GENOTYPIC DIVERSITY AND FACTORS ASSOCIATED WITH HUMAN PAPILLOMAVIRUS AMONG PATIENTS ATTENDING SPECIAL TREATMENT CENTRE (STC) IN NAIROBI COUNTY, KENYA

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(Medical Virology)

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Genotypic diversity and factors associated with Human Papillomavirus among patients attending Special Treatment Centre (STC) in Nairobi County, Kenya

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A thesis submitted in partial fulfillment for the degree of Master of Science in Medical Virology in the Jomo Kenyatta University of Agriculture and Technology

2016
DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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KEMRI, Kenya
DEDICATION

I dedicate this work to my beloved parents Mr. and Mrs. Joshuah Bett and my siblings Philemon Langat, Erick Langat, Patrick Langat and Vincent Langat for their love, moral support and encouragement during this period of my study.
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<tbody>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CCS</td>
<td>Cervical Cancer Screening</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>Creates</td>
<td>Center for Research in Therapeutic Sciences</td>
</tr>
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<td>CVR</td>
<td>Centre for Virus Research</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Food and Drug Administration</td>
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<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
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<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICC</td>
<td>Invasive Cervical Cancer</td>
</tr>
<tr>
<td>Jkuat</td>
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<tr>
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<td>Kilo Dalton</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
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<tr>
<td>LCR</td>
<td>Long Control Region</td>
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<td>---------</td>
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<td>Open Reading Frames</td>
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<td>Polymerase Chain Reaction</td>
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<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RRP</td>
<td>Recurrent Respiratory Papillomatosis</td>
</tr>
<tr>
<td>SIL</td>
<td>Squamous Intraepithelial Lesion</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for Social Science</td>
</tr>
<tr>
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<td>Scientific Review Committee</td>
</tr>
<tr>
<td>STC</td>
<td>Special Treatment Centre</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually Transmitted Infection</td>
</tr>
<tr>
<td>URR</td>
<td>Upstream Regulatory Region</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VIA</td>
<td>Visual Inspection with Acetic acid</td>
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<tr>
<td>VILI</td>
<td>Visual Inspection with Lugol’s iodine</td>
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ABSTRACT

Different samples can be used for cervical screening, including Pap test or biopsy, Liquid Based Cytology, Visual inspection using acetic acid or Lugol’s iodine, and HPV testing using cervical brush. These methods require pelvic examination, which is invasive and uncomfortable for patients. Therefore there is need to use urine which is non-invasive, easy to collect and unbiased since it bypasses medical examination, socio-cultural and religious implications. The study aimed at determining genotyping diversity and assessing the factors for HPV in urine from patients attending Special Treatment Centre (STC) in Nairobi County. A laboratory based cross-sectional survey was conducted on 222 consenting men and women aged 18–49 years (mean age 32 years; SD=1.2). Participants were sampled using simple random sampling and structured questionnaires were administered. Approximately 10 ml self-collected urine samples were collected using 15ml sterile falcon tubes. Genomic DNA was extracted from 222 urine samples (45 from men (mean age 34 years; SD=2.73) and 177 from women (mean age 31 years; SD=1.33) using Qiagen DNA blood mini kit and the presence of HPV DNA was amplified using the L1 gene. Big dye terminator method of sequencing was used to determine the HPV viral genotypes. High risk HPV genotypes detected among females were: HPV-16 (10%) and 66 (7.5%), while low risk types were HPV 6 (27.5%), followed by 81 (25%), 83 (10%), 11 (7.5%), 70 (7.5%) and 54 (2.5%). The prevalent low-risk HPV type detected in males was HPV type 6 (75%) while HPV-58 (25%) was the only high risk type in males. History of sexually transmitted infections was significantly associated with HPV infection among females (P=0.002). The prevalence of HPV was high from this population. There is need to monitor HPV types in circulation since it is important for HPV vaccine development.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cervical cancer linked etiologically to HPV is ranked as the second or third leading cause of cancer death in women (Parkin et al., 1999; Baay, et al., 2004). Of the 450,000 new cases of cervical cancer diagnosed per year in the world (WHO, 1995), 80% occur in countries with limited resources (Parkin et al., 1984). Genital HPV is one of the common sexually transmitted infections (STI) in the world. Up to 80% of sexually active people will be infected at some time in their lives. Of these, approximately 10-20% develops persistent infection (Einstein et al., 2009). Persistence of HPV infections for 10-15 years causes cervical squamous intraepithelial lesions (SILs) and Invasive Cervical Cancer (ICC) (Munoz et al., 2003). HPV is also linked to genital warts in men and women and it has been identified as a risk factor for the development of cervical, anal, penile, and vulval and oropharyngeal cancers (IARC monographs, 1995; Melbye & Frisch, 1998; Chaturvedi et al., 2008; Hoots et al., 2009; Miralles-Guri et al., 2009).

Cervical HPV infection occurs frequently within the year of first sexual contact and its prevalence decreases with increasing age in women (Smith et al., 2008). Therefore, progression of the cervical cancer lesions is slow and most of these lesions are asymptomatic but can be detected early through cervical cancer screening (CCS) (Youngkin, 2004). On the other hand, early detection of cervical cancer can lead to a positive prognosis that can result in decreased mortality and reduced health costs though it can lead to social and psychological suffering of individuals or family members as well as significant community adverse effects if left unattended to (WHO, 2006a; Leung & Leung, 2010).
To date, more than 120 HPV genotypes have been characterized. Over 40 of these infect the epithelial and mucosal lining and other areas (de Villiers et al., 2004).

