSPE	50 ml
20% SDS	5 ml

Make up to 1litre with distilled water and store at room temperature until required.

Conjugate solution

1X wash solution	100 ml
Amplicaor SA-HRP	0,3 ml

Make up solution just before required.

Citrate solution

20X citrate solution	50 ml

Make up to 1 liter with distilled water and store at room temperature until required.

Appendix B.

CCR 5 Primers

LLF Forward Primer: 5' – ACC AGA TCT CAA AAA GAA CT – 3'
LLR Reverse Primer: 5' – CAT GAT GGT GAA GAT AAG CCT CAC A – 3'

HPV Nested consensus Degenerate Primers

Primer 1 (Outer forward primer): 5'- CAG GAT GG(T,G,C)GA(T,C)ATG GT -3'

Primer 2 (Outer reverse primer): 5'- CAT (A,G)TT(A,T,G)GTA CTG CG(A,T)G- 3'

Primer 3 (Inner forward primer):5'- TG(T,C)AAA TAT CC(A,T)GAT TAT (T,A)T-3'

Primer 4 (Inner reverse primer): 5'- GTA CTG(C,G)G(T,A)GTG GTA TC

Nucleotides encompassed in brackets show the position of the degeneracies. The universal codes for the degeneracies are:

v = T,G,C

w = T,C

x = A,T

y = A,T,C

z = G,C

Appendix C

Molecular weight markers VI and VIII. The numerals positioned to the right of the molecular weight markers indicate the size of the fragments in base pairs.