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GENITAL HUMAN PAPILLOMAVIRUS INFECTION IN MALES UNDERGOING VOLUNTARY MEDICAL CIRCUMCISION FOR HIV INFECTION PREVENTION IN SELECTED CLINICS IN LUSAKA CITY

By

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The University of Zambia

Lusaka

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DECLARATION

I, **PETER D. JULIUS** this 14th day of June 2016, declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references.

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CERTIFICATION OF APPROVAL

DISSERTATION TITLE: GENITAL HUMAN PAPILLOMAVIRUS INFECTION IN MALES UNDERGOING VOLUNTARY MEDICAL CIRCUMCISION FOR HIV INFECTION PREVENTION IN SELECTED CLINICS IN LUSAKA CITY

This dissertation for **PETER D. JULIUS** has been approved as partial fulfilment of the requirements for the award of the **Master of Medicine in Pathology** at the University of Zambia.

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ABSTRACT

Background

Human papilloma virus (HPV) infection is the most common sexually transmitted infection that commonly affects both males and females, yet much of the information about it centres on women because it is associated with cervical cancer, the most common malignancy in women of child bearing age. In males, most HPV infections are harmless and have no symptoms, however in some people the infection may persist and lead to diseases of the genital area including ano-genital warts or cancer and one may pass it to a female partner without even knowing it.

Objectives

To study the prevalence and determinants of Human papillomavirus (HPV) infection among males going for voluntary medical male circumcision for HIV infection prevention in Lusaka, Zambia.

Methods

Males seeking Voluntary Medical Circumcision for HIV infection prevention and living in communities in Lusaka were recruited into the study. Baseline demographic and lifestyle data was collected and the penile foreskin specimens were tested for HPV DNA

Results

Overall HPV infection prevalence was 71.9%. The risk factors for HPV infection included being sexually active, multiple sex partners and age. Viral wart changes was the common abnormal histologic finding in the foreskins and it was associated with HPV infection in this study.

Conclusion

HPV infection among participants going for voluntary medical male circumcision for HIV infection prevention in Lusaka is high. Age and sexual activity appear to be risk factors for HPV infection.

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DEDICATION

I would like to dedicate this work to my father, Clifford Julius and mother, Margaret Chikobela, who always took pride in their work and new the value of leaving a positive mark, whether in a professional or interpersonal context.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
CDC	Centre for Disease Control and Prevention
CDK	Cyclin dependent kinase
CIN	Cervical Intraepithelial Neoplasia
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic Acid
E	Early
FDA	Food and Drug Administration
FFPE	Formalin Fixed Paraffin Embedded
HIV	Human Immunodeficiency Virus.
HPV	Human Papilloma Virus
Hr	High Risk
L	Late
Lr	Low Risk
MOH	Ministry of Health
PCR	Polymerase Chain Reaction
PeIN	Penile intraepithelial neoplasia
PI	Principle Investigator
SOP	Standard operating procedure
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TLR	Toll like receptor

INTRODUCTION

Human papillomavirus (HPV) infection is the most common sexually transmitted infection that commonly affects both males and females, yet much of the information about it centres on women because it is associated with cervical cancer, the most common malignancy in women of child bearing age. In males, most HPV infections are harmless and have no symptoms, however in some people the infection may persist and lead to diseases of the genital area including ano-genital warts or cancer and one may pass it to a female partner without even knowing it. Studies have suggested that circumcision may reduce common sexually transmitted infections such as HPV, human immunodeficiency virus (HIV) and urinary tract infections as well as penile cancers that may be associated with these infections. Little is known, however, about HPV and its associated risk factors for infection among asymptomatic HIV negative males in Lusaka, Zambia.

1.0 LITERATURE REVIEW

1.1: HUMAN PAPILLOMA VIRUS (HPV)

Human papilloma viruses (HPV) are double stranded DNA viruses from the papilloma virus family that are capable of infecting humans. HPV infection is considered to be the most common sexually transmitted infection worldwide (Schiffman and Castle, 2003), with nearly 80 million people - about one in four - are currently infected in the United States (Center for Disease control, 2015). Most sexually active women and men will be infected at some point in their lives and some may be repeatedly infected (WHO, 2015) through sexual contact or skin to skin contact.

HPV establish productive infections only in keratinocytes of the skin or mucus membranes of humans. The infections are classified as latent (asymptomatic), subclinical or clinical. Most HPV infections are latent and are only detected with tests for viral DNA. Clinical lesions are grossly apparent as benign papilloma or

premalignant lesions that may progress to cancers of the cervix, vulva, vagina, penis, oropharynx and anus (Chouhy et al., 2013).

More than 170 HPV types have been completely sequenced (Chouhy et al., 2013)and at least 200 additional viruses have been identified and await sequencing and classification. HPV have been divided into five genera: Alpha papilloma virus, Beta papilloma virus, Gamma papilloma virus, Mu papilloma virus and Nu papilloma virus as shown in figure 1.

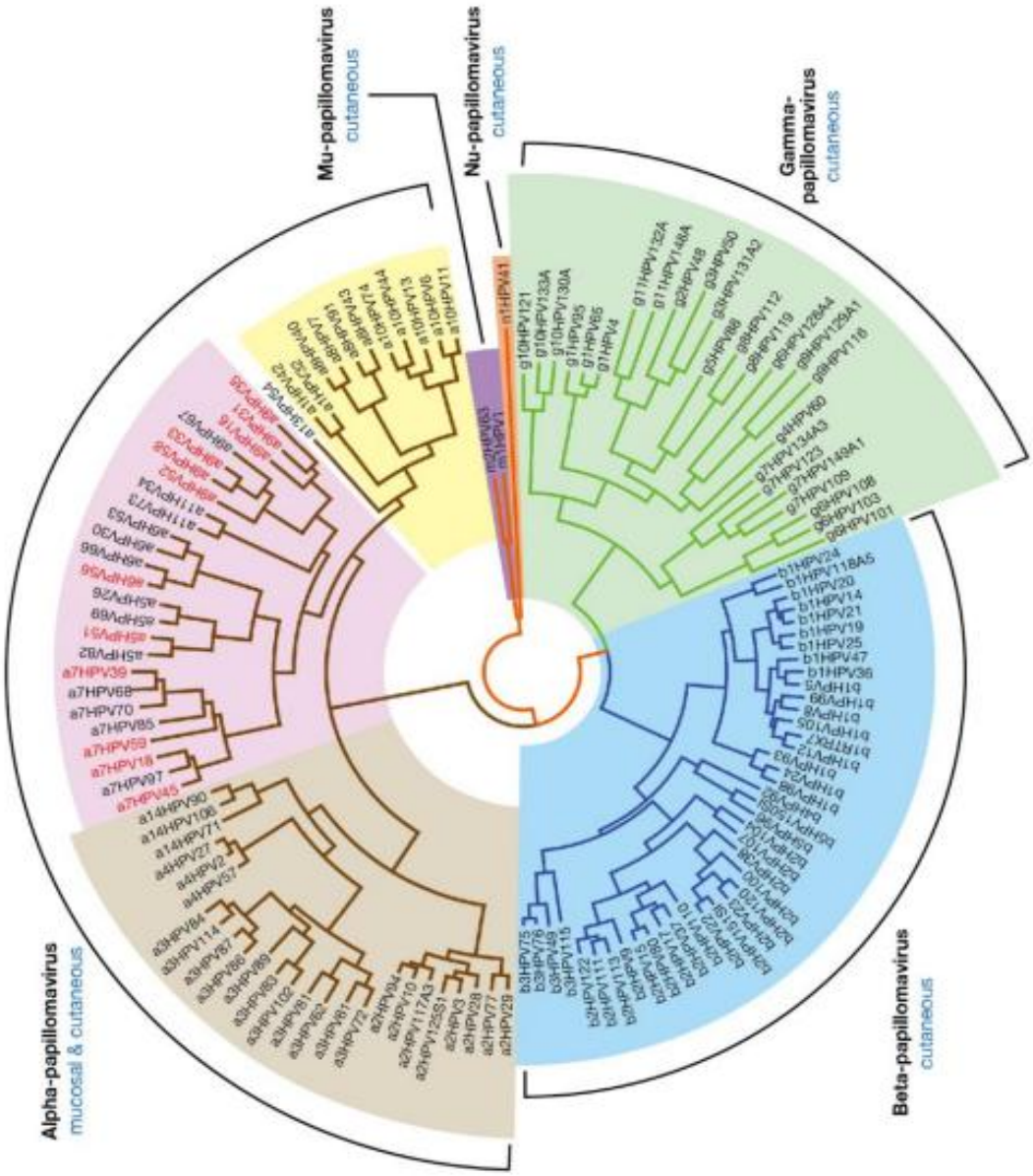

Figure 1. Evolutionary Relationship between Human Papillomaviruses, Source (Doorbar et al., 2012).

Human Papillomaviruses comprise five evolutionary groups with different epithelial tropisms and disease associations. The Alpha papillomaviruses include the low-risk mucosal types (many of

which are within the orange shaded branch) that cause genital warts, and the high-risk mucosal types (contained within the branch highlighted with pink shading) that can cause cervical neoplasia's and cancer.

HPV has been associated with 97.7% of cervical cancers, 69.9% of vaginal cancers, 47% of penile cancers, 40% 0f vulva cancers, 35.6% of oropharyngeal cancers and 23.5% of oral cavity cancers (Lenzi et al., 2013).

1.1.1: Classification of Human Papilloma Viruses

HPV is a member of the papovirus group, reclassified as the papillomaviridae family and based on their viral genome heterogeneity, papilloma viruses have been classified into genera, species and types as shown in figure 1. The largest genus is that of the alpha-papilloma viruses and it includes 59 HPV types. These cause mucosal and cutaneous lesions in both humans and primates (Doorbar et al., 2012).

Papilloma viruses have been classified epidemiologically into High risk (Hr-HPV) and Low risk according to their association with cancer (Table 1).

Table 1. Epidemiologic classification of HPV types

Epidemiologic classification	**HPV types**
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Probable high risk	26, 53, 66
Low risk	6, 11, 40, 42,
Undetermined risk	34, 57, 83

Source: (Muñoz et al., 2003).

The Low risk HPV types cause benign papilloma (warts) while the High risk types have been implicated in the genesis of squamous cell carcinomas of the anogenital region and the head and neck region. Cancers have been associated with persistent infection by Hr-HPV (Muñoz et al., 2003).

The current classification system is based on similarities in genomic sequences. The classification generally correlates with the three clinical categories applied to HPV:

- Anogenital or mucosal
- Non genital or cutaneous
- Epidermodysplastic verruciformis (EV).

1.1.2: Natural History of HPV infection

HPV infections are transmitted sexually or by direct contact between an infected individual's epithelium (skin or mucosa) and the epithelium of another individual. The infection can also be transmitted vertically from the mother to the new born baby by contact between a child and the maternal genital tract during delivery. It is also hypothesized that the HPV infection can result by oral mucosal contact. Studies have also shown HPV transmission between hands and genitals of the same person or sexual partners (Longworth et al., 2004)

.

In the sexual transmission of HPV in males, the virus principally infects the basal keratinocyte of the epidermis, presumably through disruptions of the skin or mucosal surface. At this location the virus remains latent in the cell as a circular episome in low copy numbers. Autoinoculation of virus into opposed lesions may occur. The papilloma virus at this point is thought to replicate through two modes. The virus replicates outside the host genome (episomal replication) in the basal cells in a stable manner. The vegetative or run away replication occurs as the epidermal cells differentiate and migrate to the surface. The virus undergoes replication and maturation and is shed in exfoliated cells in high copy numbers at the superficial keratin layer. Virus production occurs predominantly at the epithelial surface because most viral genes are activated when the infected keratinocytes leaves the basal layer (Longworth et al., 2004)

.

The characteristic feature of productive papilloma virus infection is koilocytosis. Koilocytes are HPV infected keratinocytes that exhibit a high degree of nuclear atypia characterized by a shrunken (raisinoid), pyknotic nucleus with a characteristic perinuclear clearing (halo). This is probably due to viral replication that is confined to the nucleus.

Most HPV infections (90%) are subclinical and transient (Burk et al., 1996, Cubilla et al., 2008, Figueroa et al., 1995). The virus is thought to clear spontaneously by the shedding of virus infected keratinocytes within 4 to 20 months(Trottier and Franco, 2006)with more than half clearing the virus by 12 months. However, people with specific persistent infections may develop clinical lesions that may include benign papilloma's (warts), or premalignant-lesions that may progress to cancer. In particular, HPV 6 and HPV 11have been shown to be associated with warts while HPV 16 and HPV 18 are known to cause 70% of cervical cancer cases(Longworth et al., 2004)

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In cancers, the HPV genome has been shown to be integrated in the host genome, while in benign warts, the HPV genome is maintained in a non-integrated episomal form, suggesting that integration of viral DNA is important for malignant transformation. The viral integration site into the host chromosome is random, hence HPV infection has no consistent association with a proto-oncogene. The pattern of viral integration is clonal. It interrupts the viral DNA within the E1/E2 open reading frame, leading to loss of E2 viral repressor and over expression of the oncoprotein E6 and E7 (Longworth et al., 2004)

.

1.1.3: Biology of Human Papilloma Viruses

The HPV is a small (55nm), non-enveloped, double stranded DNA (ds DNA) virus that infects the cutaneous and mucosal tissue. The virus contains a double stranded closed circular DNA genome, associated with histone like proteins and protected by an icosahedral capsid formed by two late proteins L1 and L2 (figure1)(Lie and Kristensen, 2008).

The viral genome consists of a single molecule containing approximately 8000 base pairs and harbours an average of 8 open reading frames (ORF). The HPV genome is functionally divided into three regions as shown in figure 4.

- A non-coding upstream regulating region (URR) or long control region (LCR). This has a regulatory function of the transcription of the E6 and E7 viral genes.

- The early region (E). This consists of six ORF: E1, E2, E4, E5, E6 and E7. This region encodes six non-structural proteins involved in viral replication and oncogenesis.
- The late (L) region encodes the two structural proteins (L1 and L2). See figure 4. (HPV genome).

The genes code for different proteins and each of these play a specific role as shown in table 2.

Table 2. The HPV proteins and their functions

E1	Ubiquitin-activating enzymes, acts as a DNA helicase and is involved in viral DNA replication(Hughes and Romanos, 1993).
E2	Ubiquitin-conjugating enzymes are transcription activators. Control of viral transcription, DNA replication, and segregation of viral genomes(Hughes and Romanos, 1993).
E3	Ubiquitin-protein ligases, permit degradation of target proteins and thereby enhance the efficiency of replication.
E4	Favour and support the HPV genome amplification, besides regulating the expression of late genes, controlling the virus maturation, and facilitating the release of virions
E5	Enhance the transforming activity of E6 and E7; promotes fusion between cells, generating aneuploidy and chromosomal instability; Contribute to immune response evasion.
E6	Oncoprotein. Bind and degrade the tumour-suppressor protein p53, inhibiting apoptosis; interact with proteins of the innate immune response, contributing to immune evasion and persistence of virus; Activate the expression of telomerase (Frattini et al., 1996, Kajitani et al., 2012).
E7	Oncoprotein. Bind and degrade the tumour-suppressor protein pRB; Increase cdk activity; affects the expression of S phase genes by directly interacting with E2F factors and with histone deacetylases; Induce a peripheral tolerance in cytotoxic T lymphocytes (CTL) and downregulate the expression of TLR9, contributing to immune response evasion (Frattini et al., 1996, Kajitani et al., 2012).
L1	Major capsid protein; contains the major determinant required for attachment to cell surface receptors. It is highly immunogenic and has conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus.
L2	Minor capsid protein; L2 contributes to the binding of virion in

	the cell receptor, favouring its uptake, transport to the nucleus, and delivery of viral DNA to replication centres. Besides, E2 helps the packaging of viral DNA into capsids

The E1, E2, E4 and E5 proteins produced are required for viral DNA replication while E6 and E7 Oncoproteins regulate cell cycle control, maintain HPV viral genome and co-operate to transform and immortalize infected cells aided by E3 ubiquitin ligase. The L1 and L2 proteins are needed for production of viral particles (Frattini et al., 1996, Kajitani et al., 2012).

Infection occurs when the virus accesses the basal epidermal layer and the virus is endocytosed. Following endocytosis, viral capsid dis-assembly occurs and viral genome transfer into the nuclei of mitotic keratinocytes occurs with the aid of L2 protein. The viral genome is maintained in episomal form during the normal life cycle and replicates in synchrony with the host cell DNA under the mediation of E1, E2, E4 and E5 viral proteins. In most high grade lesions and cancers, the viral genome is integrated into the host genome through the disruption of the E2 reading frame, while in benign warts, the viral genome is maintained in a non-integrated episomal form. This suggests that integration of viral DNA is important for malignant transformation. The viral integration site into the host chromosome is random, hence HPV infection has no consistent association with the proto-oncogene. The pattern of viral integration is clonal. It interrupts the viral DNA within the E1/E2 open reading frame, leading to their over expression. The oncoproteins E6 and E7 are then free to inhibit the Rb and the p53 tumour suppressor pathways(Lie and Kristensen, 2008).

1.2 PENILE TUMOURS

Tumours of the penile are uncommon(Kumar et al., 2014). The frequent tumours of the penis include carcinomas and condyloma acuminatum (warts).

1.2.1 Condyloma acuminatum

Condyloma acuminatum (also known as genital wart) is a benign epithelial tumour that is HPV- related and the lesions have a predilection for the anogenital area. It is highly contagious and is transmitted through skin or mucosal contact during genital, oral or anal sex with an infected partner. HPV 6 and 11 are the most frequent identified serotypes (Buechner, 2002, Nuovo et al., 1990, Giuliano et al., 2008c), although others (16, 18, 30, 31, 32, 42, 43, 44, 51, 52, 53, 54, and 55) have been identified as well (Shah, 1992, Clark, 1987, Cobb, 1990). Condyloma may appear as flat topped warts on the shaft of foreskin, small polyps on the glans or urethral meatus or larger cauliflower- like tumours that may be confused with cancer.

1.2.2: Penile Cancers

Most penile cancers are squamous cell carcinomas (SCC) and most originate from the squamous mucosa of the glans, urethral meatus, foreskin (prepuce) and the skin covering the penile shaft (Kumar et al., 2014). Penile carcinomas occur in two forms: a pre-invasive form (carcinoma in-situ) and an invasive variety.

Invasive SCC of the penis is an uncommon tumour in the developed world, accounting for 0.5% of all cancers in males in the United States of America, with the average age of patients being 60 years. However, it is more common in the less developed countries and it accounts for up to 10% of cancers in men in parts of Africa, Asia and South America (Nordenvall et al., 2006, Zhu et al., 2007).No single agent has been identified as a cause of penile cancer; however, it is associated with poor genital hygiene and with high risk HPV infection. Current interest centres on the possible influence of an accumulated keratin debris and inflammatory exudate (smegma) that accumulate beneath the prepuce, as most patients have had phimosis since early age, suggesting that prolonged contact between smegma and the mucosa may play a role. Other risk factors for penile cancers include phimosis (Madsen et al., 2008, Daling et al., 2005, Tseng et al., 2001), genital warts, HPV infection(Rubin et al., 2001, Gregoire et al., 1995, Cubilla et al., 2010), lichen sclerosis (Minhas et al., 2010, Micali et al., 2006) and immune suppression (Bleeker et al., 2009). Circumcision early in life is protective from penile cancer as cancer is uncommon in males who are circumcised at birth.

Evidence of HPV infection is found in 40-45% of penile carcinomas (Pow-Sang et al., 2010). Studies have shown a correlation between viral presence and tumour morphology (Gregoire et al., 1995, Cubilla et al., 2008). Tumours that show histologic features of Basaloid type, mixed waxy basaloid type, and condylomatous types are associated with HPV in 80-100% of cases. HPV is usually negative in usual type, sarcomatoid, papillary, verrucous and pseudo hyperplastic carcinomas (Cubilla et al., 2008, Gregoire et al., 1995).

1.2.3: Penile Intraepithelial Neoplasia

Penile Intraepithelial Neoplasm (PeIN) may be sub classified as differentiated (simplex), warty, basaloid and warty/ basaloid (mixed) (Pfannl et al., 2008, Cubilla et al., 2008) as shown in table 3.

Table 3. Histologic classification of Penile Intraepithelial Neoplasia.

Differentiated
Undifferentiated • Warty • Basaloid • Mixed warty-Basaloid • Others. Small spindle, or clean cells, pagetoid or pleomorphic
Mixed Differentiated and Undifferentiated

Differentiated PeIN tends to affect the skin of older patients were as PeIN with warty and /or basaloid features preferentially affects the glans of younger patients. Other features of differentiated PeIN include the following; HPV unrelated, anatomic predilection for foreskin, preferential association with lichen sclerosis and it is precursor of keratinizing and well differentiated variants of penile squamous cell carcinoma. Morphologically, differentiated PeIN shows epithelial thickening, parakeratosis, elongation of rete ridges, prominent bridges, enlarged nuclei, prominent nucleoli and basal cell atypia. The clinicopathologic features of undifferentiated PeIN include, a tendency to affect younger patients, association with HIV infection, anatomic predilection for glans penis and precursor lesion for HPV

related penile tumours (condylomatous, basaloid and mixed variants)(Pfannl et al., 2008, Cubilla et al., 2008).