HPV have been categorized according to the risk they pose on various epithelial sites. For instance, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, 69, 73, 59 and 82 have been classified as high risk (HR)/oncogenic types while HPV type 6, 11, 42, 43 and 44 have been classified as low risk (LR) types (Sargent et al., 2008; Bouvard et al., 2009; Trottier & Burchell, 2009). The LR type’s causes low grade cervical lesions, genital warts and Recurrent Respiratory Papillomatosis (RRP) (Sargent et al., 2008).

1.2 Statement of the problem

It is estimated by the World Health Organization (WHO) that there are 530,000 new incidences of cervical cancer every year and 270,000 deaths in the world. Eighty five percent of these deaths occur in developing countries including Kenya (Forman et al., 2012).

Currently, different types of samples can be used for screening cervical cancer such as conventional cervical cytology (Papanicolaou-Pap smear) or biopsy and Liquid Based Cytology (LBC), visual inspection using acetic acid or Lugol’s iodine (VIA-VILI), and HPV testing using cervical brush (Tota et al., 2014). However, screening by these methods requires pelvic examination, which is invasive, uncomfortable for patients and tedious for the health care providers (Mandelblatt et al., 2002).

Moreover, the success of cervical cancer prevention programs in low resource settings has been hindered by women’s educational backgrounds, misconceptions and prejudices. Due to these challenges, the disease is often identified in the late stage, resulting in higher rates of cervical cancer incidence and mortality (Sankaranarayanan et al., 2001; Cuzick et al., 2008; Tornesello et al., 2014). Therefore, to overcome these problems there is need to use alternative tools such as HPV testing on urine which may increase
screening coverage among men and women. This is due to the fact that urine method of sampling is a non-invasive, easy to collect, readily available sample and unbiased because it bypasses medical examination, as well as socio-cultural and religious implications.

1.3 Justification

Previous Kenyan studies have found high HPV prevalence for instance; Yamada et al. (2008) reported a high prevalence of 49% among HIV positive women and 17% among HIV negative women respectively. According to Ngugi et al. (2011), the prevalence of HPV was 21.3% among women. In addition, HPV types 16 and 18 have prevalence of 43.7% and 17.2% in Kenya respectively compared to the world’s 54.4% and 16.5%. HPV 45 which is also a high risk type has a high prevalence of 18.3% in Kenya compared to the global prevalence of 4.4% (WHO, 2010). Therefore, this means that more data are currently needed on the prevalence, genotype distribution and natural history of genital HPV infections in men and women, as well as the transmission patterns of HPV infection between sexual partners (Barrasso et al., 1987; Burk et al., 1996). Men are important reservoirs of HPV and may play an important role in the transmission of HPV infection to women, and in the perpetuation of the HPV-associated disease in the general population (Hagensee et al., 2004).

Knowledge of the genetic diversity of HPV among males and females is not only important in the study of the natural history of HPV, but also in the possible design of region-specific HPV screening tests and vaccines. The study aimed to determine the prevalence of HPV, factors associated with HPV infection and genetic diversity of HPV among males and females attending Special Treatment Centre (STC) in Nairobi County.
1.4 Research Questions?

1. What are the socio-demographic and behavioural characteristics of men and women attending Special Treatment Centre (STC) in Nairobi County?
2. What is the genetic diversity of HPV detectable in urine specimens from men and women attending Special Treatment Centre (STC) in Nairobi County?
3. What are the factors associated with HPV DNA infection among men and women attending Special Treatment Centre (STC) in Nairobi County?

1.5 Hypothesis

1.5.1 Null hypothesis

There is no difference in genetic diversity of HPV detectable in urine from men and women attending Special Treatment Centre in Nairobi County.

1.6 Objectives

1.6.1 General objective

To determine the genotypic diversity and factors associated with Human papillomavirus in urine from patients attending Special Treatment Centre (STC) in Nairobi County, Kenya

1.6.2 Specific objectives

1. To determine the socio-demographic and behavioural characteristics of men and women attending the Special Treatment Centre (STC) in Nairobi County.
2. To determine the genetic diversity of HPV in urine specimens from men and women attending the Special Treatment Centre (STC) in Nairobi County.
3. To determine the factors associated with HPV DNA infection among men and women attending a Special Treatment Centre (STC) clinic in Nairobi County.
CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of papillomaviruses

Papillomaviruses are ubiquitous and have been detected in a wide variety of animals, humans and birds. They are species-specific (Zur Hausen, 1999). HPVs fall into a number of distinct groups or genera, and the lesions they cause have different characteristics. The two main HPV genera are the α-alpha and β-beta papillomaviruses, with approximately 90% of currently characterized HPVs belonging to one or other of these groups (Harwood & Proby, 2002; Pfister, 2003). Alpha papillomaviruses comprises the largest group and most of the genital/mucosal HPV types belong to this group. The α-papillomaviruses also include cutaneous viruses such as HPV2, which causes common warts and the beta genus contains those that are associated with the development of cutaneous tumours (de Villers et al., 2004). The remaining HPVs come from three genera (Gamma, Mu and Nu) and generally cause cutaneous papillomas and verrucas that do not progress to cancer (Pfister, 1992; Shamanin et al., 1994).

2.2 HPV genotypes

More than 100 types of HPV have been characterized on the basis of DNA sequence divergence (Benard, 2005). Furthermore, HPVs are DNA viruses classified as either cutaneous or mucosal types due to the epithelial sites they colonize (IARC, 2011). The cutaneous types are epidermitropic while mucosal types are highly epitheliotrophic (Burd, 2003). Mucosal (anogenital and oral) HPV types are classified into HR and LR HPV types, depending on their potential to induce invasive cancer and precursor lesions HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 have been regarded as high risk (HR-HPV) and types 26, 53, and 66 as potentially high risk (probably HR-
HPV (Munoz et al., 2003). It’s now known that HPV 16 and 18 is the causative agent of approximately 70% cervical cancer cases (Muñoz et al., 2004).