Basaloid PeIN is morphologically characterised by a replacement of the normal epithelium by small, uniform cells with round nuclei and scant cytoplasm. Numerous mitoses and apoptotic cells are usually present: warty PeIN shows a spiky surface with parakeratosis. The normal epithelium is replaced by markedly pleomorphic cells showing prominent koilocytosis. Mixed-basaloid lesions show a mixed feature. Warty and basaloid PeIN are HPV related lesions and usually over express p16 (also known as cyclin-dependent kinase inhibitor 2A or multiple tumour suppressor 1 is a tumour protein, and in humans, is encoded by the *CDKN2A*gene. Over-expression of p16 in penile or cervical specimens, detected by immunohistochemistry is highly correlated with oncogenic transformation caused by persistent high-risk HPV infections) (Pfannl et al., 2008, Cubilla et al., 2008).

1.2.4: Squamous Cell Carcinoma in situ

Historically, carcinoma in situ of the penis was clinically described in two forms: Bowen disease and Eythroplasia of Queyrat. Bowen disease appears as leucoplakia involving the shaft of the penis and scrotum. It is usually seen in patients 35 years and above. It is commonly associated with HPV 16. Bowen disease transforms into infiltrating squamous cell carcinoma in about 10% of patients, usually over a span of many years. Erythroplasia of Queyrat manifests as single or multiple, shiny, erythematous plaques on the glans and foreskin. It is associated with HPV 16 and is a precursor of invasive squamous cell carcinoma (Rubin, 2011; Goljan, 2011). Bowenoid papulosis appears as multiple papules unlike the single lesions of Bowen disease. It is a variant of carcinoma in situ, however, it has no predisposition for invasion (Goljan, 2011).

1.2.5: Invasive Squamous Cell Carcinoma

Invasive penile cancer is a malignant growth found in the skin or in the tissues of the penis. It is a rare malignancy that typically presents as a painless lump or ulcer. It usually arises from the epithelium of the inner foreskin (prepuce) or the glans penis.

Around 95% of penile cancers are usually squamous cell carcinomas, although there are other types of carcinoma such as Markel cell carcinoma, small cell carcinoma, melanoma and other rare ones. The invasive squamous cell carcinomas are classified into the following histologic subtypes as shown in table 4 below(Hakenberg et al., 2014).

Table 4: Histologic subtypes of penile carcinomas, their frequency and outcome

Subtype	**frequency (% of cases)**	**Prognosis**
Common SCC	48-65	depends on location, stage and grade
Basaloid carcinoma	4-10	poor prognosis, frequently early inguinal nodal metastasis
Warty carcinoma	7-10	good prognosis, metastasis rare
Verrucous carcinoma	3-8	good prognosis, no metastasis
Papillary carcinoma	5-15	good prognosis, metastasis rare
Sarcomatoid carcinoma	1-3	very poor prognosis, early vascular metastasis
Mixed carcinoma	9-10	heterogeneous group
Pseudohyperplastic carcinoma	<1	foreskin, related to lichen sclerosus, good prognosis, metastasis not reported
Carcinoma cuniculatum	<1	variant of verrucous carcinoma, good prognosis, metastasis not reported
Pseudoglandular carcinoma	<1	high grade carcinoma, early metastasis, poor prognosis
Warty-basaloid carcinoma	9-14	poor prognosis, high metastatic potential (higher than in warty, lower than in basaloid SCC)
Adenosquamous carcinoma	<1	central and peri-meatal glans, high grade carcinoma, high metastatic potential but low mortality
Mucoepidermoid carcinoma	<1	highly aggressive, poor prognosis
Clear cell variant of penile carcinoma	1-2	exceedingly rare, associated with HPV, aggressive, early metastasis, poor prognosis, outcome lesion dependent, frequent lymphatic metastasis

Source: (Hakenberg et al., 2014)

The diagnosis of penile cancer is made based upon biopsy of the lesion and its pathology is similar to squamous cell carcinoma of the oropharynx, female genitalia (cervix, vagina and vulva) and anus, and they share some of the natural history.

It is not known how often squamous cell carcinoma is preceded by premalignant lesions (Table 5), and up to one-third of the premalignant lesions will transform to invasive squamous cell carcinoma.

Precursor lesions generally progress from low-grade to high-grade lesions. HPV related penile cancer follow a sequence from squamous hyperplasia to low grade PeIN to high grade PeIN (carcinoma in situ—Bowens disease, Erythroplasia of Queyrat and Bowenoid papulosis) and finally to invasive carcinoma of the penis (Bleeker et al., 2009). However, in some cases non dysplastic lesions may progress directly into cancer as seen in condyloma accuminatum, flat penile lesions (Bleeker et al., 2009) and lichen sclerosis (seen in HPV negative cancers) (Bleeker et al., 2009).

Primary penile cancer variants that have not yet been included in the WHO classification include: pseudo hyperplastic carcinoma, carcinoma cuniculatum, pseudoglandular carcinoma and warty Basaloid carcinoma (Hakenberg et al., 2014).

Table 5. Premalignant Penile Lesions (Precursor Lesions).

1. Lesions sporadically associated with squamous cell carcinoma of the penis
   - Cutaneous horn of the penis
   - Bowenoid papulosis of the penis
   - Lichen sclerosis (balanitis xerotica obliterans)

2. Premalignant lesions
   - Intraepithelial neoplasia grade III (PeIN)
   - Giant condylomata (Buschke-Löwenstein)
   - Erythroplasia of Queyrat
   - Bowen disease
   - Paget disease (intra-dermal ADK)

The presenting signs and symptoms of invasive penile cancer may vary but commonly include:

Redness, rash, foul smelly discharge, pain, non-healing ulcer or growth, change in colour, phimosis or bleeding from penis or prepuce (Maddineni et al., 2009, UK, 2016).

1.2.6: Risk factors for developing penile cancer

The risk factors for developing penile cancer include:

—infections:

- HPV infection: infection with HPV is the most important risk factor for penile cancer(Pow-Sang et al., 2010). HPV is responsible for about 40% of penile cancers diagnosed annually in the United States of America (Reis et al., 2010, Morris et al., 2011).
- HIV/AIDS: the immune suppression which is as a result of HIV/AIDS makes the immune system less able to fight early stage cancer. HIV positive men have eightfold increased risk of developing penile cancer (Seu, April 2009).
- Genital warts: males with genital or perianal warts have a 3.7 times risk for penile cancer when present for at least two years before cancer diagnosis and about half the men with penile cancer also have genital warts which are caused by HPV.

—Hygiene and injury

- Poor penile hygiene increases ones risk for penile cancer (Minhas et al., 2010, Reis et al., 2010).
- Smegma accumulation beneath the foreskin increases the risk for penile cancer (Morris et al., 2011, Seu, April 2009) probably through chronic irritation and associated inflammation (Seu, April 2009).
- Balanitis is associated with about 3.1 times increased risk for cancer(Pow-Sang et al., 2010).
- Phimosis is both a risk factor and a symptom for penile cancer. (Pow-Sang et al., 2010).

- Paraphimosis is a risk factor for cancer probably through chronic irritation and ulceration.

—Others:

- Age: penile cancer is commonly seen in males above the age of 50 with 4 out of 5 males diagnosed with penile cancer being over the age of 55 (Reis et al., 2010).
- Lichen sclerosis is commonest risk factor for cancer in elderly people that develop penile cancer in the absence of HPV infections.
- Smoking: tobacco chewing or smoking increases the risk for penile cancer by 1.5-6 times.
- Ultraviolet (UV) light: exposure to UV light treatment and a drug known as psoralen increases risk for penile cancer (Minhas et al., 2010).

1.2.7: Prevention of Penile Cancer

Practices that help prevent penile cancers include:

1. HPV vaccination such as Gardasil or cervarix may reduce the risk of HPV infection, and, consequently penile cancer (Bleeker et al., 2009, Minhas et al., 2010).
2. Genital hygiene which involves washing the penis, scrotum and prepuce daily to prevent balanitis.
3. Cessation of smoking (Pow-Sang et al., 2010).
4. Prevention of phimosis that can be achieved by practicing proper hygiene and by retracting the foreskin on a regular basis.
5. Prevention of paraphimosis is achieved by not leaving the foreskin retracted for prolonged periods of time.
6. Circumcision particularly during infancy or in child hood may provide some protection against penile cancer.

Three vaccine types are available to prevent HPV types that cause most cervical cancers as well as some cancers of the penis, anus, vulva, vagina and oropharynx, as

well as genital warts. These vaccines are given in three shots over 6 months. The vaccines are type specific and hence they will not offer protection against HPV types not included in the vaccine type (Paavonen et al., 2007, Paavonen et al., 2009).

Three vaccines are currently on the market:

Cervarix: HPV types 16 and 18

Gardasil: HPV 6, 11, 16, 18 (VAX, MERCK)

Gardasil 9: HPV types 6, 11, 16, 18, 31, 33, 45, 52, 58 (VAX, MERCK)

The above vaccines have been licensed for use in girls aged 11 and 12, but it is also recommended for females age 13 through 26 years of age who have not yet been vaccinated or completed the vaccine series.

Gardasil and Gardasil 9 are licensed for use in boys aged 9-12 years. The recommended routine vaccination is for boys aged 11 or 12 years with doses for most effectiveness. However, it can still be administered to males aged 13 through 21 years who have not yet been vaccinated or not received the three doses (Center for Disease control, 2015). All the three vaccines have been licensed by the Food and Drug agency (FDA) and approved as safe and effective by CDC (Center for Disease control, 2015).

The HPV vaccine was introduced for the very first time on the immunization program in Zambia on 27th May, 2013 and it targeted girls only in three districts in the initial place of 2013/2014(Nora, 2015).

1.3 Epidemiology of HPV

1.3.1: HPV infection in males

HPV is the most common sexually transmitted infection and it is spread from one person to another through close skin to skin or mucosal contact especially during vaginal, anal or oral sexual activity. Much of the information about HPV infection centres on women because the virus significantly increases their risk for getting cervical cancer (CDC, 2015).

Numerous cross sectional studies and prospective cohort studies have been conducted in many different populations to determine the prevalence of genital HPV infections in males. A systematic review of global literature demonstrates a wide range of HPV DNA prevalence among men in different countries. The results show a variation with geographic region and level of development and it ranges from 6.9% in less developed countries and 14.2% in sub-Saharan Africa to 15.5% in India (De Sanjosé et al., 2012).

Systematic review of papers summarizing global data on genital HPV in men showed a wide range of prevalence rates both between and within high and low risk groups and by geographic region.

A review by Smith et al of populations at high risk of HPV infection, such as sexually transmitted infection (STI) clinic attendees, HIV positive males, and partners of females with HPV infection or abnormal cervical cytology showed that the prevalence was high among all sexual active males in all regions and had considerable variation, from 1% to 84% among low risk men and from 2% to 93% among high risk men(Smith et al., 2011).

A systematic review of literature on HPV prevalence among asymptomatic men in studies in which multiple anatomic sites of specimen were evaluated ranged from 1.3% to 72.9% (Dunne et al., 2006), (see figure 2). The results tabulated show the results of prevalence studies in which PCR was used to detect the presence of the virus in samples collected from the glans penis, sulcus and scrotum. The study population reviewed included the general population (workers, students), patients attending sexually transmitted diseases (STD) clinics, healthy participants, military personnel and partners of women with HPV related disease. The results obtained showed a wide range of prevalence rates both between and within the study groups.

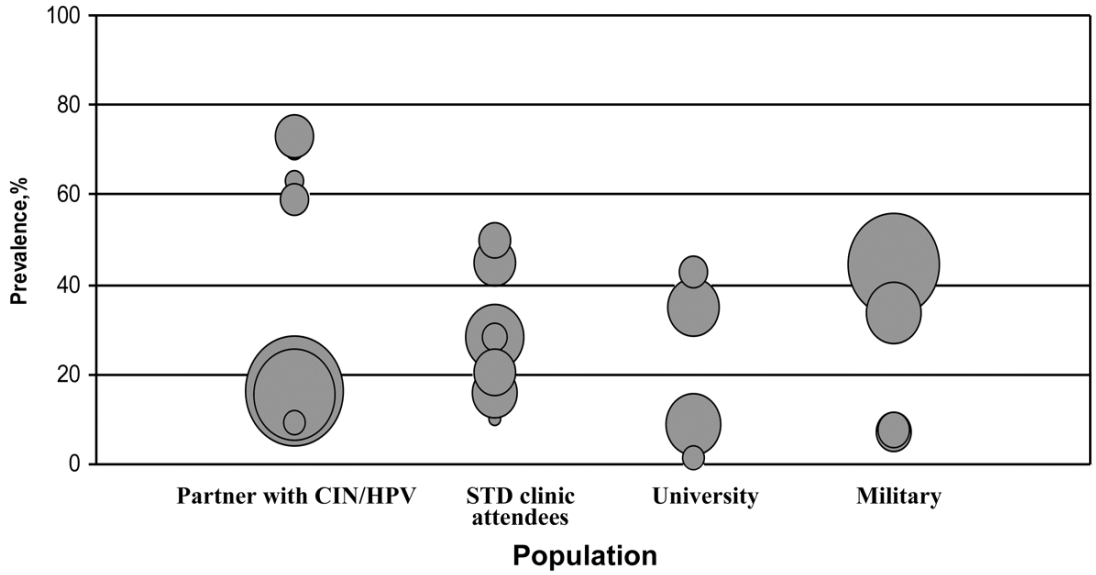


Figure 2. Human Papillomavirus (HPV) prevalence in asymptomatic men of various populations. Source: (Dunne et al., 2006).Each circle represents a study, the size of the circle indicates the no. of males tested, and the centre of the circle is the point estimate of prevalence. CIN, cervical intraepithelial Neoplasia; STD, sexually transmitted disease.

A review by the Rome Consensus Conference (Lenzi et al., 2013) summarized the main prevalence studies (Table 6) conducted using PCR method to detect the presence of HPV in multiple samples collected from different anatomical sites that included the glans, penis, sulcus and scrotum. The study population reviewed varied and included general workers, students, STD clinic attendees and partners of women with HPV related diseases such as Cervical Intraepithelial Neoplasia (CIN). The prevalence rates results ranged from 6% to 45% for studies that did not take into consideration partners with HPV positivity and this group included University students, military recruits and healthy sexually active males. However, the prevalence rate was higher (50% to 70%) in males whose female partners had CIN. The mean prevalence was 45% for males that were recruited from STD clinics.

Table 6. Main prevalence studies conducted using the PCR method

Population type (n° males examined)	Country	Prevalence of positive HPV DNA (PCR test)	Prevalence of positive HPV DNA tests (high-risk genotypes)
Population (290) (Giuliano et al., 2007)	USA	30.0%;	16.6%;
Military recruits (285) (HIPPELäINEN et al., 1994)	Finland	16.5%;	- -
University students (317) (Weaver et al., 2004)	USA	32.8%;	14.5%;
Students and industrial workers (114) (Lazcano-Ponce et al., 2001)	Mexico	36.0%;	16.7%;
Military recruits (337) (Kjaer et al., 2005)	Denmark	33.8%;	- -
Military men (1030) (Lajous et al., 2005)	Mexico	44.6%;	34.8
University students (381) (Shin et al., 2004)	South Korea	8.7%;	4.2%;
Subjects attending STD clinics (235) (Wikström et al., 2000)	Sweden	13.2%;	8.1%;
Subjects attending STD clinics (198) (Svare et al., 2002)	Denmark	44.9%;	- -
Subjects attending STD clinics (393) (Baldwin et al., 2003)	US	28.2%;	12.0%;
Subjects attending STD clinics (85)	Netherland	28.2%;	- -

(Van Doornum et al., 1994)			
Subjects attending STD clinics (204) (Takahashi et al., 2005)	Japan	5.9%;	5.9%;
Males with female partner affected by CIN (119) (Bleeker et al., 2002)	Netherland	59.0%;	55.4%;
Husbands of women recruited in case–control studies on carcinoma of the cervix (1143) (Franceschi et al., 2002)	Thailand, Philippines, Brazil, Columbia, Spain	16.0%;	- -
Males with female partner affected by CIN (181) (Bleeker et al., 2005)	Netherland	72.9%;	58.6%;
Males with female partner affected by CIN (77) (HIPPELäINEN et al., 1994)	Finland	9.1%;	- -
Males with female partner affected by HPV (50) (Nicolau et al., 2005)	Brazil	70.0%;	- -
Males through 18–70 years from one of the three sampling sites (1,160) (Giuliano et al., 2008b, Giuliano et al., 2008a)	Brazil Mexico USA	65.2%;	12.0%; oncogenic types only

Source: (Lenzi et al., 2013).

A more recent and significant study that included the largest sample size (1160 men) from three different countries including Brazil, Mexico, USA, reported a total prevalence of 65.2% HPV positivity (Giuliano et al., 2008c).

The reviews above show that there is no information on the prevalence of genital HPV and its associated disease burden in males for Zambia (Zambia: HPV and related cancers, summary report 2010). HPV genotype studies from Zambia were in women and these had different findings. A study by Ng'andwe (Ng'andwe et al., 2007) found a prevalence rate of 72%. HPV 16 and 18 were found to be the most prevalent Hr-HPV types (21.6% each) among HIV positive and HIV negative women. However, a study by Sahasrabuddhe(Sahasrabuddhe et al., 2007), showed a relatively higher frequency of HPV type 52 (37.2%), 58 (24.1%) and 35 (20.7%) than HPV 16 (17.2%) and 18 (13.1%) among HIV positive women receiving ART at the University teaching hospital (U.T.H) in Lusaka.

A cross sectional study from South Africa that assessed samples collected from the coronal sulcus, glans penis, penile shaft and perianal regions when warts were present had an HPV prevalence of 78%. HPV was detected in 100% of patients with genital warts, 48% of males with urethral discharge, and 62% of asymptomatic participants attending HIV voluntary counselling and testing (VCT) (Müller et al., 2010). The HPV test method involved the use of the linear array HPV genotype test in all these three studies.

1.4: Circumcision

The penile foreskin (also described as prepuce) is a double layered fold of stratified squamous epithelium containing smooth muscle tissue, blood vessels, neurons and connective tissue. The outer layer is comprised of keratinizing stratified epithelium whilst the inner layer is a nonkeratinizing mucosal membrane. The foreskin covers and protects the glans penis and the urinary meatus when the penis is not erect. It is mobile and fairly retractable over the glans. The WHO suggests that the foreskin helps to maintain the glans moist, protects the developing penis in utero and enhances

sexual pleasure due to its abundant nerve endings. Other functions include protection to the thin skinned glans against injury (WHO, 2015).

Circumcision is a surgical procedure that is used to remove the foreskin. It is an ancient practice that has been performed as a religious ritual, family tradition, for personal hygiene or for preventive health care. In Zambia, circumcision has been used as a family tradition among people of the North Western province as a symbol of transition into adulthood.

The Ministry of Health (MOH) has introduced VMMC into the male reproductive health package as part of comprehensive HIV prevention strategies as well as HIV testing entry point for access to antiretroviral therapy (ART), because medical circumcision has been shown to reduce the risk of HIV transmission through heterosexual intercourse by up to 60% in the absence of high risk behaviours. The current prevalence rate of male circumcision in Zambia is 17% and MOH has a goal of a male circumcision prevalence of 50% by the year 2020. The ministry hopes to achieve this by making high quality male circumcision services accessible and available to all men and boys on a voluntary basis for free (Nora, 2015).

The inclusion of male circumcision into the male reproductive health package was necessitated by the fact that circumcision in males has been shown to protect against sexually transmitted infections and penile cancers (Bouvard et al., 2009, Nicolau et al., 2005). More important is the protection of their female partners from cervical cancer which is ten times (fold) more common in women with uncircumcised partners (Castellsagué et al., 2002, Morris et al., 2011).

It is postulated that male circumcision is associated with better hygiene, which, in turn, reduces exposure to HPV and carcinogens that may be concentrated in smegma (Larke et al., 2011, Rehmeyer, 2011).

2. STUDY SETTING AND RATIONALE

2.1 Study Setting

The study was conducted in Lusaka, the capital and largest city of Zambia. The city covers approximately 360 square kilometres. The population of Lusaka as of 2010 was about 1.7 million and its people are scattered across low-, middle- and high-income communities.

The residents of Lusaka come from all of Zambia's tribes, and there are also small communities of Asians and Europeans. Over 60% of the nearly 2 million inhabitants of Lusaka are unemployed.

Lusaka is the centre of both commerce and government in Zambia and connects to the country's four main highways heading north, south, east and west.

2.2Rationale

HPV infection is a highly prevalent STI and nearly all sexually active men and women will get HPV at some time in their life. Although most HPV infections resolve on their own without causing problems, some types can cause health problems including genital warts and cancers. Widespread efforts to study the epidemiology of HPV and its complications (genital warts and cancer) will become increasingly valuable for prevention programs such as HPV vaccination and cancer screening.

To our knowledge, there are no published data that describes prevalence of HPV infection in Zambian males. The reporting of such epidemiologic data will be important in informing for a national wide study as well as future male vaccination and screening efforts.

2.3: OBJECTIVES

Main objective

The main objective of this study was to determine the prevalence of genital HPV infection and microscopic penile foreskin lesions in males going for voluntary medical circumcision in selected clinics in Lusaka.

Specific objectives

i) To determine the proportion of genital HPV infections in the sample population.

ii) To describe any microscopic changes present in the sampled tissue.

iii) To determine the socio-demographic and behavioural risk factors for HPV infection in the sample population.

Hypothesis

The primary hypothesis was that males in Lusaka experience a high prevalence of HPV infection and that age and sexual activity are the most important predictors of HPV infection.