However, Low Risk HPV’s include types 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, 81, and candidate HPV89. Types 6 and 11 are the most common and are associated with 90% of genital warts and laryngeal papillomas (Gale et al., 1994; Brown et al., 1999; Gale, 2005; Gale & Zidar, 2006; Potocnik et al., 2007). A new HPV type is defined as showing less than 90% homology to any of the known types on the basis of the E6, E7, L1 regions. If between 2% and 10% DNA divergence is present, the two viruses are considered subtypes of the same HPV type. When they show less than 2% divergence, the viruses are considered variants (de Villiers et al., 2004).

### 2.3 HPV Genome

HPV virion has a small non-enveloped icosahedral circular DNA genome. It is approximately 8000 base pairs and contains an average of 8 open reading frames (ORFs) (Jo & Kim, 2005; Zheng & Baker, 2006). The genome is about 52-55 nm in diameter and belongs to the family Papillomaviridae (de Villiers et al., 2004; Zur Hausen, 2006). Each capsid is composed of 72 pentameric capsomers, each of which is composed of five monomers of 55kDa units that join to form a pentamer corresponding to the major protein capsid, L1 (Sapp et al., 1995).

The genome of HPV is divided into three regions, first is a non-coding Upstream Regulatory Region (URR) or Long Control Region (LCR) that has regulatory role of the transcription of E6 and E7 viral genes; Second is an early region (E), consisting of six ORFs: E1, E2, E4, E5, E6, and E7, which encodes non-structural proteins involved in viral replication and oncogenesis. The third is a late (L) region that encodes the L1 and L2 structural proteins (Fehrmann & Laimins, 2003; Jo & Kim, 2005). The LCR of 400-1,000 base pairs contains cis elements, overlapping binding sites for many different transcriptional activators and repressors, including activating protein 1 (AP1) and
nuclear factor 1 (NF-1). This region contains the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs (Apt et al., 1996).

The E6 and E7 proteins deregulate the host cell growth cycle by binding and inactivating tumor suppressor proteins, cell cyclins, and cyclin-dependent kinases (Syrjanen & Syrjanen, 1999). E1 E2, E4, E5, and E8 are involved in virus DNA replication, transcriptional control, beyond other late functions and L1 and L2 are responsible for the assembly of viral particles (Bodily & Laimins, 2011). The function of the E6 and E7 gene products during a productive HPV infection is to subvert the cell growth-regulatory pathways and modify the cellular environment in order to facilitate viral replication in a cell that is terminally differentiated and has exited the cell cycle (Syrjanen & Syrjanen, 1999).

Figure 2.1: A schematic representation of the HPV genome.
The eight early and two late genes are indicated. Below is a schematic of the HPV long-control region. E2-BS 1-4 is represented by gray boxes. The binding site for the helicase E1 at the origin of replication is shown as a black box. The TATA box and the early promoter P97/P105 are also indicated (Scheffner et al., 1994).

2.4 Epidemiology of HPV infection and cervical disease

HPV is the most common sexually transmitted virus in the world. It causes a substantial disease in both men and women especially in developing countries, where the prevalence of asymptomatic infection varies from 2 to 44%, depending on the population, severity of the injury and geographical region studied (Dunne et al., 2006; De Sanjose et al., 2007). Cervical cancer which is associated with HPV represents the second most common cancer among women in the world (WHO, 2006b; Mutyaba et al., 2007; WHO/ICO, 2010). In 2008, there were about 529,409 new cases and 274,883 deaths due to cervical cancer in women throughout the world. Of the total new cases each year, about 86% of the new cases occur in developing countries, representing 13% of female cancers (WHO/ICO, 2010).

In the United States, the incidence of new HPV infections ranges from 1-5.5 million per year and the burden of disease is estimated to be as high as 20% (Cates, 1999). Of the many genotypes of HPV, about 30 infect the genital tract through sexual contact. Genital HPV types infect primarily the cervix, vagina, vulva, penis and anus (Munoz et al., 2003).

It is estimated that up to 80% of women worldwide will be infected with at least one genotype of HPV at some point in their lives (Syrjänen et al., 1990; Koutsky, 1997). HPV infection rates are highest among young women, usually peaking soon after the age when most young women become sexually active (Smith et al., 2008). However, in men, HPV infection is evident at all ages and the risk of acquiring new HPV infection seems to remain stable over time (Smith et al., 2011). A woman’s lifetime number of sex
partners is the most important predictor of HPV acquisition although HPV is frequently acquired from a first sexual partner (Winer et al., 2008).

2.5 Transmission and clinical manifestations of HPV

2.5.1 Clinical manifestations of HPV

HPV is associated with a variety of clinical conditions ranging from innocuous lesions to cancer (Bonnez & Reichman, 2000). Most HPV infections are benign. Infection of cutaneous epithelium can cause warts such as plantar warts, common warts and flat warts on hands and feet. Warts are areas of hypertrophied skin filled with keratin and are considered to be mainly a cosmetic nuisance however, they resolve spontaneously within 1 to 5 years. Skin warts are transmitted by direct contact with an infected tissue or indirectly by contact with virus-contaminated objects. Strains that target the face make skin cancer more likely. Other strains of HPV that grow primarily in the lining of the mouth produce small elevated nodules that can develop into fatal squamous cell cancers. Focal epithelial hyperplasia of the oral cavity (Heck’s disease) is caused predominantly by HPV-13 and tends to regress spontaneously (Burd, 2003). Flat warts caused by HPV 5 are most commonly found on the arms, face or forehead. Abnormal immune function is associated with the development of a cancer called Epidermodyplasia Verruciformis (EV) (Dubina & Goldenberg, 2009).

EV is a rare chronic inherited disease believed to be associated with HPV types 5 and 8 (Majewski et al., 2002; Lane et al., 2003). It is also associated with warts on the trunk and upper extremities and can develop into invasive squamous cell carcinomas. Conjunctival papillomas and carcinomas associated with HPV have been reported. Recurrent Respiratory Papillomatosis (RRP) is primarily a disease of the larynx in young children but can also occur in adults. This results due to the fact that mothers have a history of HPV and similar HPV types cause anogenital warts which are generally associated with HPV-6 and HPV-11 and do not lead to cancer. Most of these warts are
asymptomatic and may spontaneously regress in 3-4 months, remain the same, or increase in size and number. There is also some suggestion that the disease may be acquired in utero since cases have been documented at birth after caesarean section (Cates, 1999; Burd, 2003).

2.5.2 Transmission of HPV

HPV infections are transmitted mainly through direct skin-to-skin or skin-to-mucosa contact. Though HPV is very resistant to heat and desiccation, nonsexual transmission via fomites can also occur, such as by prolonged exposure to shared contaminated clothing (Roden et al., 1997). High risk HPVs are transmitted mainly through sexual route as this mode of transmission accounts for the majority of cervical HPV infections. However, vertical transmission may occur as HPVs have been detected in virgins, infants and children born to HPV negative women and transmission from father to baby after birth, which suggests that non-sexual parent-to-child transmission of low risk HPVs may occur (Cason et al., 1998; Rintala et al., 2006).

Non-sexual transmission of genital HPV infections through modes such as skin to skin contact, fingers and sex toys has been evaluated and accounts for a very small proportion of HPV infections (Fairley et al., 1996; Coutlée et al., 1997; Winer et al., 2003). However, transmission through this mode is not common (Stevens-Simon et al., 2000).

2.6 Pathogenesis of HPV

2.6.1 HPV lifecycle

HPV replication cycle begins with entry of the virus into the cells of the stratum germinativum (basal layer) of the epithelium. HPV infection of the basal layer requires mild abrasion or microtrauma of the epidermis. Alpha-6 Integrin has been proposed as the epithelial cell receptor for HPV-6 but is not obligatory for attachment of HPV-11 or
HPV-33 (Evander et al., 1997; Joyce et al., 1997; Giroglu et al., 2001). HPV-16 and HPV-33, like many other viruses, attach to host cells via cell surface heparan sulphate (Giroglu et al., 2001). A secondary receptor or stabilizing proteoglycans may also be involved in HPV attachment (Giroglu et al., 2001). Following attachment via L1 and L2, endocytosis has been shown to take many hours. The virus usually enters via clathrin coated pits or caveolar endocytosis depending on the type of papillomavirus (Smith et al., 2007; Hindmarsh & Laimins, 2007). Once inside the host cell, HPV DNA replicates as the basal cells differentiate and progress to the surface of the epithelium. In the basal layers, viral replication is considered to be nonproductive and the virus establishes itself as a low-copy-number episome by using the host DNA replication machinery to synthesize its DNA on average once per cell cycle (Gilbert & Cohen, 1987; Flores & Lambert, 1997). In the differentiated keratinocytes of the suprabasal layers of the epithelium, the virus switches to a rolling-circle mode of DNA replication, amplifies its DNA to high copy number, synthesizes capsid proteins, and causes viral assembly to occur (Flores et al., 1999). The life cycle requires three different modes of viral DNA replication that is ‘‘estimation’’, ‘‘maintenance’’ and ‘‘amplification’’ (McBride, 2008). Expression of the E1 and E2 proteins results in viral replication so that the infected cell contains around 20-50 copies of the episome. After the initial amplification step, the viral genome must be maintained in the dividing basal cells to sustain a persistent infection. The life cycle of HPV is strictly linked to the differentiation program of the host keratinocyte. Infection within the basal layer generates a pool of infected cells. As basal cells divide, a daughter cell is produced that harbours episomal viral DNA. This is a consequence of the E2 protein tethering the viral episome to chromatin during mitosis (Skiadopoulos & McBride, 1998; de Oliveira et al., 2006). Post mitosis, one infected cell remains in the basal layer and the other migrates up through the suprabasal layers. The migrating cell begins a process of terminal differentiation. In an uninfected epithelium, the migrating cells exit the cell cycle. However papillomavirus infected cells remain mitotically active and they can undergo
some differentiation. Amplificational (or vegetative) viral DNA replication permits viral genomes to be replicated to a high copy number destined to be packaged into the capsid to yield progeny virions (McBride, 2008).

Some studies have reported that there is a switch in replication strategies of HPV in differentiated cells and that this partially explains the high copy plasmid number detected (Flores & Lambert, 1997; Rector et al., 2004).