3. METHODOLOGY

3.1 STUDY DESIGN

3.1.1. Overview

A cross sectional survey study was used to answer the primary study questions. This analysis was conducted using data collected for males recruited from five circumcision sites in Lusaka, Zambia. The study utilized information from a baseline questionnaire and corresponding HPV- DNA and histopathology test results.

3.1.2. Site

The study was conducted in Lusaka, Zambia. The study sites were purposefully chosen based on the high numbers of circumcisions conducted per month. The sites chosen included U.T.H (Adult centre of excellence), Railway clinic, M'tendere clinic, Chelstone clinic and Chawama clinic. Participants that came to any one of these sites for VMMC were approached to participate in the study by a practitioner nurse after explaining what the study was all about.

3.1.3. Target population

The target for this study was all males aged 10 years and above that presented for VMMC between January, 2014 and December 2014.

The total population for Lusaka was 1.7 million as of 2010. The males that were circumcised were all HIV negative as circumcision was part of the HIV comprehensive package of testing for the disease. Males that were found to be HIV positive were referred to the ART clinic for work-up for anti-retroviral therapy.

3.1.4. Study population

The study population for this study was consenting males aged 10 years and above that went to one of the above circumcision sites for VMMC and had no visible penile lesions.

3.1.5. Sample size calculation

There were no published data on the prevalence of HPV infections or microscopic penile lesions amongst males undergoing VMMC in Zambia. No pilot study was done prior to this project, and therefore the sample size was calculated using a simplified formula for proportions by Yamane (1967: 886)(Israel, 1992), as shown below. The formula outlines the sample size needed for the prevalence rate and the levels of precision.

$$\mathbf{n} = \frac{\mathbf{N}}{\mathbf{1} + \mathbf{N}(\mathbf{e})^{\mathbf{2}}}$$

Where $\mathbf{n}$ = Sample size

$\mathbf{N}$ =Is the population size

$\mathbf{e}$ =The level of precision.

In the year 2012, the five clinics mentioned above conducted a total of 776 circumcisions. Using the formula, a total of 264 samples were required based on a 95% confidence interval and 5% precision.

The participants of the study were enrolled using multistage sampling. The sites for participants enrolment where purposefully chosen based on the high number of circumcisions conducted on a monthly bases. Out of the total circumcisions that took place during the study period in the selected sites, 379were approached to participate in the study and 312 gave consent and were handled as shown in figure 3 below.

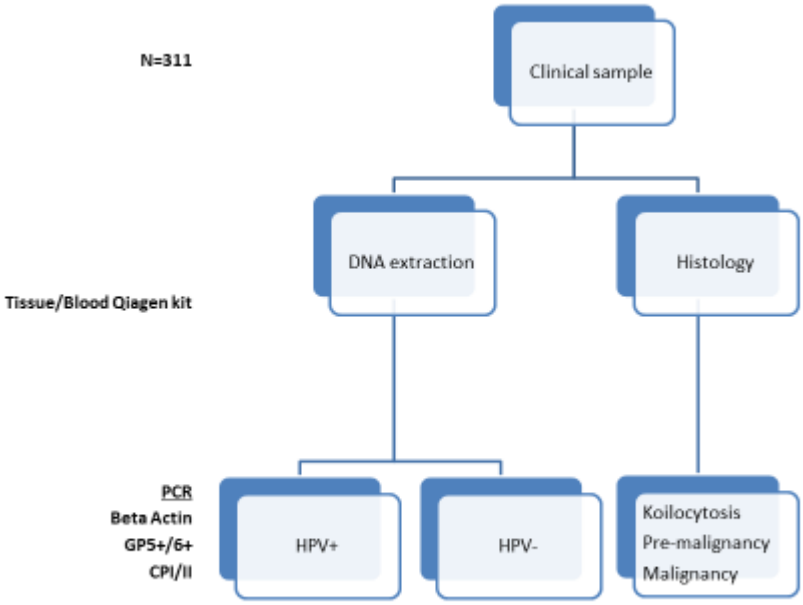


Figure 3. Genital HPV infection Experimental Design

A cohort 311 HIV negative foreskin formalin fixed, paraffin embedded tissues were studied. Genomic DNA was extracted from the tissues. The quality of the extracted DNA was checked using Beta (β) active primers. PCR amplification to detect HPV infection was done using GP 5+ / GP 6+ and CP I/ CP II primers. Histologic analysis to determine the presence of inflammation, premalignant and malignant microscopic lesions was done whilst blinded for HPV infection detected by PCR.

The participants were stratified in different age groups so as to have equal numbers across the age strata as shown below in table 7.

Table 7.Participant's age group stratification

Age range (years)	Total enrolled	# enrolled/ clinic
10—14	55	11
15—19	55	11
20—24	55	11
25—30	55	11
≥30	55	11
Total	**275**	**275(5 clinics)**

The participants for each age range were enrolled using multistage (stratified) sampling for participation into the study. Clients that agreed to participate in the study were enrolled.

3.1.6. Eligibility criteria

Males were eligible to participate in the study if they:

i) Were 10 years of age and above
ii) Undergoing VMMC in the study sites
iii) Had no visible penile lesions
iv) Had no history of receiving the HPV vaccine.

3.1.7. Subject recruitment

The sample frame constituted of all males aged 10 years and above presenting at one of the study clinics for VMMC between January2014 and June 2014. These circumcision sites are all located in Lusaka, the capital city of Zambia. M'tendere and Chawama clinics are based in communities of low social economic status while Chilenje and Railway clinics are based in medium social economic status communities. UTH receives clients from across the city. Qualified nurse and clinical officer practitioners systematically asked all HIV negative males aged 10 years and above about their willingness to participate in the study after receiving information

about the study (Appendix 1), and, if they were interested, determined their eligibility.

Written informed consent was obtained from all eligible subjects by means of standardised consent form (Appendix 2). Consent from a guardian was obtained for eligible subjects below the age of 18 years. Participation was voluntary and it could be withdrawn at any time without having to give reasons. The consenting participants were asked questions from a standardized questionnaire (Appendix 3) by a nurse or clinical officer practitioner, who recorded the responses on the questionnaire form. The questionnaire collected information about socio-demographic and behavioural characteristics. Foreskin specimens were collected and fixed in 10% Neutral buffered formalin (10% NBF) and embedded in paraffin for both HPV- DNA testing and histologic assessment.

3.1.8. Ethical considerations

Ethics approval was obtained from Ethics and Sciences (EREs) Institutional Research Board (IRB). (Appendix 4). Permission was also sought for from the Lusaka District Health Management team (Appendix 6) and the management of U.T.H (Appendix 7), which provides services to the participants. All the participant information sheets and specimens were stripped of identifiers by the nurse practitioner to blind the PI and supervisor from the study participants.

3.2 DATA COLLECTION

3.2.1. Questionnaire

A questionnaire (Appendix 3) was administered at baseline by a nurse or clinical officer practitioner. The information collected included social demographic characteristics, sexual history, marital status and a history of having received the HPV vaccine. The questionnaire was provided in English. The questionnaire was piloted at the U.T.H site to ensure its comprehensibility and ease of use as a study tool. The questionnaire was administered to males before circumcision. The males had already been tested for HIV. The group comprised HIV negative males only because all HIV positive males were referred to the ART clinic for pre-ART assessment by the VMMC service providers.

3.2.2. Sample collection

All clients who gave informed consent had their foreskin sample collected immediately after circumcision. The specimens were immersed in 10% NBF immediately for fixation. All samples together with the questionnaire and consent form were couriered to the UTH, Histopathology laboratory.

3.2.3. Laboratory specimen processing

The specimens were all processed by the principle investigator. The specimen was retrieved from the specimen container and dissected on the lid for the specimen container using a sterile blade to avoid specimen contamination. Each half was put in a separate cassette and labelled using sample number as sample A and B. The specimens were then processed using standard Histology techniques (Appendix 8-11). The specimens were then embedded in paraffin wax and stored. Blocks labelled A were submitted for preparation of histology slides. A single Histotechnologist prepared all the slides for histology. Six serial 5µm tissue sections were cut from each block (Appendix 9). The first section and the sixth sections were mounted on a glass

slide. The slides were stained with Haematoxylin and Eosin (H & E) according to SOP (Appendix 10). The slides were examined under light microscopy by a PI and a consultant pathologist. Histopathologic changes were categorised as shown in table 8 below.

Table 8. Classification of penile epithelial cellular abnormalities.

Number	Histological abnormality	Category
1.	Acute and / or chronic inflammation	No dysplasia
2.	Koilocytes / viral wart changes	No dysplasia
3.	Low grade dysplasia	Dysplasia
4.	High grade dysplasia	Dysplasia
5.	Carcinoma In-situ	Malignant
6.	Invasive squamous cell carcinoma	Malignant

3.2.4. Genomic DNA extraction

The 311 formalin fixed, paraffin embedded samples collected were all shipped to Nebraska Centre for Virology (NCV) at the University of Nebraska-Lincoln (UNL) for genomic DNA extraction and HPV testing.

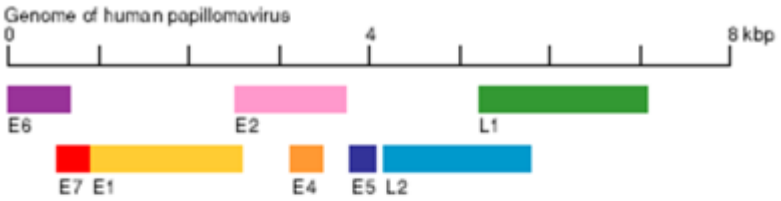
Genomic DNA was extracted according to the manufacturer's protocol by a method based on the DNeasy blood and tissue kit (Qiagen, Valencia, CA) as explained briefly:

Each of the FFPE was micro sectioned in to 5µm levels. Standard precaution was taken to prevent contamination. Two of the 5µm (up to 25mg) sections were placed in a 2ml micro centrifuge tube and treated with 1200µl of xylene to dissolve the paraffin wax from the tissue. The sample was centrifuged in a micro centrifuge for 5 minutes at room temperature and the supernatant was removed by pipetting. The xylene was removed from the sample by gently vortexing with 1200µl of 100% ethanol twice. The ethanol was removed by carefully pipetting to avoid removing the tissue pellet. The open micro centrifuge tube tissue pellet was then incubated at 37℃ for 15

minutes until the ethanol had evaporated. The dry tissue pellet was re-suspended in 180µl of ATL lysis buffer.

Tissue digestion was achieved by adding 20µl of proteinase K in the morning and evening followed by mixing, vortexing and incubation (on a rocking platform) at 56℃ for four consecutive days until the tissue was completely lysed. The digested sample was vortexed and 200µl of buffer AL was added to the sample and mixed thoroughly by vortexing. This was followed by adding 200µl of 100% ethanol and thoroughly mixing by vortexing. The mixture was pipetted into a DNeasy mini spin column placed in a 2ml collection tube and centrifuged at 8000rpm for 1 minute. The flow through liquid in the collection tube was discarded. The mini spin column was placed in a new collection tube after which 500µl of the buffer AW 1 was added and centrifuged at 8000 revolutions per minute (rpm) for 1 minute. The flow through was discarded and the mini spin column was transferred to a new collection tube. Five hundred microliters of buffer AW2 was added and centrifuged at 14000 rpm to dry the DNAeasy membrane. The flow through was discarded and the mini-spin column was placed in a clean 1.5ml micro centrifuge tube. 100µl of buffer AE was pipetted directly into the DNeasy membrane and incubated at room temperature for 1 minute and then centrifuged at 8000rpm for 1 minute to elute the extracted DNA. The extracted DNA was quantified by the Nona Drop spectrophotometer (ND-1000).

The quality of DNA samples was assessed by amplification of a 268bp region of the human β globin gene using forward primer: 5'- GCC ATG TAC GTT GCT ATC C-3' and reverse primer: 5' CCG CGC TCG GTG AGG ATC-3' HPV DNA was detected by PCR amplification using redundant primers GP 5+ /GP 6+ primers (GP 5+: 5'- TTT GTT ACT GTG GTA GAT ACT AC-3' and GP 6+: 5'- GAA AAA TAA ATG TAA ATC ATA TTC-3') amplifying 150 base pairs of the L1 region (nt 6624-6746) of the genome. The CP I /CP II primers (CP I: 5'- TTA TCW TAT GCC CAT TGT ACC AT3' AND CP II: 5'- ATG TTA ATW SAG CCW CCA AAA TT-3') target the E1 region (nt 1777-1964) and amplifying 188 base pairs of the HPV genome (figure 4). Standard precaution was taken to prevent contamination.



Redundant HPV Detection Primer Sets

Name	Sequence	nt Position	Length
Gp5+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′	6624-6649	150
Gp6+	5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′	6719-6746	
CPI	5′-TTA TCW TAT GCC CAY TGT ACC AT-3′	1942-1964	188
CPII	5′-ATG TTA ATWSAG CCW CCA AAA TT-3′	1777-1799	

Figure 4. HPV genome and redundant HPV detection primer sets

The two sets of primers (GP 5+/GP 6+ and CP I/CP II) were used on all the samples to simultaneously detect HPV so as to provide a robust result for our analysis. The thermal cycler model, TECHNE, TC-412, was used for amplification. The parameters for denaturation, hybridization, and extension were as follows: 94℃ for 1 minute, followed by 30 cycles of 95℃ for 30 second, 55℃ for 1 minute, 72℃ for 10 minutes and final hold at 4℃. The positive control constituted the HPV 16 plasmid DNA (pEF 399), whereas the negative control was nuclease free water. Amplification was determined by pre-mixing 30µl of the PCR product from each sample with 1:5µl ratio of 6x loading dye and running it by gel electrophoresis on 2% (W/V) agarose gel in 1x TAE buffer. At the end of the electrophoresis the gel was stained with 0.3% ethidium bromide (0.1 mg/µl solution) for 30 minutes. The DNA fragments on the gel were then visualised under ultraviolet light.

Beta actin (β-actin)

The parameters for thermos-cycling for human beta globin gene amplification were as shown below in figure 5.

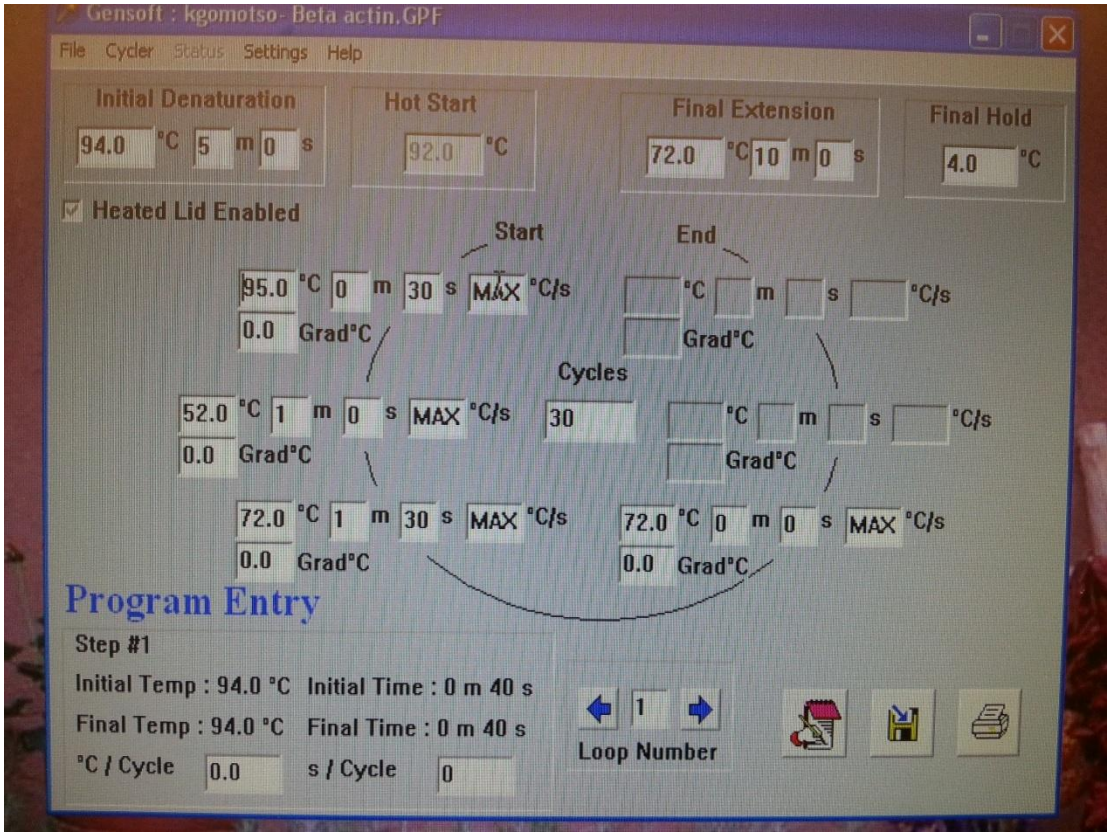


Figure 5.Thermocycling parameters for human beta globin gene amplification

The parameters for thermocycling for GP5+/Gp6+ gene amplification were as shown in figure 6 below.

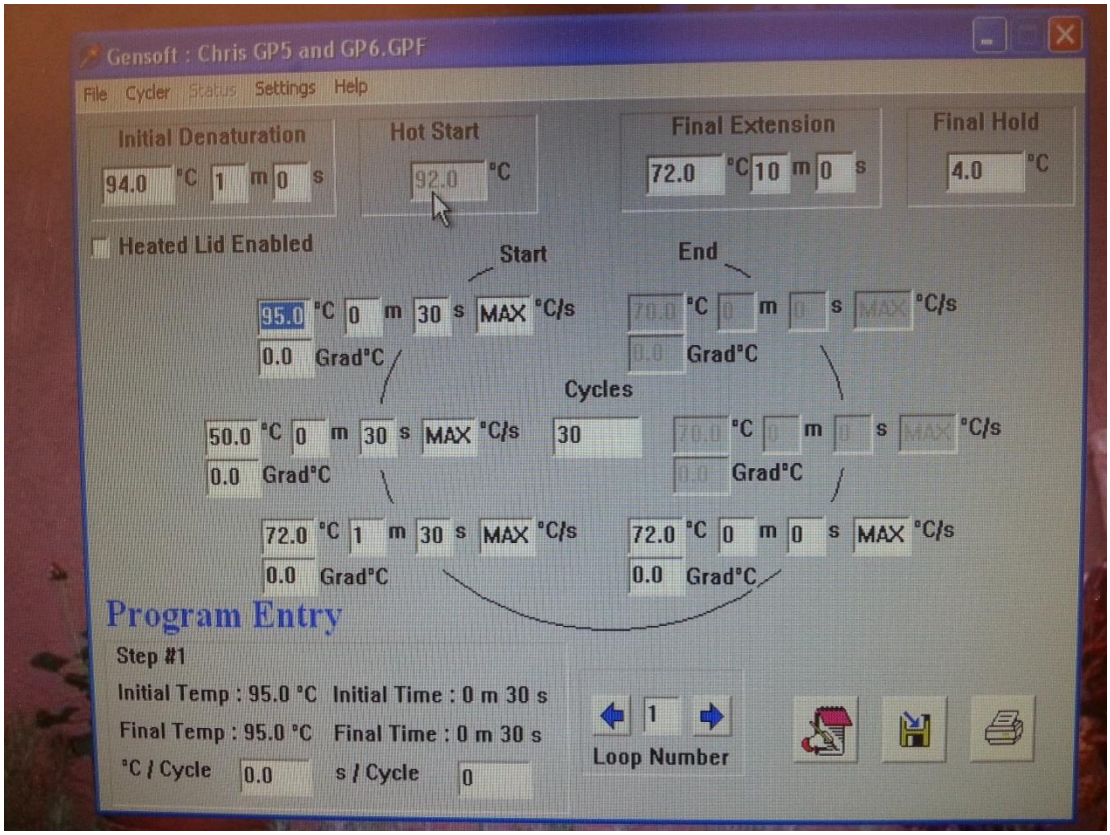


Figure 6.Thermocycling parameters for GP5+/Gp6+ gene amplification

Initial denaturation 94℃ for 1 minute

Denaturation at 95℃ for 30 seconds

Annealing at 50℃ for 30 seconds

Extension at 72℃ for 90 seconds

Final extension at 72℃ for 10 minutes

Final hold 4℃ for ∞ (infinity).

Samples were considered positive for HPV if they were positive for either GP5+/GP6+ or CPI/CPII and also for β-actin globulin. Samples were considered

HPV negative if they tested negative for GP5+/GP6+ and CPI/CPII, but were positive for β-globulin. All samples that were negative for β-globulin were not analysed for HPV and they were considered to have poor quality DNA.

3.2.6. HPV DNA multiplex PCR

Multiplex PCR was conducted using the Qiagen multiplex PCR kit (www.qiagen.com/handbooks).

Primers for genotyping of HPV were obtained from Integrated DNA Technologies (IDT) (idtdna.com).