When differentiated cells are withdrawn from the cell cycle, the virus relies on the proteins it encodes to maintain or induce a pseudo-S phase-like state in order to gain access to the cellular replication machinery. The E6 and E7 proteins modulate the cell cycle through associations with the tumour suppressors p53 and retinoblastoma (Rb) proteins respectively (Howley, 2006). The E1^E4 and E5 proteins are also translated in a differentiation dependent manner. It has been shown that E1^E4 inhibits G2 to M phase transition and that this arrest may play a role in creating an environment optimal for viral DNA replication (Davy et al., 2002; Davy et al., 2005). It has also been suggested that E5 contributes to cellular proliferation by cooperating with E7. E5 enhances E7-induced mitogenic response and this contributes to viral DNA synthesis (Bouvard et al., 1994). The L1 and L2 capsid proteins undergo expression within the granular layer of the epithelium (Barksdale & Baker, 1993). Restricting the synthesis of the L1 and L2 antigens to the upper layers of the epithelium may aid viral evasion from the immune response.

The capsid proteins assemble into the icosahedral capsid via assistance from chaperone proteins such as heat shock protein 70 (Hsp70) (Chromy et al., 2003; Buck et al., 2005; Chromy et al., 2006). Karyopherins may play a role in papillomavirus capsid assembly (Bird et al., 2008). It is unknown whether encapsidation of the viral episome takes place during or after capsid formation. The E1^E4 protein then interacts with cellular keratin
networks to assist viral egression. The nature of the epithelium allows the virion to spread through the shedding of outer most cell layers.

2.6.2 Molecular biology of High risk HPV

Cervical cancer is one of the examples that can be used to explain how viral infection can lead to malignancy or neoplasia (Figure 2.2). After infection of cervical epithelial cell, circular DNA is initially located extrachromosomally in the nucleus in benign lesions caused by HPV. LR types including, 6 and 11 remain exclusively in this episomal form. This also applies to high risk types in CIN (1) and frequently in CIN (2) and (3) however, in high-grade intraepithelial neoplasias/cervical carcinoma cell lines and invasive cancers; viral DNA is usually integrated into the host cellular genome. The product of the E1 gene is a helicase necessary for viral DNA replication. Integration of HPV-DNA disrupts or deletes the E2 region, which results in loss of its expression (Yoshinouchi et al., 1999). This interferes with the function of E2, which is normally tasked with down-regulation of the transcription of the E6 and E7 genes and DNA replication, and leads to an increased expression of E6 and E7 genes. The function of the E6 and E7 products during a productive HPV infection is to subvert the cell growth-regulatory pathways and modify the cellular environment in order to facilitate viral replication (Syrjanen & Syrjanen, 1999). The E6 and E7 gene products deregulate the host cell growth cycle by binding and inactivating two tumour suppressor proteins: the tumour suppressor protein (p53) and the retinoblastoma gene product (pRb). The HPV E6 gene product binds to p53 and targets it for rapid degradation (Thomas et al., 1999). As a consequence, the normal activities of p53 which govern G1 arrest, apoptosis, and DNA repair are abrogated. LR-HPV E6 proteins do not bind p53 at detectable levels and have no effect on p53 stability in vitro. The HPV E7 gene product binds to pRb protein resulting in the disruption of the complex between pRb protein and the cellular transcription factor E2F-1, resulting in the liberation of E2F-1, which allows the transcription of genes whose products, is required for the cell to enter the S phase of the
cell cycle (Flores et al., 1999). The E7 gene product can also associate with other mitotically interactive cellular proteins such as cyclin E (Syrjanen & Syrjanen, 1999). The outcome is stimulation of cellular DNA synthesis and cell proliferation. The E7 protein from low-risk HPV types binds pRb with decreased affinity. Next, the E5 gene product which has the transforming properties induces an increase in mitogen-activated protein kinase activity, thereby enhancing cellular responses to growth and differentiation factors. This results in continuous proliferation and delayed differentiation of the host cell (Syrjanen & Syrjanen, 1999).

The inactivation of p53 and pRb proteins can give rise to an increased proliferation rate and genomic instability and as a result the host cell accumulates more and more damage DNA that cannot be repaired, leading to transformed cancerous cells (Park et al., 1995). In addition to the effects of activated oncogenes and chromosome instability, potential mechanisms contributing to transformation include methylation of viral and cellular DNA, telomerase activation, and hormonal and immunogenetic factors (Park et al., 1995; Hegde, 2002).
2.7 Methods for the detection of HPV virus and infection

The diagnosis of HPV can be inferred from morphological, serological, and clinical findings. For instance, productive infections, such as warts, virus particles about 50 nm in diameter can be detected by electron microscopy and by immune detection of the virus capsid late proteins (L1 and L2) (Villa & Denny, 2006). Immunological detection of HPV in human cells or tissues has been hindered by 3 challenges: the late (L1,L2), capsid proteins are only expressed in productive infections; the early proteins are often expressed in low amounts in infected tissues; and there is a lack of sensitive and specific antibodies of high quality against the viral proteins (Villa & Denny, 2006). In addition, HPV cannot be grown in conventional cell cultures, and serological assays have limited accuracy (Dillner, 1999). As infection with HPV is followed by a humoral immune response against the major capsid protein (Dillner, 1999), with antibodies remaining
detectable for many years, serology is not suitable for distinguishing present and past infections. Consequently, accurate diagnosis of HPV infection relies on the detection of viral nucleic acid (DNA and/mRNA) detection.

2.7.1 Molecular diagnostic techniques

Several genotyping methods have been developed to identify HR-HPV in Liquid Based Cytology (LBC) samples and tissue samples (Sherman et al., 1997; Depuydt et al., 2003; Arbyn et al., 2004). Molecular techniques applied for detection of HPV DNA (Hubbard, 2003) include a direct probe method using Southern blotting, Northern blotting and \textit{In situ} hybridization (ISH), signal amplification methods such as the Hybrid Capture II assay (HCII) (Lorincz, 1996), and target amplification methods by PCR (Burd, 2003). The only procedure that may be capable of recognizing all HPV types and variants present in a biologic specimen is DNA sequencing of the viral genome, either after cloning into plasmids or by direct sequencing of a PCR fragment. However, the methodology is presently labour intensive and requires expensive equipment (Villa & Denny, 2006).