The sequences of HPV primers for multiplex PCR are shown in table 9 below

Table 9. Sequences of HPV–specific primers for multiplex PCR[a]

Genotype	Primer	Primer sequence (5′–3′)	Length (no. of bases)	GC ratio (%)	T_m (°C)	Position (bp)	Amplicon size (bp)
6	PP × 6/F	GCT AAA GGT CCT GTT TCG AGG CGG CTA	27	55.6	72.1	252–278	263
	PP × 6/R	GGC AGC GAC CCT TCC ACG TAC AAT	24	58.3	71.0	491–514	
11	PP × 11/F	GCG TGT TTT GCA GGA ATG CAC TGA C	25	52.0	70.8	193–217	472
	PP × 11/R	TGC GTC TTG TTT GTC CAC CTT GTC C	25	52.0	70.3	639–664	
16	PP × 16L/F	CGC ACA AAA CGT GCA TCG GCT ACC	24	58.3	73.0	4259–4282	217
	PP × 16L/R	TGG GAG GCC TTG TTC CCA ATG GA	23	56.5	72.5	4453–4475	
	PP × 16U/F	TCC TGC AGG TAC CAA TGG GGA AGA GG	26	57.7	72.5	873–898	397
	PP × 16U/R	TGC CAT ACC CGC TGT CTT CGC TTT	24	54.2	71.5	1246–1269	
18	PP × 18/F	AAC AGT CCA TTA GGG GAG CGG CTG GA	26	57.7	73.7	1199–1224	187
	PP × 18/R	TGC CGC CAT GTT CGC CAT TTG	21	57.1	73.2	1365–1385	
30	PP × 30/F	ACG CAG ACG AAA ACG GGC CTC TGC T	25	60.0	75.0	4297–4321	249
	PP × 30/R	GGC CTA GCA GGG GAT GCG TCC ACA A	25	64.0	76.0	4521–4545	
31	PP × 31/F	GCG GTC CAA ACG CTC TAC AAA ACG CAC T	28	53.6	74.3	4173–4200	360
	PP × 31/R	GCA GGG GCA CCA ACA TCA ACA ATT CCA	27	51.9	75.3	4506–4532	
33	PP × 33/F	ACA CAG AGG CAG CCC GGG CAT TGT TT	26	57.7	76.1	1063–1088	139
	PP × 33/R	CAC GGG TTT GCA GCA CGA TCA ACA	24	54.2	73.1	1178–1201	
35	PP × 35/F	CCA TAA CAT CGG TGG ACG GTG GAC AGG	27	59.3	75.0	484–510	434
	PP × 35/R	CCA TTA CAT CCC GTC CCC TCC CCT TCA	27	59.3	75.7	891–917	
39	PP × 39/F	CCG ACG GAG TGT CCC TGG ACC ATC TTA	27	59.3	74.1	3576–3602	229
	PP × 39/R	CCA GCG TTT TTG GTT CCC TTA CCC CGT A	28	53.6	74.1	3777–3804	
45	PP × 45/F	TGT TGG ACA TCA CAC CTA CCG TGG A	25	52.0	69.7	4654–4678	205
	PP × 45/R	TCC GTA CCT GAC CCA GAA GAT GCA A	25	52.0	69.8	4834–4858	
51	PP × 51/F	CAA CTA GCA ACG GCG ATG GAC TG	23	56.5	68.1	859–881	299
	PP × 51/R	CTG CTT CGC GGG CTG ACT AGA A	22	59.1	68.1	1136–1157	
52	PP × 52/F	GGT GTT GGT GCT GGT GCT TTT GCT A	25	52.0	70.1	4025–4049	517
	PP × 52/R	CAG TTA CAG GGG GAC GAA TGG TGG A	25	56.0	70.8	4517–4541	
56	PP × 56/F	TGT TGT TTT TCC GCC ATT TTG TAC ATG CAA CC	32	40.6	74.8	7417–7448	330
	PP × 56/R	TGG CCT ACA TAG TGT ATT CTG CAA GCC AAA AC	32	43.8	71.1	7715–7746	
58	PP × 58/F	ACC ACC GAG GCC ACC AAC AAC GAA AGT	27	55.6	75.0	3392–3418	128
	PP × 58/R	CGT GGT CTA CTG TCC ACG GCG CAG TCT	27	63.0	75.4	3493–3519	
59	PP × 59/F	CCG AGC AAG ACA CCT AAG ACA GCA ACG	27	55.6	71.5	483–509	169
	PP × 59/R	TCG GAG TCG GAG TCA GGT AAT TGC T	25	52.0	68.2	627–651	
66	PP × 66/F	GCG GGC GGC TCC TAC CTC TTC CTC TTC	27	66.7	75.9	7104–7130	277
	PP × 66/R	CCA CCT AAC CTG ACA CAC ACT GCC CAA GG	29	58.6	74.9	7352–7380	
IC	PP × IS/F	TTA TCC CGA GTC CCC CAG GCC TTT CT	26	57.7	73.3		99
	PP × IS/R	TGG CTT GGC CCC AAC TTC CAT CA	23	56.5	73.4		
EC	PP × ES/F	CGT GGA TGT CCA TGA AGG ATG AAG G	25	52.0	69.7		758
	PP × ES/R	GTT CTG GGA ACA GGC GGG GAT T	22	59.1	69.5		

[a] F, forward primer; R, reverse primer; T_m, melting temperature; IC, internal control; EC, external control.

Source: (Nishiwaki et al., 2008)

Standard quality primers were purchased from IDT and dissolved to obtain a 100µm stock solution.

The multiplex 10x primer mix was made by adding 20µl of each primer to an2ml micro-centrifuge tube. A total of 18 primers were added to the tube to make a total of 360µl. A total of 640µl of TE (10mM Tris CL, 1 mM EDTA, pH 80) buffer was added to the mix to create a total volume of 1000µl, achieving a dilution of 1:50 to give a 10x primer mix (2µM of each primer). The final concentration was 2µM for each primer as shown in table 10 and figure 7below. The 10× primer mix was stored in 100µl aliquots at $-20℃$ to avoid repeated freezing and thawing.

Table 10. Preparation of 10x primer mix (containing each primer at 2µM)

Concentration of normalized primer stock	**100µM (100pmol/µl)**
Each primer	20µl (×18× primer)
TE buffer or distilled water	640µl
Total volume	**1000 µl**

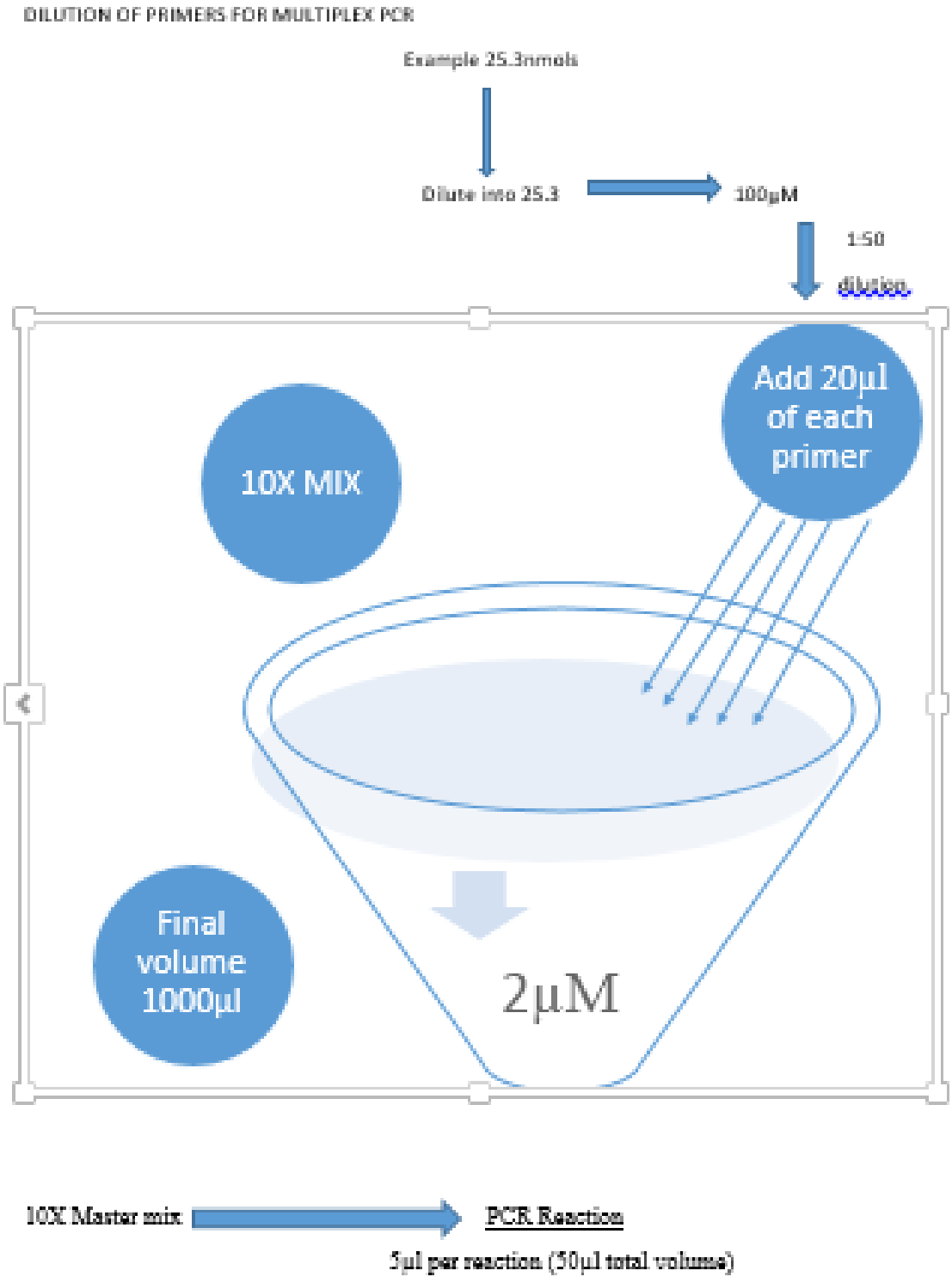


Figure 7: Dilution of primers for Multiplex PCR

HPV genotyping was accomplished using HPV multiplex PCR to identify 18 HPV types: 6, 11, 16, 18, 30, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66, 1C (15) and EC (ES).

All primers (lyophilized) were dissolved in TE buffer to get a final concentration of 1000μM. The primer and buffer mix was left for 20 minutes to let the primer dissolve completely. The primers were then quantified by using a spectrometer. The A260 (OD) of a 1 in 100 dilution of the stock solution was measured using a glass pipette with a 1cm path length, and the final concentration was measured.

The thermocycling parameters for Multiplex PCR gene amplification were as shown below in figure 8.

Only samples that tested positive for β-globin and HPV were analysed by multiplex PCR.

Protocol for HPV multiplex PCR

2× Qiagen Multiplex PCR Master Mix, template DNA, RNAse free water, and primer mix stored at $-20℃$ was thawed. Each of the above was mixed completely to avoid localised concentration of salts. A reaction mix containing all the components required for multiplex PCR except the template DNA was made as shown in table 11 below.

Table 11. HPV multiplex PCR components (Reaction mix and template DNA)

Component	Volume/reaction	Final concentration
Reaction mix		
2x QIAGEN Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see Table 2)	5 µl	0.2 µM†
RNase-free water	15 µl	
Template DNA Added at step 4	5 µl	≤1 µg DNA/50 µl
Total volume 50 µl‡	**50 µl**	

* Provides a final concentration of 3 mM MgCl2.

† A final primer concentration of 0.2 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (0.1–0.3 µM) may further improve amplification performance.

‡ For volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained.

The reaction mix was added to PCR tubes. The DNA template, samples (≤ 1 µg /50 µl reaction- the best result was at 100ng of DNA template) were added to the individual PCR tubes containing the reaction mix. The PCR tubes were placed in the thermal cycler model, TECHNE, TC- 412, for amplification with the cycling program as outlined in figure 8 below.

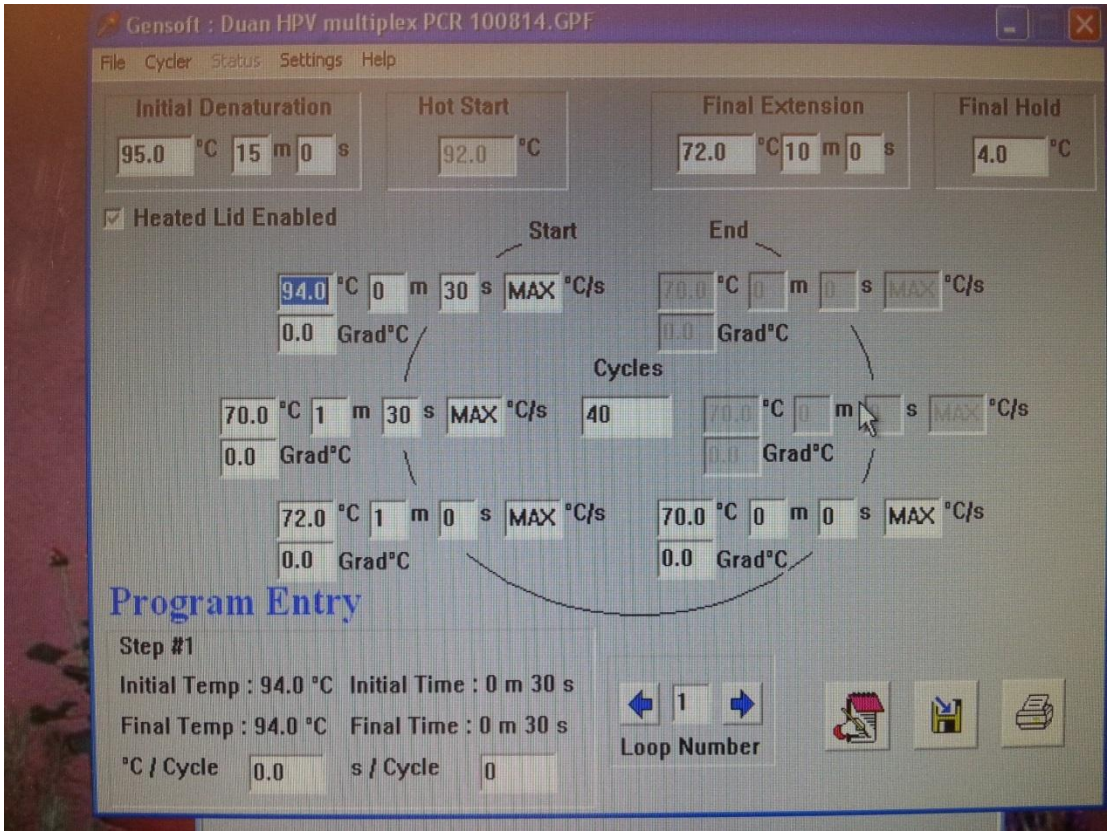


Figure 8. Thermocycling parameters for Multiplex PCR gene amplification

Initial Denaturation 95℃ for 15 minutes

Denaturation 94℃ for 30 seconds

Annealing 70℃ for 90 seconds

Extension 72℃ for 60 seconds

Final extension 72℃ for 10 minutes

Final hold 4℃ for ∞ (infinity)

Multiplex PCRs were performed in duplicate using 100ng of genomic DNA and 18 pairs of primers to amplify fragments of 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, E5 and 15.

The samples to be tested and the negative control was set up first and the positive control was set up last to prevent cross contamination of samples. The positive control constituted the HPV 16 plasmid DNA (pEF 399) whereas the negative control was nuclease free water.

The PCR product was analysed using 2% agarose gel based on guidelines from Qiagen- multiplex PCR handbook as shown in table 12 below. The sizes of the primer amplicons ranged from 99 to 758 base pairs as shown in table 9. Primers were chosen based on their corresponding PCR products that can be distinguished from one another by size shown in table 9.

As per recommendation 1× TAE buffer was used for preparation and running of the agarose gel. A ladder of 1000 base pairs was used as it could cater for all the primer amplicons

Table 12.Guidelines for Agarose Gel Analysis of Multiplex PCR Products.

Minimum difference in size of PCR products	**Maximum size of Fragments**	**Concentration of agarose**
>200 bp	2000 bp	1.3%
>100—200 bp	1000 bp	1.4—1.6%
>50—100 bp	750 bp	1.7—2.0%
20 —50 bp	500 bp	2.5—3.0%
<20 bp	250 bp	3.0—4.0%

At the end of the electrophoresis, the gel was stained with 0.3 ethidium bromide (0.1 mg/µl solution) for 30 minutes and the visualisation of the DNA fragments was performed under ultraviolet light.

A sample was considered positive for a specific HPV genotype if the PCR product generated corresponded to the amplicon size (bp) of any HPV genotype as shown in table 9. The ladder was used to determine the amplicon size generated. A sample was

considered to have multiple HPV infection if it generated more than one PCR product.

3.2.7. Data Management

At recruitment, each study participant was assigned a unique identifier (ID) by the qualified nurse practitioner. The identifier was used to link information collected from the questionnaire, and the HPV-DNA test result as well as the histopathology results. All identifying information of the participant except for the unique ID was excluded from the data bank used in statistical analysis to ensure confidentiality. Access to data collection sheets and consent forms was restricted to members of the research team.

3.3. STATISTICAL ANALYSIS

3.3.1: Inclusion in Dataset

An excel dataset was built for entering data. Participants (subject) were included in this dataset if they met the eligibility criteria for the study, completed the questionnaire, gave consent and had their foreskin sample collected. This was a descriptive study and it used descriptive statistics looking at frequencies and cross tabulations.

- SPSS version 20.0 was used for analysis of the data
- Frequencies, means and standard deviations calculated for continuous variables:
- Chi-square test for association with categorical variables was determined.
- All results were analyzed at 95% confidence interval and the p-values was considered significant if it was <0.05.

3.3.2: Study Variables

HPV status

HPV status was classified as overall positive or negative based on genomic DNA, PCR amplifying with GP 5+/GP 6+ or CP I/CP II primers. These participants were categorised as positive for "any type of HP". HPV types were classified as either HR (16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, E5, 15) or LR (6, 11) based on their oncogenic potential.

Unknown HPV types were considered of a sample tested positive for GP 5+/GP 6+ or CP I/CP II but failed to amplify by multiplex PCR. "Multiplex HPV infection" comprised a sample that tested positive for more than one HPV by multiplex PCR.

Independent variables

Information on socio demographic and lifestyle variables was collected through administration of a questionnaire. The main independent variable on which information was collected were:

Sociodemographic variables

- Age (years)
- Community facility (U.T.H, Chawama, Chilenje, M'tendere, Ridgeway)
- Marital status (single, married, divorced/ widowed).

Sexual behaviour

- Age at first intercourse (years)
- Number of sexual partners in the past 12 months (0=Nil, 1=1, 2=more than 1).

Histopathology status

Histopathology status were categorised as either No dysplasia, dysplasia or Malignant as shown in Table 8. Samples were categorised as No dysplasia if they showed no histologic abnormality and/or did show inflammation and/or HPV viral wart changes (koilocytosis). Dysplastic category was composed of samples that showed mild, moderate or severe dysplasia. The malignant category comprised cases that showed carcinoma in-situ or overt stroma invasion by malignant squamous cells.

3.3.3: Coverage of target population and selection Bias

Coverage of the target population was evaluated by using the total number of circumcisions conducted in the selected five sites in the year 2012. Overall and age specific HPV prevalence was calculated for the males for each of the five sites as well as for combined sites.

The characteristic of the study population was not compared to that of the general population of Lusaka city to evaluate for selection bias.

3.3.4: HPV DNA prevalence

The prevalence of HPV infection was calculated by overall HPV infection, type and oncogenic risk grouping. Age specific prevalence was calculated for males aged 10-14 years, 15-19 years 20-24 years, 25-29 years and 30 years or above.

The collected data was entered onto an Excel sheet and analyzed using SPSS version 20.0. Frequencies, means, standard deviations and cross tabulations were used to analyze for descriptive statistics. Univariate unconditional logistic regression was performed on all independent variables to explore their association with the outcome of HPV infection. Odds rations (ORs) and their associated 95% Confidence Intervals were calculated. Multivariate analysis examined the effect of each variable, independent of age, by generating age-adjusted ORs and 95% CIs. These were calculated for the association between each independent variable and the outcome of prevalent HPV infection, adjusted for all other variables in the model. P-values were considered significant if less than 0.05.

4. RESULTS

4.1 RECRUITMENT ELIGIBILITY

Between January 2014 and June 2014, 379 HIV negative males that came for VMMC were approached for consent, of which 312 (82.3%) consented to participate in the study. One participant reported of having received the HPV vaccine and hence he was excluded from the study. Of the 311 eligible participants 04 (1.3%) did not have a matching baseline questionnaire. These were also excluded from the study. One sample was excluded for baseline DNA test because it was negative for β globin. A total of 306 samples were finally analysed in this study compared to the minimum sample size of 264 (116%) participants that was required for this study.