2.7.1.1 Hybrid Capture II

The Hybrid Capture II system (hc2, Digene Corp., USA) is a non-radioactive signal amplification method based on the hybridization of the target HPV-DNA to the labelled RNA probes in solution (Lorincz, 1996; Bozzetti et al., 2000). The resulting RNA-DNA hybrids are captured onto microtitre wells and detected by a specific labelled monoclonal antibody and a chemiluminescent substrate, providing a semi-quantitative measurement of HPV-DNA. Two different probe cocktails are used, one comprising probes for five low-risk genotypes 6, 11, 42, 43 and 44 and the other containing probes for 13 high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. This assay has become the standard in many countries therefore; it is widely used in many clinical studies and has FDA approval. However, hc2 has some limitations as it only
distinguishes between the high-risk and low-risk groups but does not permit identification of specific HPV genotypes. The detection limit of approximately 5000 genome equivalents makes it less sensitive than PCR (Smits et al., 1995; Cope et al., 1997) and cross-reactivity of the two probe cocktails (Castle et al., 2002; Poljak et al., 2002) can reduce the clinical relevance of a positive result. Nevertheless, hc2 has been shown to be robust and reproducible screening assay in the world (Castle et al., 2004a). Trials of the automated third generation Hybrid Capture assay were recently introduced but it is based on biotin-labelled oligonucleotide sequences for capture of target regions, in order to effectively reduce the cross-reactivity (Castle et al., 2004b).

2.7.1.2 Direct hybridization assays

For HPV genome analysis, hybridization in solid phase, such as Southern blot for DNA molecules and Northern blot for RNA molecules, are excellent procedures that can generate information of quality, but are time consuming and require large amounts of highly purified nucleic acids. In addition, hybridization in solid phase requires well-preserved, ideally full-size molecules, and therefore cannot be done with any biologic specimen particularly those derived from fixed tissues in which DNA degradation is often observed. The procedure is also technically cumbersome and time consuming, and therefore not amenable for large-scale population studies. Therefore, nucleic acid amplification methods have been developed to increase the sensitivity as well as the specificity of HPV DNA detection (Nuovo et al., 1992).

Hybridization is based on the use of labelled probes that specifically hybridize to intracellular HPV DNA. Although the sensitivity of this method is limited, it permits localization of HPV infection in the sample and possible co-localization with other markers. Identification of HPV genotypes would require the use of type-specific probes in multiple in ISH experiments ((Sato et al., 1997). ISH has been used to detect
messenger RNA (mRNA) as a marker of gene expression where levels of viral proteins are low (Stoler et al., 1997).

2.7.1.3 Polymerase Chain Reaction (PCR)

The sensitivity and specificity of PCR-based methods can vary, depending mainly on the target gene, the spectrum of HPV types amplified and ability to detect multiple types. Consensus or general PCR primers have been designed to amplify a wide spectrum of HPV genotypes by targeting a well conserved region of either L1 (Hildesheim et al., 1994) or E1 (Tieben et al., 1993) within the HPV genome. Several consensus PCR primer sets have been developed to amplify these regions: GP5 (TTTGTACTGTGGTAGATAC) and GP6 (GAAAAATAAACTGTAAATCA) and its modified version of GP5+ (5- GAA AAATAA ACT GTA AAT CAT ATT C -3) and GP6+ (5- TTT GTT ACT GTG GTA GAT ACT AC -3) PCR system (de Roda Husman et al., 1995; Jacobs et al., 1999) uses a single pair of consensus primers to amplify a 150 bp region in the L1 gene allowing the identification of 30 HPV genotypes. This method, characterized by high sensitivity and specificity, is useful in predicting high-grade CIN (Jacobs et al., 1999) in spite of documented inter-laboratory variation of results (Elfgren et al., 2005); The MY09 (5- CGT CCA AAA GGA AACTGA TC -3) and MY11 (5- GCA CAG GGA CAT AAC AAT GG -3) pair of degenerate primers (Hildesheim et al., 1994) identifies high-risk HPV genotypes by amplifying a 450 bp sequence in the conserved L1 region. The disadvantage of this design is that synthesis of degenerate oligonucleotides is not highly reproducible and can result in a high batch-to-batch variation. Improved primer design with the development of 2 sets of non-degenerated PGMY09/11 primers has led to improved consistency and better sensitivity for a large number of HPV genotypes (Gravitt et al., 2000). However, when compared to other methods for HPV DNA detection, MY09 (5- CGT CCA AAA GGA AACTGA TC -3) and MY11 (5- GCA CAG GGA CAT AAC AAT GG -3) primer system has been shown to generate a large number of discrepancies (Peyton et al., 1998) as well as more
multiple HPV positivities (Kornegay et al., 2001); Short PCR fragment (SPF10) system primers amplify a 65 bp sequence from a highly conserved region of the viral L1 gene (Kleter et al., 1998). It is designed to discriminate a broad spectrum of HPV types in an Enzyme Linked Immunosorbent Assay (ELISA) format or reverse line blot hybridization (line probe assay). The use of a defined mixture of non-degenerated primers has the advantage that the oligonucleotides can be synthesized with high reproducibility and PCR is performed at optimal annealing temperatures and it is useable for less preserved specimens. The short amplicon allows the test to achieve a high analytical sensitivity, but has the disadvantage of lacking in HPV type discrimination (Ifnner & Villa, 2003).