4.2. CHARACTERISTIC OF STUDY POPULATION

Table 13. Sociodemographic characteristics of the study population (N=306)

VARIABLE	N	PROPORTION (%)	% ANY HPV-DNA
CIRCUMCISION SITE			
• M'TENDERE CLINIC	48	15.7	52.1
• U.T.H	71	23.2	78.9
• RAILWAY CLINIC	46	15.0	91.3
• CHILENJE CLINIC	41	13.4	73.2
• CHAWAMA CLINIC	100	32.7	67.0
TOTAL	**306**	**100**	**71.9**
AGE RANGE			
• 10-14	57	18.6	50.9
• 15-19	66	21.6	66.7
• 20-24	63	20.6	73.0
• 25-29	66	21.6	84.8
• 30 AND ABOVE	54	17.6	83.3
TOTAL	**306**	**100**	**71.9**
MARITALSTATUS			

• SINGLE	230	75.2	68.7
• MARRIED	74	24.2	81.1
• DIVORCED/WIDOWED	2	0.6	100
TOTAL	**306**	**100**	**71.9**
RECEIVED HPV VACCINE			
• YES	1		
• NO	306		
• NO RESPONSE	0		
HIV STATUS			
• REACTIVE	0	0	
• NON REACTIVE	306	100	

4.2.1. Socio demographic Characteristics

The socio demographic characteristics of the study population showed that of the 306 participants that were finally included in the study and whose samples were analysed, 48 (15.7%) were from Railway clinic, 41 (13.4%) from U.T.H., 46 (15.0%) from Railway clinic, 41 (13.4%) from Chilenje clinic and 100 (32.7%) were from Chawama clinic. The marital status of the participants included 230 (75.2%) singles, 74 (24.2%) married and 2 (0.6%) divorced/widowed (figure 10).The ages of the participants ranged from 10 years to 67 years (figure 9). The median age of the participants was 22years while the mean age was 22.8 years (SD9.6). These were considered in the age categories of one to five as shown in figure 13. Only one of the 312 participants that gave consent to participate in the study self-reported having had received the HPV vaccine before and he was excluded from the study. All the participants in this study were HIV negative as shown in table 13.

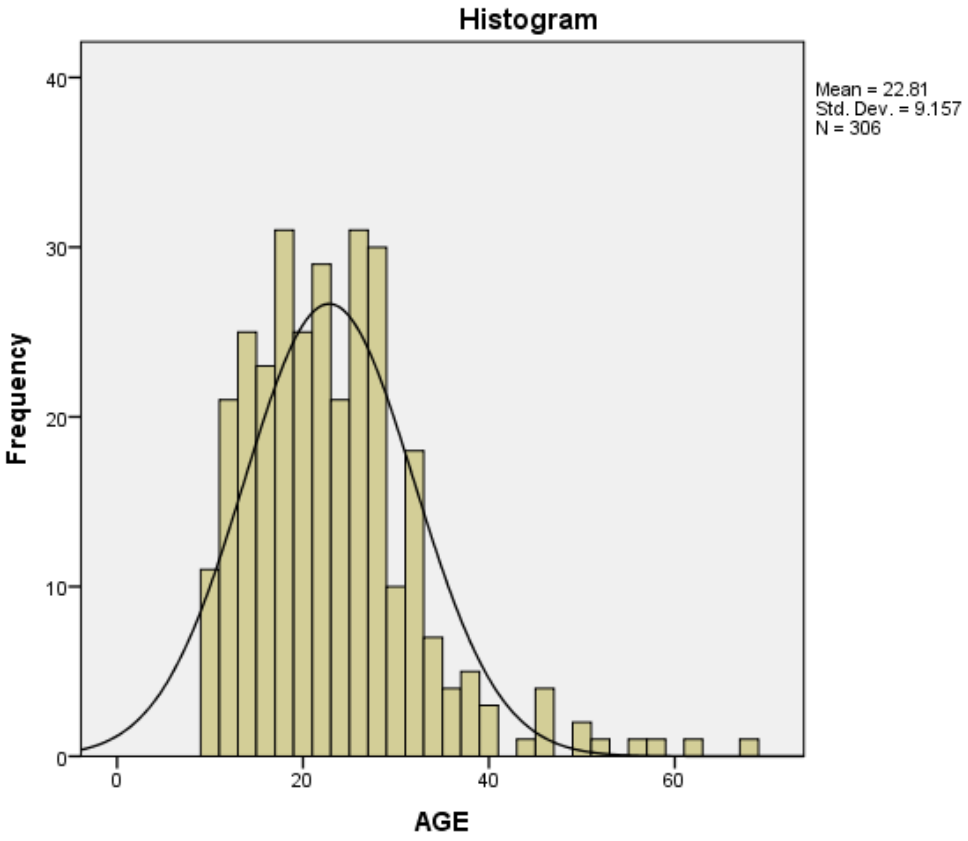


Figure 9. Age (years) distribution of participants

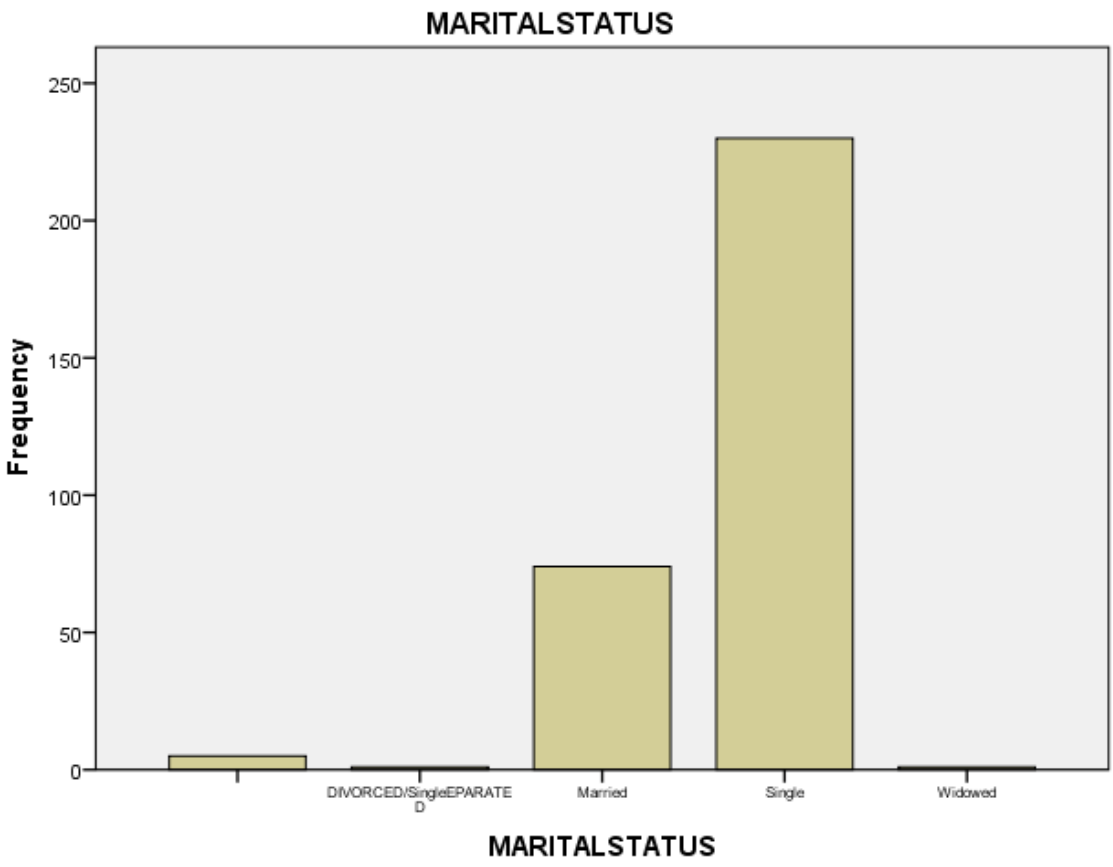


Figure 10. Marital status of participants.

4.2.2. HPV DNA Prevalence

HPV-DNA was detected in 71.9% of the participants (n =306) as shown in figure 11. Figure 12 shows representative PCR results obtained by amplification of a 288 base pair region of the E1 (nt 1777 —1964) of the HPV genomes using CP I/CP II primers. A plasmid containing the entire HPV 16 genome (pEF 399) was used as positive control, whereas the negative control was nuclease free water.

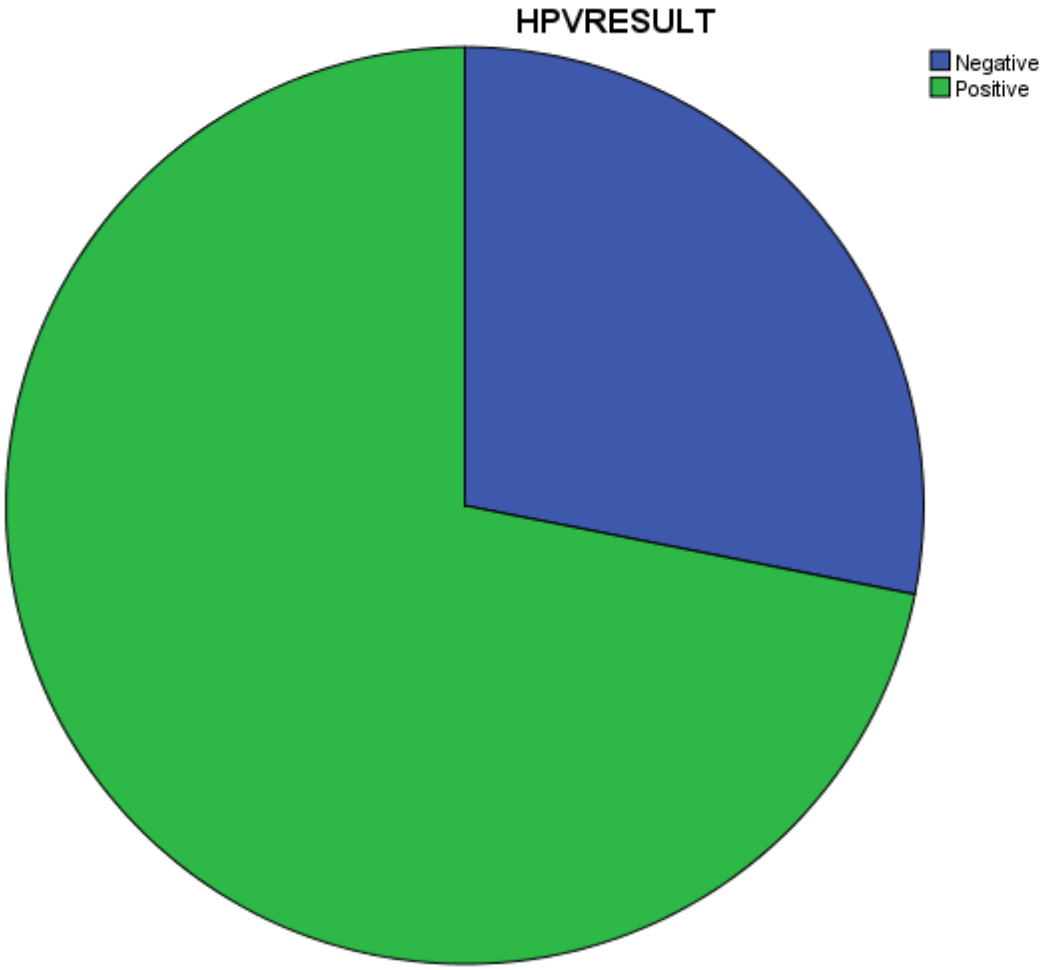


Figure 11.HPV Prevalence by DNA PCR.

The quality cellular DNA was assessed using β-actin PCR with gDNA extracted from a B-cell line used as a control. HPV-DNA was assessed by GP 5+/GP 6+ that amplified a 150bp PCR product from the HPV L1 region and CP I/CP II that amplified a 188bp PCR product from the HPV E1 region of HPV genome. The

positive control used for HPV was pEF399 while the negative control was nuclease free water. The test samples were numbered as shown above.

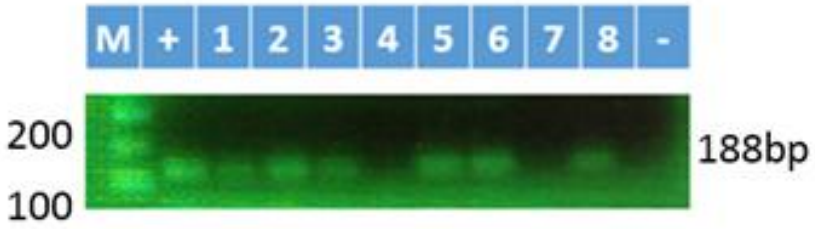


Figure 12.HPV PCR Results obtained after amplification of 188bp of HPV E1 region of DNA using CPI/II primers.

The cellular DNA status was assessed by β-Actin polymerase chain reaction. Nuclease free water was used as a negative (-) control, while pEF399 was used as a positive control (+). Samples 1, 2, 3, 5, 6 and 8 came out positive while 4 and 7 were negative. M lane represented the ladder.

The HPV prevalence rates for the recruitment sites showed that Railway clinic had the highest prevalence rate of 91.3% (n=41) followed by U.T.H at 78.9% (n=71). The other sites had the following prevalence rates: Chilenje clinic 73.2% (n=41), Chawama clinic 67% (n=100) and M'tendere clinic 52.1% (n=48) as shown in figure 13 below.

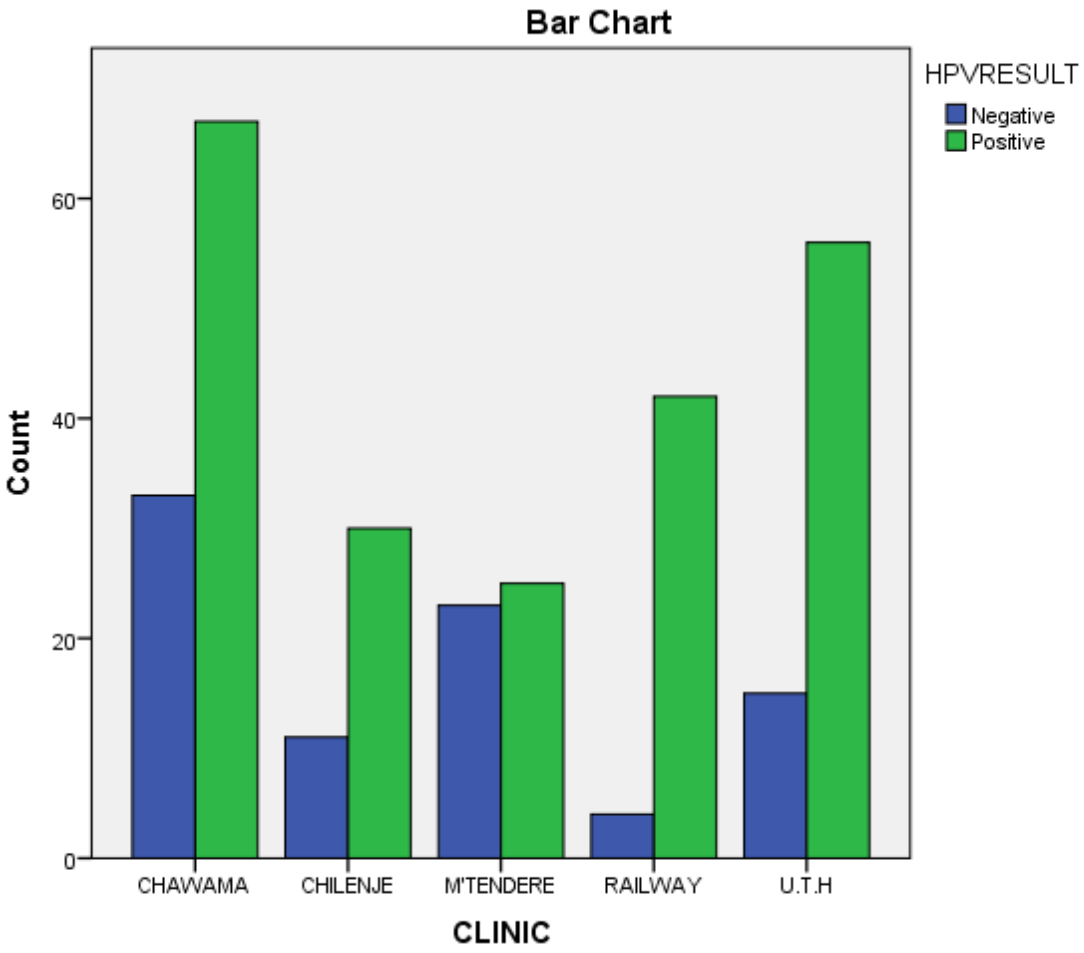


Figure 13. HPV Prevalence by recruitment site. The positive control used for the β-active PCR amplification was gDNA extracted from a B-cell line.

The prevalence of HPV infection was 81.1% and 68.7% for the married and singles respectively as shown in figure 14.

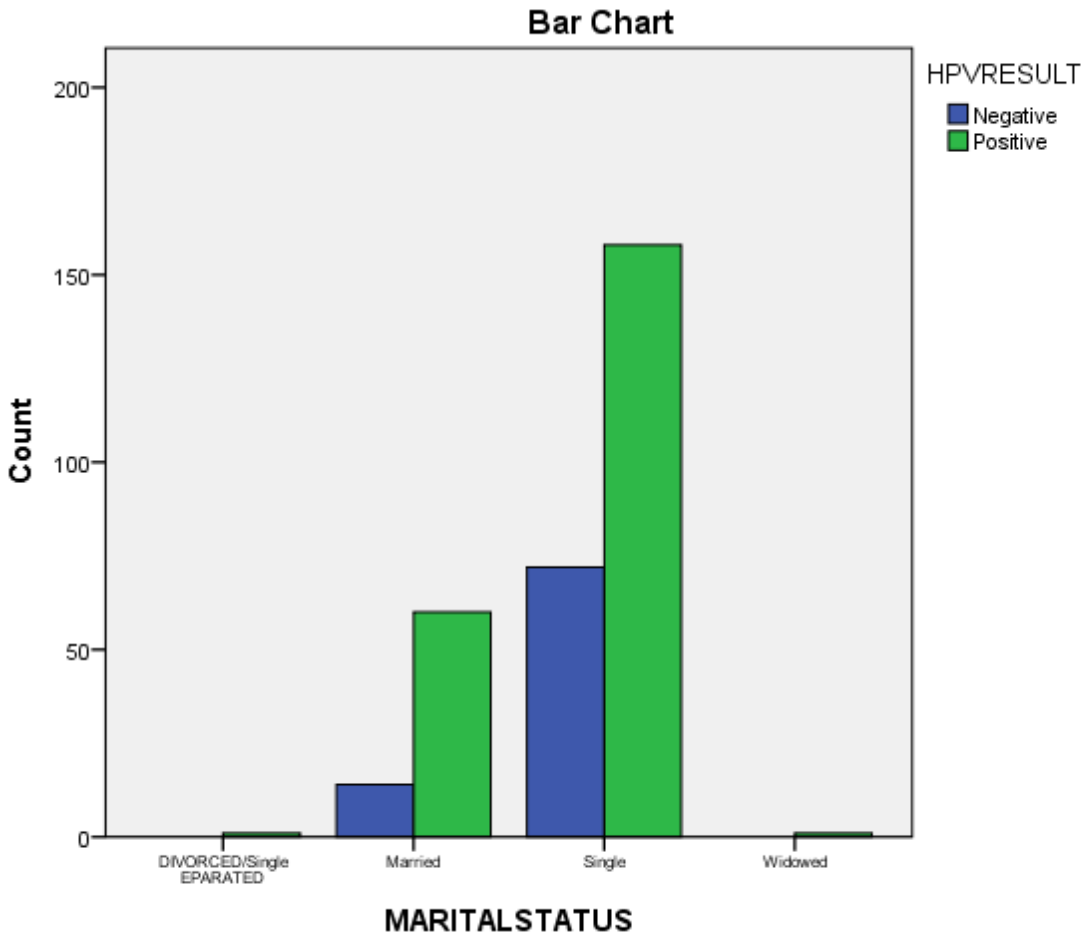


Figure 14. HPV result vs marital status of participants

The participants were grouped into age bands of 5 year ranges with category 1 representing 10 to 14 years, category 2 representing 15-19year olds, category 3 representing 20-24 year olds, category4 representing the 25 – 29 year old and category five representing participants who were 30 years and older. Table 14 shows the age categories with the actual head counts, the % within the HPV DNA test result and the HPV result within the age category. The results showed the prevalence of HPV infection was highest among the age range of 25—29 years at 25.5% and lowest

among males aged 10—14 at 13.2%.Category 4 also showed the highest HPV prevalence within the age category at 84.8%.

Table 14. Age category vs HPV result cross tabulation

HPVRESULT * AGECAT Crosstabulation

			AGECAT					
			1	2	3	4	5	Total
HPVRESULT	N	Count	28	22	17	10	9	86
		% within HPVRESULT	32.6%	25.6%	19.8%	11.6%	10.5%	100.0%
		% within AGECAT	49.1%	33.3%	27.0%	15.2%	16.7%	28.1%
		% of Total	9.2%	7.2%	5.6%	3.3%	2.9%	28.1%
	P	Count	29	44	46	56	45	220
		% within HPVRESULT	13.2%	20.0%	20.9%	25.5%	20.5%	100.0%
		% within AGECAT	50.9%	66.7%	73.0%	84.8%	83.3%	71.9%
		% of Total	9.5%	14.4%	15.0%	18.3%	14.7%	71.9%
Total		Count	57	66	63	66	54	306
		% within HPVRESULT	18.6%	21.6%	20.6%	21.6%	17.6%	100.0%
		% within AGECAT	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	18.6%	21.6%	20.6%	21.6%	17.6%	100.0%

KEY: HPV Result: N = negative, P = positive. Age Cat = Age category. Age Cat 1 = 10 to 14 years, Age Cat 2 = 15-19 years, Age Cat 3 = 20-24 years, Age cat 4 = 25 – 29 years and Age Cat 5 = 30 years and older.