2.8 Risk factors for cervical cancer and HPV

HPV infections of specific pathological strains have been shown to act as initiators of cervical cancer. It is also influenced by many environmental and genetic factors, age of first sexual debut, high parity, contraceptives, health care and nutrition/diet, tobacco smoking and also co-infection by other sexually transmitted agents such as Chlamydia Trachomatis and Herpes Simplex Virus type 2 (Mortazavi et al., 2002; Castellsagué, 2008).

2.8.1 Diet and nutrition

Folate deficiency has been associated with increased susceptibility to HPV infection and risk of invasive cervical cancer (ICC) (Gostout et al., 2003). Another study found out that circulating vitamin B12 levels were inversely associated with HPV persistence (Weinstein et al., 2001). Increased intake of carotenoids, total vitamin A and retinol have been associated with a lower risk of developing precancerous and cancerous lesions (French et al., 2000, Yeo et al., 2000). Vegetable consumption and circulating cis-lycopene may be protective against HPV persistence (Sheriff et al., 2001).
2.8.2 Age and age of sexual debut

Most cervical cancers arise at the squamo-columnar junction between the columnar epithelium of the endocervix and the squamous epithelium of the ectocervix. At this site, there are continuous metaplastic changes. The greatest risk of HPV infection coincides with greatest metaplastic activity. Greatest metaplastic activity occurs at puberty and first pregnancy and declines after menopause (Burk et al., 1996). Increasing age is linked to decreasing acquisition of anogenital HPV infection as a corollary of fewer new partners and, possibly, immunity to previously cleared infections (Burchell et al., 2006; Dunne et al., 2006). However, cervical cancer is more common in women older than 35 years, suggesting infection at a younger age and slow progression to cancer. Persistence of infection is more common with the high-risk oncogenic HPV types and is an important determinant in the development of cervical cancer (Burk et al., 1996).

2.8.3 Sexual activity

The type and pattern of sexual activity, especially in teenagers, is a major factor in determining whether a person becomes infected with HPV. As a result of relaxed attitudes about sexuality among adolescents in many cultures, the number of sexual partners that teenagers have before the age of 20 can be many, and each of their partners also may have had multiple sexual partners. As a consequence, this pattern of sexual activity increases their risk of exposure to Sexually Transmitted Infections; especially HPV. Condom usage may not adequately protect individuals from exposure to HPV since HPV can be transmitted by contact with infected labial, scrotal, or anal tissues that are not protected by a condom (Burd, 2003).

2.8.4 Immune suppression

The primary immune response to HPV infection is cell mediated; therefore, conditions that impair cell-mediated immunity such as renal transplantation or Human
Immunodeficiency Virus (HIV) disease increase the risk of acquisition and progression of HPV (Cubie et al., 2000; Torrisi et al., 2000; Calore et al., 2001).

Suppression of the immune system due to HIV infection is also a significant risk factor because it makes the cells lining the lower genital tract (vulva, vagina and cervix) more easily infected by the cancer-inducing types of HPV (Stentella et al., 1997). Other conditions that cause immunosuppression include those requiring chronic corticosteroid treatments, such as asthma or lupus (McDonald, 1999). Women also increase their risk for Cervical Intraepithelial Neoplasia (CIN) by engaging in other behaviours such as use of recreational drugs, drinking alcohol and smoking cigarettes that are known to suppress the immune system. The latter is particularly important because while a decrease in smoking among men has occurred, the number of women who smoke has increased dramatically in recent years especially in teenage girls (McDonald, 1999).

Clinical studies involving HIV positive women have consistently demonstrated a lot more prevalent and incident infections of any HPV type compared to high-risk HIV negative women (de Vuyst et al., 2003). Prospective cohort studies have also consistently reported an increased incidence of Squamous Intraepithelial Lesion (SIL) in HIV positive compared to HIV-negative women (Hawes et al., 2003).

Some studies have suggested that HIV infection is the strongest risk factor for cervical cancer independent of the usual demographic and behavioural risk factors (Wright et al., 1994; Franco et al., 2001). There are several mechanisms of how HIV could interact with HPV infections:

First, a substantial proportion of cervical HPV DNA detected in HIV positive women might reflect reactivation of previously acquired quiescent infections, rather than recent sexual transmission (Strickler et al., 2005). Secondly, HIV may have a direct viral to viral interaction with HPV, given that both viruses infect macrophages (Clarde & Chetty, 2002). Thirdly, in vitro studies have indicated that expression of HIV tat protein
may increase the expression of HPV E1 and L1 viral genes (Dolei et al., 1999) and HPV-16 E7 transcription (Vernon et al., 1993).

### 2.8.5 Co-infection with other STI’s and viruses

Sexually transmitted viruses may serve as cofactors in the development of cervical cancer. It has been postulated that coinfection with HSV type 2 may play a role in the initiation of cervical cancer (Zur Hausen, 1982).

Co-infection offers the opportunity for these viruses to interact with HPV. Putative oncogenes and transforming factors have been proposed for CMV and HHV-6, but epidemiologic and in vitro data are not conclusive of a causal association with cervical cancer (Razzaque et al., 1993; Shen et al., 1993; Chen et al., 1994; Romano et al., 1995). Recent studies using PCR to detect CMV, HHV-6, and HHV-7 in women with abnormal cervical cytologic test results indicate that these viruses are only bystanders rather than cofactors in the development of cervical cancer (Chan et al., 2001).