4.2.3. Sexual Behaviour Characteristics

Table 15.Determinants of HPV infection among VMMC participants (Univariate and Multivariate analysis) (N=306)

	Variable	Univariate			Multivariate		
		OR	CI	P-value	AOR	CI	P-value
1.	**Current Age category**						
	10 – 19 years	1	1	1	1	1	1
	20 – 29 years	0.39	(0.17-0.87)	0.02	0.94	(0.14-6.51)	0.95
	≥30 years	0.60	(0.26-1.35)	0.21	0.37	(0.11-1.24)	0.11
2.	**Report of Sex before**						
	No	1	1	1	1	1	1
	Yes	2.49	(1.49-4.15)	<0.001			
3.	**Age range at first sex**						
	10 – 19 years	1	1	1	1	1	1
	20 – 29 years	1.00	(0.51-1.97)	1.00	1.58	(0.59-4.23)	0.37
4.	**Marital status**						
	Single	1	1	1	1	1	1
	Married	1.99	(1.04-3.78)	0.03	1.03	(0.37-2.75)	0.95
5.	**Multiple sex partners**						
	No	1	1	1	1	1	1
	Yes	0.42	(0.21-0.86)	0.02	0.45	(0.22-0.94)	0.03

Table 15 shows the sexual behaviour characteristics of the study population. Results from the study population shows that of the 306 participants recruited, 194 (63.4%) reported previously having had sexual intercourse (table 15), with 69 (35.6% self-reporting to have had sex with more than one sex partner in the past twelve months. The mean age at first intercourse was 18.7 (SD 3.42) years for participants (n=189) that reported previously having had sexual intercourse. The reported age at first sexual intercourse in the study population ranged from 10 to 28 years. Overall HPV prevalence was not significantly different for participants whose age was less than 18

years at first sexual intercourse (77.2%) against those 18 years and above (79.5%) figure 15. Twenty two percent of the study population reported having had more than one sexual partner in the past twelve months. The prevalence of HPV prevalence was 84.4% among males that reported having had one sexual partner in the last twelve months against 69.6% for males that reported to have had more than one sexual partner in the last twelve months.

Determinants of HPV infection: Univariate analysis of determinants of HPV infection on sociodemographic and lifestyle variables showed several factors to be significantly associated with prevalent HPV infection (Table 15). Indicators of sexual activity were highly associated with the outcome of HPV infection: a self-report of being sexually active, history of being married, and multiple sexual partners in the last 12 months. Multivariate analysis showed that variables which showed a significant effect in unadjusted analyses did not retain an effect when adjusted for age apart from a history of multiple sex partners.

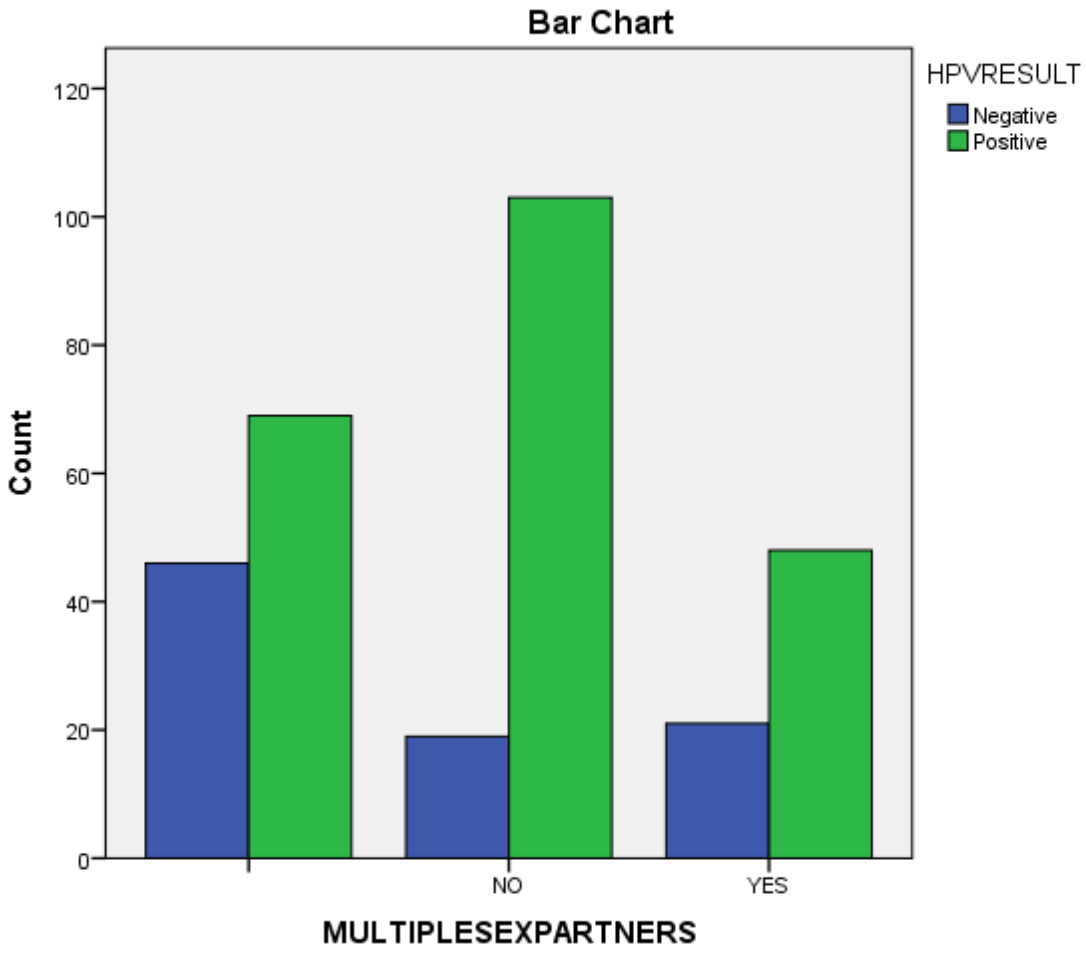


Figure 15. Self-reported multiple sex partners in last 12 months vs HPV test result.

4.2.4 Histopathological findings of foreskin samples

Table 16. Histology results by HPV status (N=306)

Histology	Overall N (%)	% Any HPV DNA by PCR
Histologically Unremarkable +/- inflammation	126 (41.2)	31.7
Koilocytosis	180 (58.8)	100
Low grade dysplasia	0 (0.0)	0.0
High grade dysplasia	0 (0.0)	0.0
Carcinoma In-situ	0 (0.0)	0.0
Invasive Cancer	0 (0.0)	0.0

Haematoxylin and Eosin stained sections of foreskin samples were examined under light microscopy to look for viral induced lesions. Viral wart changes (koilocytosis) was present in 58.8% (n=306) of the analysed samples (table 16). Samples that had viral wart changes were all positive for HPV by PCR. Of the 220 samples that were positive for HPV by DNA PCR, 81.8% showed koilocytosis. None of the samples that were negative for HPV by DNA PCR showed koilocytosis. None of the examined tissues showed high grade dysplasia or malignancy. The mean age of males with koilocytosis was 24.2 years while that for males with normal cytology was 20.5 years.

Males that self-reported to be married had a higher percentage of koilocytosis (64.9%) compared to their single counterparts (56.5%). Kiolocytosis was reported to be higher in males that self-reported to previously having had sexual intercourse (64.4%) while the participants that reported not having had sexual intercourse 49.1% koilocytosis. Koilocytosis was found in 70.5% of participants that only reported to have had one sexual partner in the past twelve months. Participants that self-reported to have had more than one sexual partner in the past twelve months had koilocytosis in 53.6% of the foreskins. The common histologic findings are shown in figure 16.

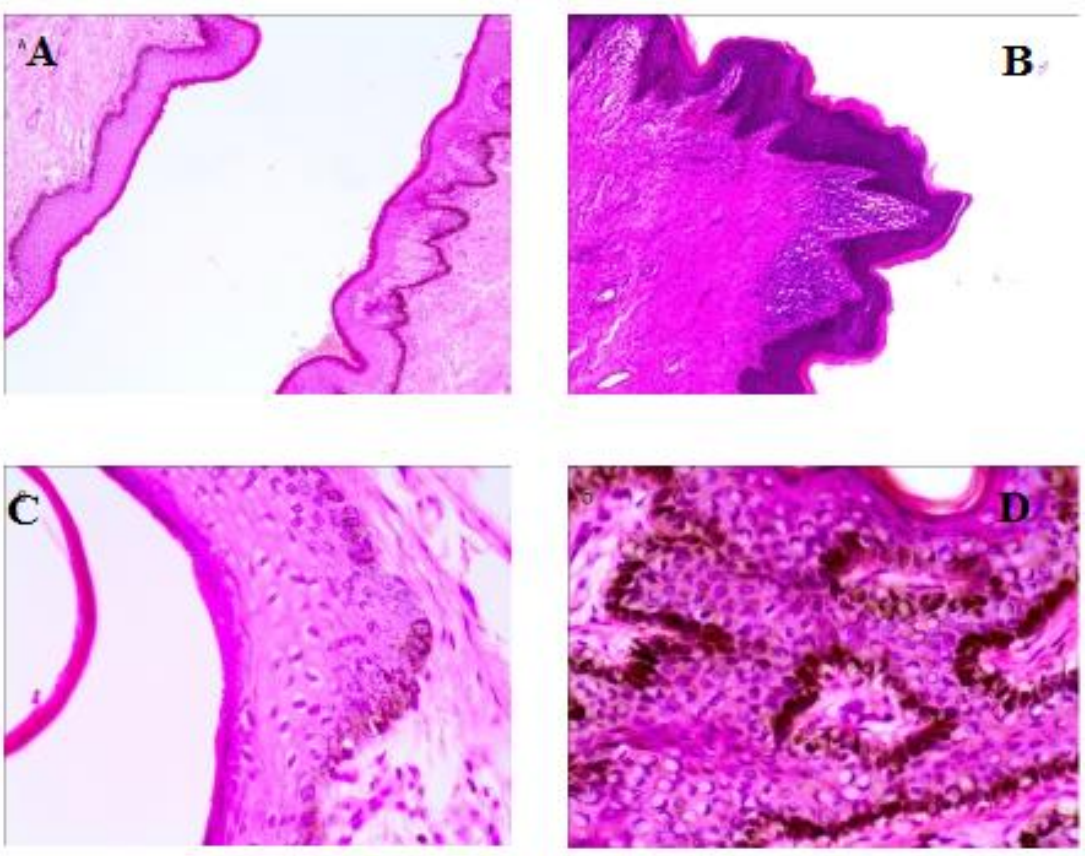


Figure 16. Histology of foreskins. A: Shows normal foreskin in upper left corner and koilocytosis in lower right corner. B: Shows non-specific chronic inflammation. C and D: Show HPV viral wart changes called koilocytosis.Images were captured at x20 power.

4.2.5 HPV Genotype Determination by Multiplex PCR

The extracted genomic DNA was amplified using Multiplex PCR to amplify 18 HPV genotypes including (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89, 1539)using primers shown in table 9. The genomic DNA failed to amplify by multiplex PCR. The results seen on the gel showed smearing of the amplicon showing that the quality of the extracted DNA was poor. Amplification with single primers like GAPDH

failed to amplify showing that only primers that were less than 300 base pairs were able to amplify by PCR. Primers that had an amplicon that was 300bp were able to amplify in a single PCR reaction, but those above 300bp failed to amplify. The multiplex PCR failed to amplify as the 18 primers had amplicons ranging from 99 to 758 base pairs showing that our DNA had fragmented to sizes that could only amplify by smaller primers.

5. DISCUSSION

The data presented here represent a cross sectional analysis of genital HPV infection in males undergoing voluntary medical male circumcision for HIV prevention in selected clinics in Lusaka, the capital and largest city in Zambia. The results reported are: 1) age specific HPV- DNA prevalence, 2) Socio-demographic and behavioural determinants of HPV infection and 3) HPV infection and microscopic outcomes. To our knowledge, this will be the first published report of its kind for the Zambian male population. The results of the study represent a starting point for understanding the burden of HPV infection amongst males in Lusaka and Zambia in general. The results may be used to determine if the study can be conducted on a larger scale.

In the present study, we used the consensus primer sets GP5- GP6 and GP5+- GP6+ from the HPV L1 gene and CPI-CPII from the E1 gene to detect for the presence of HPV infection and multiplex PCR using HPV type specific primers for amplification of each E7 gene to detect HPV types, namely, HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89, 1539 in a novel one shot method to perform multiple amplifications in a single reaction mixture.

5.1 Age Specific HPV Prevalence

Prevalence of genital HPV-DNA in males going for VMMC in Lusaka city was 71.9% overall and was 31.7% in males with microscopically unremarkable foreskin tissues. The estimate of overall prevalence is similar to reports in other studies across the globe such as in South African males (Müller et al., 2010), asymptomatic males whose female partners had CIN/HPV (Dunne et al., 2006, Bleeker et al., 2005, Nicolau et al., 2005), and populations at high risk of HPV infection as well as those with low- risk (Smith et al., 2011).

HPV prevalence rates were found to be higher in communities of middle income status at 91.3% and 73.2% for Railway and Chilenje clinics respectively and lower in communities of low social economic status at 67% and 52.1% for Chawama and

Mt'endere clinics respectively. The University Teaching Hospital that receives clients from all over Lusaka city had a prevalence of 78.9%. This difference may probably be due to the availability of disposable income to solicit for sex among our respondents. HPV prevalence was higher in participants above the median age (79.5%) and it was shown to be higher among the married at 81.1% when compared to the single participants (68.7%). The above data agrees with other studies which show that HPV infection among males peaks in the age range above 20 years because more males are sexually active at that time (Weinstock et al., 2004). The high HPVprevalence in our study participants is in keeping with the fact that HPV is the most commonly sexually transmitted infection (Bruni et al., 2010) and all our participants had not received any of the available HPV vaccines to prevent HPV infection.

HPV prevalence rate was 81.1% among the married participants whilst it was 68.7% among the singles. The higher percentage among the married participants can be attributed to the fact that they are sexually active as abstinence from sexual activity is the only reliable method for preventing genital HPV infection (Davis et al., 2004). Limiting the number of sexual partners can reduce the risk for HPV infection. However, even persons with only one lifetime sexual partner can get HPV. Partners who have been together tend to share HPV and it is not possible to determine which partner transmitted the original infection. Having HPV does not mean a person or their partner is having sex outside the relationship (Center for Disease control, 2015). HPV positivity among the singles can be attributed to sexual activity as most infections are common among sexually active persons as transmission is by direct contact, usually sexually with an infected person. However, it may also be transmitted by non-sexual routes, but this appears to be uncommon (Center for Disease control, 2015). The high HPV cannot be attributed to the lack of usage of condoms as condoms only lower the chances of acquiring and transmitting HPV when consistently and correctly used because HPV can infect areas not covered by a condom (Center for Disease control, 2015).

5.2 Histopathological findings of foreskin samples

Nearly all sexually active people will get HPV at some time in their life, however, most of these infections will be asymptomatic and will completely go away and not cause any health problems (Schiffman and Castle, 2003). However, if the infection persists, HPV may cause men to develop warts, pre- cancers or cancers (Zur Hausen, 2002). It is still not fully understood why some people develop health problems from HPV infection while others don't, but low immunity and the HPV type acquired have been implicated as some of the drivers (Bleeker et al., 2009, Pow-Sang et al., 2010).

All the participants in our study had no visible penile/ foreskin lesions (ulcer/ growth). Histology did not show any pre- cancerous or cancerous lesions. This is consistent with the fact that most HPV infections will be asymptomatic and will cause no disease at all (Schiffman and Castle, 2003). Of the samples analysed, 58.8% showed viral wart changes that were consistent with koilocytosis. All the samples that had koilocytes were positive for HPV by DNA- PCR. HPV infection and koilocytosis was higher among males that self-reported to have been married or who had previous sexual contact. This is consistent with data that shows risk factors for HPV infection to be sexual activity (Giuliano et al., 2008a). However, having one sexual partner in the last 12 months was associated with a higher risk for HPV infection (84.4%) and subsequent development of koilocytosis (70.5%) whilst it was lower at 69.6% and 53.6% among males that self-reported to have had more than one sexual partner in the last 12 months from recruitment. This is in contrast to literature that shows that multiple sexual partners are associated with higher risk for HPV infection (WHO, 2015). This finding may be attributed to the fact that one will only acquire infection if their partner/ partners are infected. The other reason is that it may be due to persistence or a longer time taken to clear the infection. One fact that cannot be definite is the accuracy on the self-reported data which may be affected by recall bias or withholding of information.

However, multiple sexual partners in the last twelve months was associated with HPV positivity as shown by a p value of less than 0.05.

5.3 Multiplex PCR

We used multiplex PCR to amplify 18 products in a single reaction tube using DNA extracted from preserved FFPE foreskin tissues. Samples tested included all samples that were positive for β globin that was used as a positive control for amplifiable DNA and tested positive for GP 5+/ GP6+ and/ or CP I / CP II. Each assay included positive controls for PCR contamination (water blanks taken through extraction and reagent water) and low positive HPV control. All our tested samples failed to amplify and came out as a smear on the agarose gel electrophoresis while the controls came out as expected. Several re-runs were done and the same results were obtained. Trouble shooting was done using different primers, reagents and different primer concentrations but this did not yield any results. The samples were then amplified using primers with different amplicon sizes including Glyceraldehyde 3- phosphate dehydrogenase (GAPDH), a ubiquitously expressed enzyme of a 37KDa that catalyses the sixth step of glycolysis, GP 5+/ GP 6+ (150bp), β- active (281bp) and CP I/ II (188bp). GAPDH, the primer with a relatively large target failed to amplify. Further review of literature on the quality of DNA extracted from other tissues showed similar challenges with amplification of DNA obtained from FFPE preserved tissues. Bonin(Bonin et al., 2003) reported difficulties with PCR amplifications with amplicons > 300bp when he analysed archival FFPE post-mortem tissues. Other researchers suggest that the relatively large target of 450bp in the HPV L1 region spanned by the LA PGMY primer system (Gravitt et al., 2000), might be the upper limit for amplification of DNA from FFPE tissue samples. This had led to researchers suggesting that HPV genotyping tests that target smaller amplicons such as 5PF (65bp) or GP5+/ 6+ (150bp) might overt the problem and are best suited when analysing FFPE tissues (Dal Bello et al., 2009, Tan et al., 2010). The primers that were used had amplicons that ranged in size from 99bp to 753bp as shown in table 9. The large primers could have interfered with the PCR amplification.

While DNA from archived FFPE tissue can be used for papilloma virus genotyping, formalin fixation has been noted to cause damage to nucleic acids, induce protein to

protein, and protein to nucleic acid cross linking which can impair PCR amplification (Plenat et al., 2006, Cannavo et al., 2012).

The protein to nucleic acid cross linkages makes it difficult to separate DNA from histones and to obtain pure nucleic acids at extraction (Srinivasan et al., 2002, Gilbert et al., 2007). The fixation of tissue also leads to the fragmentation of nucleic acids, such that PCR methods that amplify a smaller portion of the viral genome are most effective (Ren et al., 2001, Kocjan et al., 2011). Other factors that may have contributed to the failure of multiplex PCR for our study include paraffin acting as a physical barrier affecting the DNA yield and amplification efficiency in subsequent PCR amplification (Steinau et al., 2011). The protocol we used for nucleic acid extraction from FFPE tissue incorporated a pre- treatment with xylene and ethanol to physically remove the paraffin wax (Goelz et al., 1985). This step has the risk of accidentally removing small tissue fragment during the process and it may leave some residues with eluted DNA due to incomplete removal. Formalin fixation has also been shown to fragment DNA into units up to 600bp(Dietrich et al., 2013).

Studies using DNA extracted from fresh tissue and fresh frozen tissue have shown better results with multiplex PCR (Karlsen et al., 1996, Gheit et al., 2007). The next study will focus on using fresh tissue for DNA extraction and Multiplex PCR so as to understand the HPV genotypes that are common among the Zambians.

5.4 HPV Infection and Histological Outcomes

Of the males with available histology results (n= 306), 58.8% had an abnormal outcome of koilocytosis and none of them had low grade, high grade or cancerous lesions. Koilocytosis was only present in males that were HPV DNA positive. This was as expected because HPV infection is recognized as the biological cause of koilocytosis.

Univariate and Multivariate Analysis

Univariate analysis of determinants of HPV infection on sociodemographic and lifestyle variables showed several factors to be significantly associated with prevalent HPV infection. Indicators of sexual activity were highly associated with the outcome of HPV infection: a self-report of being sexually active, history of being married, and multiple sexual partners in the last 12 months. Age was indirectly associated with HPV infection. Multivariate analysis showed that variables which showed a significant effect in unadjusted analyses did not retain an effect when adjusted for age except for multiple sex partners in the last 12 months. These results are consistent with the literature which, across many studies, shows age and markers of sexual activity to be the most consistent risk factors for HPV infection [21].

5.5 Limitations

The primary limitations of the study include the fact that the study population was relatively small; the analysis was cross sectional in nature; there was data missing for some participants; and the failure to genotype the HPV infections using multiplex PCR.

5.5.1 Non Participants and Selection bias

Males were recruited when they presented for voluntary medical circumcision. Thus, the males who agreed to participate are likely to be different from non-participants and these included those who declined to participate, those who are HIV positive and those from the general population of the study communities on important characteristics. Unfortunately it was not possible to formally assess participation rates, differences between participants and non-participants, or the degree to which selection bias was influenced by the make- up of the study population. Although the number of participants who were approached and who agreed to participate in the study was not accurately recorded, the nurse practitioners estimated participation to be on the order of 80- 90%.

5.5.2 Cross- Sectional Data

The cross- sectional nature of the data presented here implies that observed age-specific HPV- DNA prevalence should be interpreted with caution. The pattern may either represent a biological phenomenon or a cohort effect. The cross sectional analysis of factors associated with prevalent HPV infection does not in any way attempt to investigate causality and should not be interpreted to do so. It should be noted that this study sought to detect HPV- DNA, not to differentially detect latent, active or persistent infection, hence the nature of the infections that were detected cannot be characterized more specifically.

5.5.3 Missing Data

Six (1.9%) of the recruited participants had their questionnaire and data sheet missing, hence these were excluded from the study. Missing data was not considered a serious limitation for this study since we recruited more participants (n= 312) than the calculated sample size of 264 hence our recruitment was 118%.

5.5.4 Lack of HPV genotype

The analysed data lacks HPV genotype distribution because of the failure to amplify the DNA by multiplex PCR due to degradation of DNA by the formalin fixative used. Hence the results will not show the burden of Hr- HPV within the male population as well as multiple infections.

5.6 Strengths

Having discussed limitations of this study, it is important to recognize the specific strengths of the study. Despite limitations in assessing selection bias, the study population is reasonably representative of the target population. The active education of communities by the Ministry of Health in Zambia to go for voluntary medical male circumcision for HIV prevention may entail that, we expect that our sampling procedure produced a study population that is roughly representative of the general population.

The cross sectional nature of the analysis limits the way in which it can be interpreted, however, this prevalence data represent a useful baseline picture of HPV infection in the study population. A longitudinal study in future work will allow for the evaluation of other important questions including persistence of infection, time of clearance of infection and frequency of new infections. To our knowledge this is the first study examining genital HPV infection among males in Zambia. It is relevant to study this population and address the questions raised since sub-Saharan has the highest burden of cervical cancer and our population is at high risk of STI's including HPV infection.

6. CONCLUSION

This study represents the first analysis of HPV prevalence, determinants of infection and histologic findings among males going for voluntary medical circumcision in Lusaka, Zambia. The data provides a foundation on which to expand understanding of the burden of genital infection among males in Zambia, a sub-Saharan country with a high burden of cervical cancer.

The results did show a high prevalence of HPV in males going for VMMC for HIV infection prevention. The youngest male with genital HPV infection was 10 years old and infection was highest in males above the age of 18 years. The risk factors for genital HPV infection were sexual activity, and multiple sex partners. None of our study participants had precancerous or cancerous lesions.

Our results for the multiplex PCR suggest that PCR using DNA from FFPE tissues suffers from inhibition of amplification of larger amplicons, which can be attained using primers with smaller amplicons. We recommend that we use other methods for DNA extraction such as heat pre-treatment or perform multiplex PCR on DNA extracted from fresh frozen tissue or fresh tissue.

The data from this research not only strengthens the understanding of HPV infection as experienced in males in Lusaka, but may also expand knowledge on the burden across the country.

A baseline understanding of the burden of HPV infection, type specific HPV prevalence and determinants of Hr- HPV infection among males will allow an informed evaluation of infection prevention strategies such as introduction of the HPV vaccine among males as well as the natural history of infection and screening for HPV associated lesions.

HPV infection was detected in 71.9% of the foreskin samples (n= 306), and it was associated with being sexually active as well as a self-report of having had more than one sexual partner in the previous twelve months. HPV infection was indirectly

associated with age and was highest among males aged fifteen to twenty four years. Koilocytosis was the most prevalent histologic finding and it was associated with HPV infection.

7. RECOMMENDATIONS

- We recommend a population based study to investigate the types of Hr-HPV infections present within the Zambian male population.
- There is need to understanding the relationship between HPV infection and disease in males, including the development of genital warts, penile intraepithelial neoplasia and invasive penile carcinoma
- Male vaccination against HPV should be considered to reduce anogenital HPV infection and its associated disease, which occur in both males and females.

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APPENDIX

APPENDIX 1: INFORMATION SHEET

Information Sheet

Principle investigator: Dr Peter Julius. University of Zambia, School of Medicine. Department of Pathology and Microbiology. Phone No. **0976080999 CO-PI: Dr Victor Mudenda.** University Teaching Hospital; Department of Pathology and Microbiology. Phone No. **0955750646. CO-PI: Dr Isaac Zulu.** University Teaching Hospital; Department of Surgery. Phone No. **0966825230.**

A) Purpose of this study

Human Papillomavirus (HPV) is a virus that causes genital warts and is normally sexually transmitted. HPV infection is detected by collecting samples of cells from the genital region that can be obtained during circumcision. The sample is then examined to determine the presence of HPV. If HPV is detected, further analysis is conducted to classify the type of HPV. When HPV infection is present for a long time, it may cause ano-genital cancer in some males. HPV infection in males is normally subclinical making them a reservoir for infection for the females. HPV infection and associated disease can be prevented by vaccination. We are doing this study to investigate how many males have the infection at the time of circumcision. The information obtained will be used to determine if the infections levels among the study participants as well as asymptomatic males in Lusaka city is high. The results can then be used as a basis to determine if the study can be carried out at national level. The subsequent information obtained can then be used to determine if the Zambian males can also receive the prophylactic HPV vaccine that is currently being given to females only. This vaccine will protect males and their partners against HPV associated cancers.

B) Procedure

If you agree to participate in this study, you will be asked to complete a self-administered questionnaire before circumcision. A nurse will accompany you in the process. If you feel uncomfortable to complete the questionnaire at once, you can always make arrangements with the nurse for another visit. After circumcision, your foreskin specimen will be collected for the study. It will be sent to the pathology laboratory within the U.T.H for processing, DNA extraction and analysis for Human papillomavirus. The specimens will be kept for the length of this study after which they will be disposed of. We will also need to review your medical file to collect further information concerning your health status.

C) Risks and benefits

There is no additional risk related to this study as we will only collect your resected foreskin. You will not have more visits to the clinic; you will only spend a little more time to fill out the questionnaire. Your participation will help in developing preventive strategies for HPV infection.

D) Participation

Your participation in this study is **of your own free will**. You can decline participating in this study. This will not affect your health care treatment in any way. You will also get a copy of this consent form. You have the right to ask the nurse any questions about the study before and after accepting to participate.

E) Confidentiality

In order to ensure your privacy and confidentiality, your name will not appear on any study record or results presented by the research team. Instead a patient identification number will be assigned to you and will appear in all your records. Only the nurse and the researchers in U.T.H will have access to the study number. You understand that all information about you and your results will be treated in the same confidential manner as other medical records and you will not be identified in any subsequent reporting of results.

APPENDIX 2: CONSENT FORM

The University Teaching Hospital

Department of Pathology and Microbiology

Study of human Papillomavirus in male's undergoing circumcision in Lusaka city.

VOLUNTARY CONSENT

By signing this form, I acknowledge having received and read a copy of the information paper concerning this study. I have had the opportunity to ask any questions I may have about this study, and they have been answered to my satisfaction. I agree to participate in this study and I understand that I may withdraw this agreement at any time. I understand that my decision whether or not to participate will not change any healthcare I might receive or my legal rights. I also understand that all information will be kept strictly confidential. My file will be coded and kept in place where only the research team will have access

1) I agree to complete the questionnaire on the risk factors for HPV infection

Yes:____ ; No:____ ;

2) I agree for my foreskin specimen to be collected for the Human papillomavirus study and be kept for the length of this study after which it can be disposed of.

Yes:____ ; No:____ ;

Signature:_____

Write your name in block letters:_____

Date:_____

Telephone number:_____ Participant number ----------

Provider section

I recognize having offered to the participant a copy of this consent form and copy of the information document.

Provider ID number: _____

Signature of the Provider: _____

Date:_____

APPENDIX 3: QUESTIONNAIRE

HPV- PREV STUDY

5.3 DEMOGRAPHIC AND BEHAVIORAL DATA QUESTIONNAIRE

Research Coordinator Section

Date:…… **Centre: Mt'endere Clinic**

Participant Number: …… **Specimen Number:** ……

1. **Age** ……
2. What is your current marital status?
   - ❖ Single.
   - ❖ Married.
   - ❖ Divorced/separated.
   - ❖ Widowed.
   - ❖ Living with partner (not married but living with partner)
3. Have you ever had sex? Yes …… No ……
   If the answer is no, go to question 6
4. How old were you when you first had sex? ……
5. Have you had more than one sexual partner in the past one year? ……
6. Do you have any other specific health problems: Yes……NO……
   If Yes, specify: ……
7. Have you ever been vaccinated against HPV? Yes…… No ……
8. Comments (please, write down any comment you want about a specific item or about the study in general):

APPENDIX 4: ETHICS CLEARANCE

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 966 765 503
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

12th December, 2013

Ref. No. 2013-Aug-020

The Principal Investigator
Dr. Peter D. Julius
University Teaching Hospital
Dept. of Pathology and Microbiology
P/Bag RW 1X,
LUSAKA.

Dear Dr. Julius,

RE: Prevalence and genotype spectrum of genital Human Papilloma Virus infection in males undergoing circumcision in Lusaka City: A cross sectional study.

Reference is made to your corrections dated 28th November, 2013. The IRB resolved to approve this study and your participation as principal investigator for a period of one year.

Review Type	Ordinary	Approval No. **2013-Aug-020**
Approval and Expiry Date	Approval Date: 12th December, 2013	Expiry Date: 11th December, 2014
Protocol Version and Date	Version-Nil	11th December, 2014
Information Sheet, Consent Forms and Dates	• English.	11th December, 2014
Consent form ID and Date	Version-Nil	11th December, 2014
Recruitment Materials	Nil	11th December, 2014
Other Study Documents	Questionnaires.	11th December, 2014
Number of participants approved for study	264	11th December, 2014

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

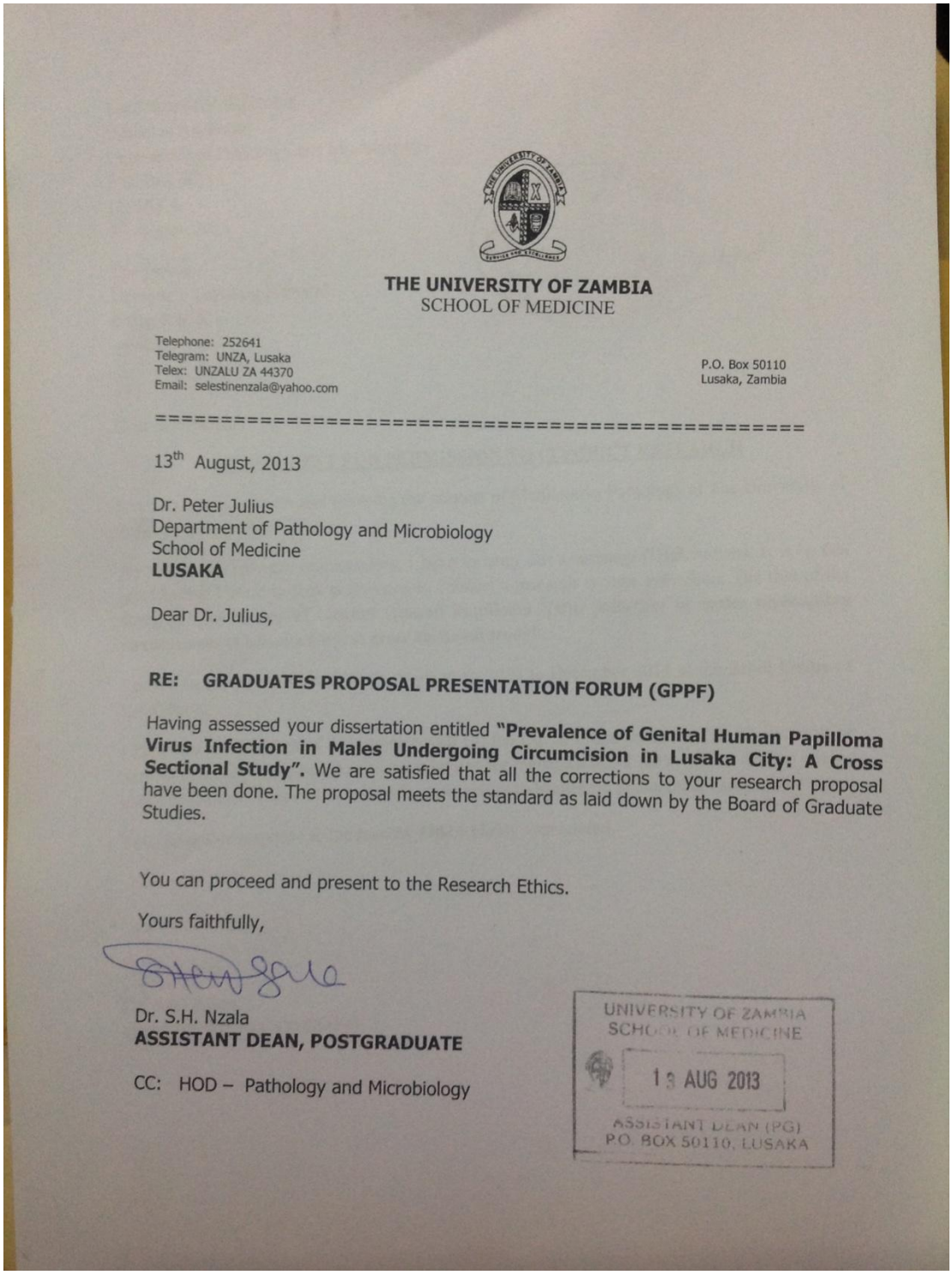
Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

PgDr. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

APPENDIX 5: CONSENT FROM HEALTH PROVIDER



THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE

Telephone: 252641
Telegram: UNZA, Lusaka
Telex: UNZALU ZA 44370
Email: selestinenzala@yahoo.com

P.O. Box 50110
Lusaka, Zambia

==========

13th August, 2013

Dr. Peter Julius
Department of Pathology and Microbiology
School of Medicine
LUSAKA

Dear Dr. Julius,

RE: GRADUATES PROPOSAL PRESENTATION FORUM (GPPF)

Having assessed your dissertation entitled **"Prevalence of Genital Human Papilloma Virus Infection in Males Undergoing Circumcision in Lusaka City: A Cross Sectional Study".** We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE

CC: HOD – Pathology and Microbiology

UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE
13 AUG 2013
ASSISTANT DEAN (PG)
P.O. BOX 50110, LUSAKA

THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE

Telephone: 252641
Telegram: UNZA, Lusaka
Telex: UNZALU ZA 44370
Email: selestinenzala@yahoo.com

P.O. Box 50110
Lusaka, Zambia

==========

09th August, 2013

Dr. Peter Julius Peter
Department of Pathology and Microbiology
School of Medicine
LUSAKA

Dear Dr. Julius,

RE: GRADUATES PROPOSAL PRESENTATION FORUM (GPPF)

Having assessed your dissertation entitled **"Prevalence of Genital Human Papilloma Virus Infection in Males Undergoing Circumcision at the University Teaching Hospital, Lusaka: A Cross Sectional Study".** We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Mrs D. Sitali
ACTING ASSISTANT DEAN, POSTGRADUATE

CC: HOD – Public Health

UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE
09 AUG 2013
ASSISTANT DEAN (PG)
P.O. BOX 50110, LUSAKA

8. SUBMITTED BY:

PRINCIPAL INVESTIGATOR/CO-PI (NAME): **Dr Peter D. Julius**

DATE: 26th August, 2013 SIGNED _____

REPUBLIC OF ZAMBIA
UNIVERSITY TEACHING HOSPITAL
28 AUG 2013
HEAD OF PATHOLOGY
& MICROBIOLOGY DEPT
P/BAG RW1X LUSAKA

Contact Address: University Teaching Hospital. Department of Pathology & Microbiology. P/Bag RW 1X. Lusaka

Tel. No. 0976080999 / 0953089637 Fax No. _____

Email: pmatashij@gmail.com

Full name of Head of Department/Institution: **Dr. Trevor Kaile.**

University of Zambia. School of Medicine. Department of Pathology and microbiology. P.O Box 50110. Lusaka.

Signed: _____ Date: 28/08/13

HEAD OF DEPARTMENT/INSTITUTION

UNIVERSITY OF ZAMBIA SCHOOL OF MEDICINE
DEPARTMENT OF PATHOLOGY
2013 -08- 28
UNIVERSITY
TEACHING HOSPITAL
P.O. BOX 50110, LUSAKA

Full name of Supervisor: **Dr Victor Mudenda**

(FOR STUDENTS ONLY)

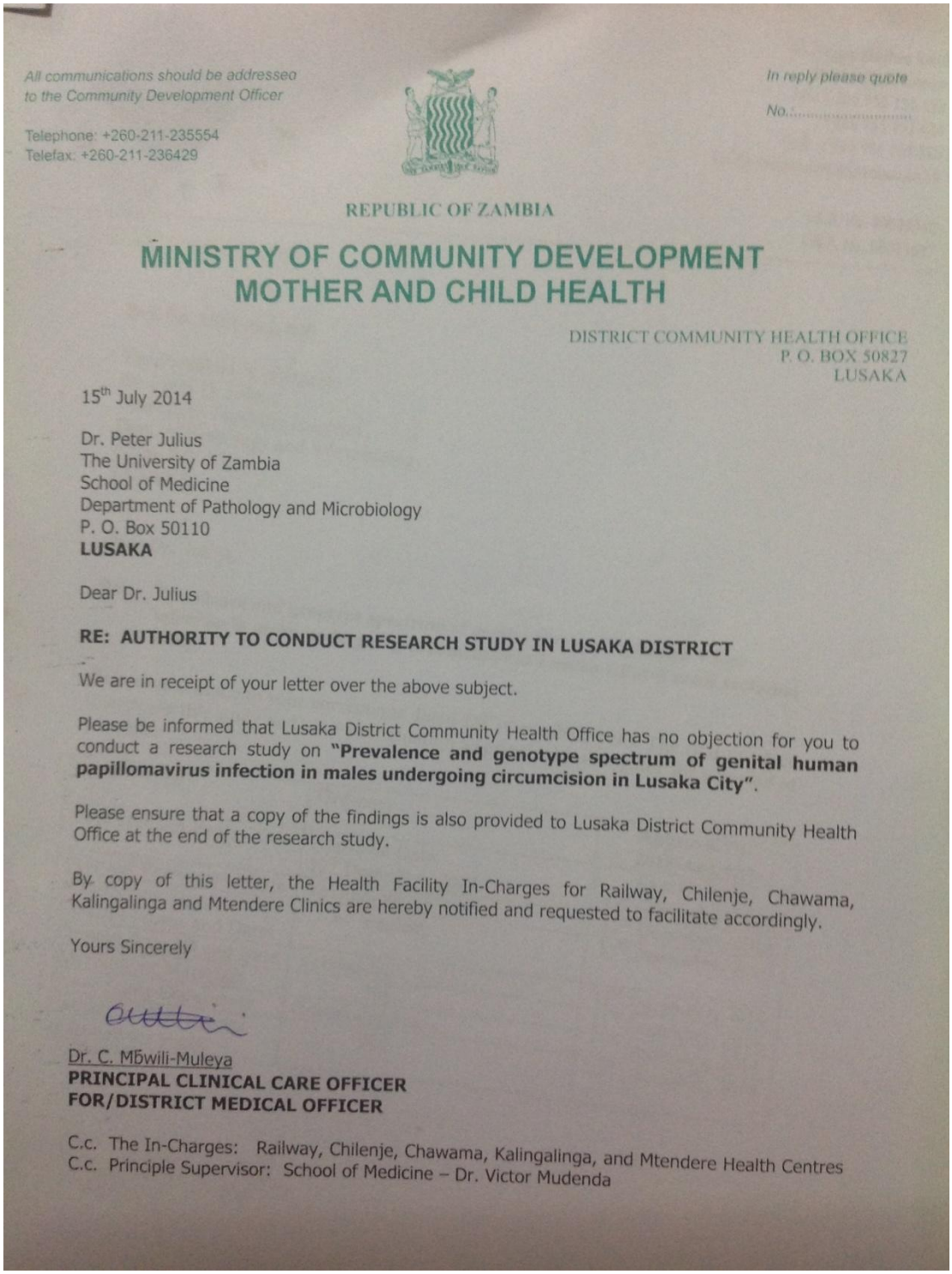
Please note that by signing this document you are endorsing that as the supervisor, you have read/cleared his proposal.

Signed: _____ Date: _____

SUPERVISOR

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
UNIVERSITY TEACHING HOSPITAL
28 AUG 2013
HEAD OF PATHOLOGY
& MICROBIOLOGY DEPT
P/BAG RW1X LUSAKA

APPENDIX 6. AUTHORITY FROM LUSAKA DISTRICT HEALTH MANAGEMENT TEAM



All communications should be addressed to the Community Development Officer

Telephone: +260-211-235554
Telefax: +260-211-236429

In reply please quote

No.:.....

REPUBLIC OF ZAMBIA

MINISTRY OF COMMUNITY DEVELOPMENT
MOTHER AND CHILD HEALTH

DISTRICT COMMUNITY HEALTH OFFICE
P. O. BOX 50827
LUSAKA

15th July 2014

Dr. Peter Julius
The University of Zambia
School of Medicine
Department of Pathology and Microbiology
P. O. Box 50110
LUSAKA

Dear Dr. Julius

RE: AUTHORITY TO CONDUCT RESEARCH STUDY IN LUSAKA DISTRICT

We are in receipt of your letter over the above subject.

Please be informed that Lusaka District Community Health Office has no objection for you to conduct a research study on **"Prevalence and genotype spectrum of genital human papillomavirus infection in males undergoing circumcision in Lusaka City"**.

Please ensure that a copy of the findings is also provided to Lusaka District Community Health Office at the end of the research study.

By copy of this letter, the Health Facility In-Charges for Railway, Chilenje, Chawama, Kalingalinga and Mtendere Clinics are hereby notified and requested to facilitate accordingly.

Yours Sincerely

Dr. C. Mbwili-Muleya
PRINCIPAL CLINICAL CARE OFFICER
FOR/DISTRICT MEDICAL OFFICER

C.c. The In-Charges: Railway, Chilenje, Chawama, Kalingalinga, and Mtendere Health Centres
C.c. Principle Supervisor: School of Medicine – Dr. Victor Mudenda

APPENDIX 7. U.T.H PERMISSION LETTER

The University of Zambia
School of Medicine
Department of Pathology and Microbiology
P.O. Box 50110
LUSAKA
7th August, 2013

The Director
University Teaching Hospital
P/Bag RWIX
Lusaka

REPUBLIC OF ZAMBIA
27 AUG 2013
UNIVERSITY
P/BAG RW 1X LUSAKA
No objection

Dear Sir/Madam

RE: REQUEST FOR PERMISSION TO CONDUCT RESEARCH

I 'am a postgraduate student pursuing the masters of Medicine in Pathology at The University of Zambia, School of Medicine.

As part of the program requirements, I have to carry out a research (Dissertation). It is in this premise that I write to seek permission to conduct a research at your institution. The title of the study is **"Prevalence of Genital Human Papilloma Virus infection in males undergoing circumcision in Lusaka City: A cross sectional study".**

I intend to carry out the study from September, 2013 to December 2014 at the Adult Centre of Excellence.

I hope that the findings of this research will fill the gap in information for Zambia that will be used to make evidence based recommendations for management of HPV related disease among males in Zambia. This will in turn reduce variability in healthcare management and related costs.

Your favorable response to the request will be highly appreciated.

Yours sincerely,

Dr. Peter Julius
COMPUTER NO. 531004997

APPENDIX 8. STANDARD TISSUE PROCESSING PROCEDURE

1. Introduction

Microscopic analysis of cells and tissues requires the preparation of very thin; high quality sections (slices) mounted on glass slides and appropriately stained to demonstrate normal and abnormal structures.

Most fresh tissue is very delicate and easily distorted and damaged and it is thus impossible to prepare thin sections from it unless it is chemically preserved or "fixed", and supported in some way whilst it is being cut. Broadly there are two strategies that can be employed to provide this support.

1. The tissue can be frozen and kept frozen while the sections are cut. These sections are called "frozen sections".

2. Alternatively the tissue specimen can be infiltrated with a liquid agent that can subsequently be converted into a solid that has appropriate physical properties which will allow thin sections to be cut from it. Paraffin wax is such an agent. This produces so-called "paraffin sections".

2. Purpose

To produce the tissue blocks that will be able to produce quality paraffin sections while retaining the tissue morphology and architecture.

3. Principle

Tissue processing is the next step after fixation. The tissue sections will go through the process of dehydration, clearing and infiltration, exposing tissues to various chemicals for certain processes. During tissue processing, one step will build upon another. Although many references include the

embedding process in tissue processing, embedding is really a separate process after paraffin infiltration.

4. Equipment, Reagents, Supplies, Personal Protective Equipment (PPE)

Equipment	Reagents	Supplies	PPE
STP 120 Tissue Processor	10% Formalin Ethyl Alcohol Xylene Paraffin Wax	Tissue Cassettes	Lab coat Gloves Proper PPE

5. Specimen

Biopsies or necropsies or any tissue sample removed from the human body and submitted for pathological review. All specimens submitted should be in a recommended fixative and in the ratio of the specimen to fixatives of 1:10. The specimen should have been completely fixed prior to processing.

6. Safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to. Additional special requirement as described in Histopathology Laboratory Safety Policy Manual.

7. Calibration procedures

NA

8. Procedure Step-by step

	Tissue Processor Program	
Step #	**Reagent**	**Duration**
1.	10% Formalin	2 Hours
2.	10% Formalin	2 Hours
3.	70% Alcohol	2 Hours
4.	80% Alcohol	2 Hours
5.	Absolute Alcohol	2 Hours
6.	Absolute Alcohol	1 Hour
7.	Absolute Alcohol	1 Hour
8.	Xylene (Para-methylbenzene)	3 Hours
9.	Xylene (Para-methylbenzene)	2 Hours
10.	Xylene (Para-methylbenzene)	1 Hours
11.	Paraffin Wax	2 Hours
12.	Paraffin Wax	2 Hours
Total Hours		**22 Hours**

9. Quality Control

The tissue processing cycles and reagents require specific quality control (QC) measures. The proper changing or rotation of the processing reagents

should be on a set schedule in most cases. Documentation of these changes or rotations must be recorded.

As well, a hydrometer must be used to check the strength of the diluted alcohols and recorded. Care must be taken to ensure that the correct reagent is poured into the correct reagent container to avoid processing errors. Temperatures of the paraffin's should be checked and recorded daily. Preventative maintenance schedules should be established at least once or twice annually. The quality and even grade of the processing reagents should be adhered to for the proper processing of the tissues.

10. Calculation of results

NA

11. Reference range/Test Interpretation

NA.

12. Alert

Ensure that the tissue specimens are completely fixed prior to processing.

13. Notes, Limitations and Anything Else

New tissue processor instruments are often installed, a processing program loaded and tissue specimens processed. However, there must be a validation process before a tissue processor is put into use to identify and record, for example, the tissue processor platform, what tissue types will be processed, which processing protocol or cycle will be implemented, technical training for each operator, the workflow process, reagents, processing cycles, etc.

One method to validate the tissue types is to use a multi-tissue block and variety of tissues in one block, all the same size. This is not perfect but can save time. If two processing cycles are available, both will need to be

validated. The thickness also needs to be validated. Standardization will play a role in the validation process. Upon completion, the slides need to be reviewed by both technical staff and pathologists, then documented.

14. Reference

a) http://laboratory-manager.advanceweb.com/Archives/Article-Archives/Tissue-Processing-Protocols.aspx
b) http://www.leicabiosystems.com/pathologyleaders/an-introduction-to-specimen-processing/
c) Bancroft, Theory and Practice of Histological Techniques, 6eISBN-13: 978-0443102790

APPENDIX 9. MICROTOME MAINTENANCE AND USE (2235 LEICA ROTARY MICROTOME).

15. Purpose

To describe procedures to be followed for the use, inspection, cleaning, calibration/standardization, testing, and maintenance of 2235 Leica Rotary Microtome.

Equipment	Reagents	Supplies	PPE
- Forceps	N/A	- Microtome Blades - Kim Wipe/Tissue - Slides - Pencil	- lab coat - Nitrile gloves

16. Specimen

N/A

17. Special safety precautions

Before use, check to make sure that:

I. Microtome is on a stable surface that is free of vibrations.
II. Microtome is not in direct sunlight or near a heat source.
III. Place blocks to be sectioned on cold plate before clamping the cassette in readiness to be cut.
IV. Turn on the Flotation bath (water bath) on the Work Station.
V. Label slides to correspond with block labels.

18. **.Procedure Step-by step for tissue cutting**

Step #	Instruction
13.	Be sure to lock the hand wheel and cover the edge of the blade with the blade protector when not in use. Firmly clamp in the pre-frozen paraffin block onto the specimen clamp specimen before installing the blade.
14.	Turn the rotary handle/hand wheel to move the specimen to the back, utmost position and lock by moving the lock key on your right. CAUTION: Avoid the edge of the blade, it is very sharp.
15.	Insert a microtome blade into the knife holder. CAUTION: microtome blades are very sharp; handle with care. Always have knife guard in place when not sectioning.
16.	Adjust the specimen's surface position so it is parallel to the blade's edge.
17.	If the display windows is showing a different thickness, adjust to the standard thickness using a section thickness knob. The standard cutting thickness is 3µm used in histopathology.
18.	The rotary handle/hand wheel on right-hand side of microtome is locked. Unlock it to start moving the specimen clamp towards the blade holder.
19.	Start trimming by moving the hand wheel on an up and down movement at a 90^{o} angle while moving the course wheel gently on forward movement or clockwise.
20.	Trim until the tissue is fully exposed. Stop turning the course wheel, and then turn the hand wheel clockwise (360^{o} angle rotation to cut the ribbons).The speed of hand wheel rotation should match the rigidity of the specimen.
21.	Select thin nice sections using a forcipes and gently place your ribbons in the water bath. Get your slide and fish the ribbons, label,

	wipe the water beneath the slide and place on a hot plate to fix/melt the excess wax.

19. Quality Control

• Have an authorized microtome technician service the instrument at least once a year.
• After the warranty period ends, a successive maintenance contract should be made to ensure the service continues.
• Clean the microtome daily.
• Lubricate the moving parts monthly with lubricating oil (one or two drops is enough).
• Maintenance should be done by the authorized technicians. Self-repair could void the warranty and can cause extra expense later.

20. Reference range/Test Interpretation

N/A

21. Notes, Limitations and Anything Else

CLEANING

Attention!*REMOVE THE BLADE.
• Be sure to lock the hand wheel before cleaning.
• Brush off any residue with a dry brush.
• Remove the blade base and the blade holder for cleaning.
• Do **NOT** use detergent containing acetone and benzene to clean the instrument; it will damage the paint on the surface of the instrument.
• Only mild cleaners or gentle lye should be used for cleaning the instrument.

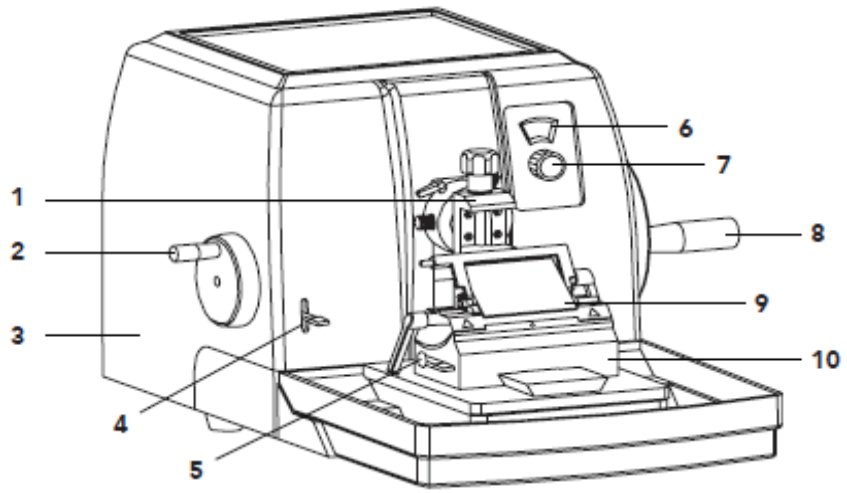
22. Reference

- http://www.bu.edu/EHS
- www.TannerScientific.com/UserManualTN5500
- www.boeckeler.com/MR-2/MR-3RotaryMicrotomeUserGuide

APPENDIX 10. MAYER'S HAEMATOXYLIN AND EOSIN (H&E) STAINING PROTOCOL

2235 LEICA ROTARY MICROTOME

2235 Leica Rotary Microtome.



1 - Standard Specimen Clamp
2 - Course Feed
3 - Enclosure
4 - Trimming Lever
5 - Lock Lever
6 - Display Window
7 - Section Thickness Knob
8 - Hand Wheel
9 - Pressure Plate
10 - Blade Holder Assembly

The **Model Titan 5000** is a manually operated microtome powered by rotating the hand wheel. The guidelines for the vertical and horizontal movement of

23. Purpose

Haematoxylin and Eosin (H&E) staining denotes staining of nuclei by oxidized Haematoxylin (haematin) through mordant (chelate) bonds of metals such as aluminium, followed by counter staining by eosin, which stains in varying shades the different tissue fibres and cytoplasm. A general tissue demonstration picture is produced and serves as the main diagnostic technique.

Haematoxylin solutions are complex and will contain one or more of the following substances forming a composite solution bearing the originator's name.

Alum:	Aluminium potassium sulphate or aluminium ammonium sulphate. This is the mordant of which aluminium cation is the main reactant.
An acid:	This aims at making staining more precise ("accelerator").
An oxidizing agent	To give speedy conversion of Haematoxylin to haematin.
Glycerol:	This slows the oxidation of naturally oxidizing Haematoxylin.

Since the H&E stain is performed routinely on the majority of tissue that goes through this lab, it is essential to have an excellent quality control program for staining. Adherence to this previously prescribed quality control measure is assurance to this desired end.

Haematoxylin:

Haematoxylin, a natural dye, which was first used about 1863, is without a doubt the most valuable staining reagent used in histologic work. It has little affinity for tissue when used alone, but in combination with aluminum, iron, chromium, copper, or tungsten salts, it is a powerful nuclear stain and chromatin stain. It has polychrome properties, which may be brought out with proper differentiation. The active coloring process, known as “ripening”, takes several days or weeks unless it is hastened by the addition of an oxidizing agent, such as mercuric oxide, hydrogen peroxide, potassium permanganate, sodium perborate, or sodium iodate.

The most common formulas for staining with Haematoxylin are combinations with aluminum in the form of alum.

The H & E is an effective stain for demonstrating the major histological structures, particularly nuclei which are most important structures in the viewing of histological sections for pathological changes. Since the H & E is almost always the initial stain used, it will invariably provide clues as to which other stains are required.

It is believed that the H&E is a “salt dye” mechanism of staining. Positively charged Haematoxylin will stain negatively charged tissue components, and negatively charged Eosin will stain positively charged tissue components.

There are two methods of staining when Haematoxylin is employed; they are progressive and regressive staining. Progressive staining is accomplished by employing a solution, which contains an excess of aluminum salts or acids, thus increasing the selectivity for nuclei. After staining with Haematoxylin, the slides are washed well in water and then counterstained.

Regressive staining on the other hand is accomplished by over-staining in a relatively neutral solution of Haematoxylin, then removing the stain from the other constituents with acid alcohol or other differentiating agent. The subsequent neutralization is accomplished by means of

ammonia water, lithium carbonate, or buffered solution, and then the counterstain is applied.

Eosin:

Eosin, an acid synthetic dye, stains cytoplasm with different degrees of intensity. It stains readily and brilliantly after Zenker fixation. The reason for this is that heavy metals such as mercuric salts combine with acid groups and therefore increase acidophilia. A longer time is required to stain formalin-fixed tissue. Eosin is one of the most valuable counterstains known. Besides its widespread used as a counterstain for Haematoxylin or other basic (nuclear) dyes, it is used in a large number of blood stains, such as Giemsa, Jenner, and Wright stain. It is used in both aqueous and alcoholic solutions. It is usually employed in 0.5% and 1.0% solutions. However, when eosin (or Phloxine) is applied before an aniline dye such as methylene blue, a strong 2.5% to 5.0% solution must be used. Over-staining is necessary here, since the subsequent treatment in methylene blue extracts much of the acid stain. Some of the eosin stain is removed from the tissue rather rapidly when it is dehydrated in 95% alcohol and more slowly in absolute alcohol. Therefore, you must allow for this by slightly over-staining the sections and rapidly dehydrating them in 95% alcohol and the absolute alcohol.

The choice of Haematoxylin and counterstain is a matter of personal preference. The University Teaching Hospital Histology Laboratory uses Mayer's Haematoxylin unless otherwise stated.

24. Equipment, Reagents, Supplies

Reagents	Supplies	Equipment	PPE
Mayer's Haematoxylin	Forceps	Rotary Microtome	Gloves

1% Aqueous Eosin	Kim wipe or Tissue	Floatation bath	Lab Coats
1% Acid Alcohol	Frosted end slides	Hot plate	
Scott's Water		Vari-stainer	
Ethyl Alcohol			
Xylene			
Tap water			

25. Specimen

Formalin Fixed Paraffin-Embedded tissue blocks

26. Special safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to. Additional special requirement as described in Histopathology Laboratory Safety Manual.

27. Procedure Step-by step

This procedure is used for both the automated Vari-stainer and the manual method. For the automated equipment, the program has been set according to this SOP.

Step #	Instruction
22.	Take sections to water (Deparaffinise in two changes of xylene 2 minutes each, wash in 2 or 3 changes of absolute alcohol 2 minutes then in running tap water for 2 minutes)
23.	Stain with an Mayer's Haematoxylin solution for the 15 minutes
24.	Wash in running tap water for 2 to 5 minutes.
25.	Differentiate in 1% acid alcohol for 20-30 seconds
26.	Blue in running tap water for 10 minutes or any of the bluing solutions for 2-3 seconds

27.	If bluing agent has been used, rinse in tap water briefly (10 dips)
28.	Stain in 1% aqueous eosin for 5 minutes.
29.	Rinse in tap water 3-5minutes.
30.	Dehydrate in 3 changes of absolute alcohol for 2 minutes each
31.	Clear in xylene in 3 changes for 2 minutes each.
32.	Mount in D.P.X Mountant media using the 24 x40 mm coverslips For Mounting a. Apply 1-2 drops off a small spatula to tissue, avoiding air bubbles. b. Place coverslip at edge of slide at an angle c. Allow the capillary rising action of the cover glass against the slide to cover the tissue.
33.	Leave to air dry (Mountant to solidify)

28. Quality Control

a. Tonsil and cervical biopsy, on one slide should be stained daily and analysed microscopically to ensure proper preparation of reagents.

Nuclei……**blue with some metachrosis**

Cytoplasm……**shades of pink, identifying different tissue components**

b. All slides are inspected for quality according to the Quality Control procedure for Histologic Stains.

c. Haematoxylin should be filtered to remove the metallic sheen that can cause precipitates on the stained slides.

29. Reference range/Test Interpretation

N/A

30. Notes, Limitations and Anything Else

N/A

31. Reference

1) Theory and Practice of Histological Techniques, 6eISBN-13: 978-0443102790
2) http://www.ihcworld.com/_protocols/special_stains/HE_Mayer.htm
3) http://www.cgcconnect.com.au/forums/topic/hematoxylin-and-eosin-he-staining-protocol/

APPENDIX 11. SLIDE MOUNTING PROCEDURE

32. Purpose

To provide the maximum degree of transparency to stained tissue sections.

33. Principle

Mounting is achieved by using a medium that has a refractive index which is approximate to that of dried protein i.e. between 1.53 and 1.54.The refractive index of a mounting medium may change on drying due to evaporation of solvents.

Air bubbles should not be permitted to remain under coverslips since these air bubbles tend to expand.

34. Equipment, Reagents, Supplies, Personal Protective Equipment (PPE)

Equipment	Reagents	Supplies	PPE
NA	DPX Mountant	Coverslips (preferably 24 x 40mm or larger) Slides of interest Filter paper/ Kim wipes Orange sticks or Mountant dropper	Lab Coat Nitrile Gloves

35. Specimen

Stained slides.

36. Safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to.

Engage the use of proper body mechanics to prevent work related musculoskeletal related disorders. REFER TO THE HISTOPATHOLOGY SAFETY HANDBOOK (HIS-SFT-v1) FOR DETAILS.

37. Calibration procedures

NA

38. Procedure Step-by step

Step #	Action
34.	Remove slide from xylene and wipe off excess xylene and let it lie on a flat surface (Do not allow tissue on the slide to dry).
35.	Place 3-4 drops of mounting medium on the glass slide (with stained tissue) and lower the cover slip onto the slide by turning it right side up until it comes into contact with the edge of the slide then continue lowering it down until it completely covers the stained section on the slide.
36.	Gently tease bubbles from the coverslip with an applicator stick.
37.	Allow the slide to dry on a flat surface
OR	
38.	Place 3-4 drops of mounting medium on the coverslip and spread over the surface using a dropper or stick
39.	Remove slide from xylene and drain off excess xylene but do not allow tissues on the slide to dry
40.	Lower slide onto the coverslip by turning it right side up until it comes into contact with the edge of the coverslip then continue lowering it down until it completely covers the coverslip.
41.	Allow to dry on a flat, clean surface (Preferable an absorbable paper like a filter paper)

39. Quality Control

Always check for the presence of air bubbles macroscopically and microscopically and remount before taking the slides to the pathologist. QUALITY CONTROL checks must be documented in the SLIDE EVALUATION CHECKLIST (HIS-FM-013v1)

The mounting medium must not be too thin or too thick as this may disturb the quality of the staining.

Remember to wipe off unwanted tissues before mounting

40. Calculation of results

NA

41. Reference range/Test Interpretation

A well mounted slide must be clean, free from air bubbles or debris and without any mountant dripping from the sides.

REFER TO SLIDE EVALUATION CHECKLIST QC SHEET (HIS-FM-013v1)

42. Alert/critical values, where appropriate

NA.

43. Notes, Limitations and Anything Else

a) To prevent the possibility of contaminating the dropper or application stick with excess cells, the dropper should never touch the surface of the cells.
b) A few drops of Xylene can be added to the dispensed mountant to get rid of bubbles.
c) Coverslips must always be wiped clean before use (with a safety caution) and wipe off excess xylene from the bottom of the slide

44. Reference

Bancroft J.D. Stevens, A. Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 1982.

Bancroft, J.D.; Cook, H.C. Manual of Histological Techniques, Churchill, Livingstone, London, 1984.

Bancroft JD, Gamble M, Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 2008.

Cook H.C.; Manual Histological Demonstration Techniques; Butterworth's, 1974.

Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.