Cervical infections with other STIs such as Chlamydia Trachomatis and HSV 2 may increase susceptibility to HPV infection by cervical inflammation or micro abrasions in the epithelium resulting from sexual intercourse which allow HPV direct access to basal epithelial cells (Moscicki et al., 2001). It is also possible that STIs could enhance oncogenic effect of an already established HPV infection by influencing local immune response (Schmauz et al., 1989).

Coinfection with adeno-associated virus is associated with a significantly reduced risk of cervical neoplasia (Coker et al., 2001). Adeno-associated virus replication gene product Rep78 disrupts the transcription of the HPV-16 E6 and E7 oncogenes by interfering with the binding of the TATA binding protein to the p97 core promoter in the LCR region of the HPV genome (Su et al., 2000). This interference does not require HPV gene products.
2.8.6 Hormonal contraception and parity

Women at the highest risk of developing cervical cancer are those who have had seven or more full term pregnancies (Kim et al., 2000). In an IARC multicentre case control study that compared women with nulliparous women, the ODDS ratio for developing cervical cancer was 2.31 for one (1) or two (2) pregnancies, 3.73 for three (3) or four (4) pregnancies, 5.0 for five (5) or six (6) pregnancies and 8.29 for seven (7) or more pregnancies (Kim et al., 2000).

The LCR of HPV contains sequences similar to the glucocorticoid responsive elements that are inducible by steroid hormones such as progesterone (the active component of oral contraceptives) and dexamethasone. Long-term use of oral contraceptives has been associated with the development of high-grade cervical cancer (Brisson et al., 1994; Adam et al., 2000). The use of oral contraceptives is associated with an increased incidence of cervical electropion, which means that the squamo-columnar junction, the site where HPV infection preferentially induces neoplastic lesions is more exposed to HPV oncogenes E6 and E7 (de Villiers, 2003). Estrogens and progesterone may also lead to cervical cells directly increasing their proliferation and thus stimulating the transcription of HPVs. However, most studies have not reported an association between the acquisition of HPV infections and the use of oral contraceptives independent of sexual behaviour (Green et al., 2003).

2.8.7 Cigarette smoking

It is not clear whether cigarette smoking independently influences susceptibility and/or infectivity of HPV, although tobacco-related carcinogens of cigarette smoking may directly damage the host DNA that include tumour suppressor genes resulting in the development of cervical cancer (Haverkos et al., 2003). In studies that have found a positive association between smoking and the acquisition of HPV infections, a positive association was attenuated after controlling for sexual behaviour (Koutsky & Kiviat,
1999; Winer & Koutsky, 2004). Only one prospective study found a significant positive association between current cigarette smoking and incident HPV infections even after controlling for sexual behaviour (Winer et al., 2003). While smoking could truly increase susceptibility to HPV infection, it may be just a proxy measure to unmeasured sexual behaviours (Phillips & Smith, 1994). Nicotine and the by-products of smoking are thought to increase a woman’s relative risk for cervical cancer because they concentrate in the cervical mucus and decrease the immune capability of langerhan’s cells to protect cervical tissue from invading oncogenic factors, such as HPV infection.

Local immune suppression induced by smoking and the mutagenic activity of cigarette components have been demonstrated in cervical cells and may contribute to persistence of HPV or to malignant transformation similar to that seen in the lung (Phillips & Nishe, 1993; Yang et al., 1996; Villa, 1997). It appears that smoking is the most important risk factor independent of HPV infection for higher grades of cervical disease (Adams et al., 2000). Smoking shows little or no relationship to low grades of cervical disease.

2.8.8 Genetic predisposition

Another risk factor for the development of cervical cancer is having a blood relative (mother or sister) with cervical cancer. Magnusson et al. (1999) compared the incidence of dysplasia and CINS in relatives of women with disease and in age matched controls.

Genetic predisposition was found to be even a greater component in cervical cancer (Magnusson et al., 1999). Genetic heritability was found to account for 27% of the effect of underlying factors for tumour development. Heritability could affect many factors contributing to the development of cervical cancer, including susceptibility to HPV infection, ability to clear HPV infection, and time to development of disease. The effect of shared familial environment was shown to be small at 2% and was found only between sisters and not between mother and daughter.
2.8.9 Male circumcision

For a long time, male circumcision was associated with the prevention of common sexually transmitted diseases (STDs) (Cook et al., 1994). A pooled analysis of the IARC data confirmed that circumcised men not only had a substantially lower risk of penile HPV infections than uncircumcised men, but also that their partners had a lower risk of HPV infections and a lower risk of developing cervical cancer (WideroffSchiffman et al., 1999).

The protective effect was more pronounced among women whose male partners engaged in high-risk sexual behaviour. Further evidence of the protective effect of male circumcision comes from two recent randomized controlled trials. One trial showed that circumcision of adolescent boys and men in a rural Ugandan population reduced the prevalence of HPV infection by 35% (Tobian et al., 2009).

Another trial conducted in South Africa demonstrated a significant reduction in the prevalence of urethral HR-HPV infection after male circumcision (Auvert et al., 2009). The reduction of HPV infection by means of circumcision may involve anatomical factors, cellular factors, or both. First, the retraction of the foreskin over the penile shaft during intercourse exposes the inner preputial mucosa to vaginal and cervical fluids through micro tears resulting from sexual intercourse particularly those that occur in the frenulum. The micro tears probably facilitate the access of HPV to the basal epithelium (Szabo & Short, 2000). Secondly, in uncircumcised men, the inner mucosa of the foreskin is lightly keratinized, which may facilitate the access of HPV to underlying basal epithelial cells. Thirdly, in circumcised men, keratinization of the surgical scar probably deters HPV infection from accessing the basal epithelia (Szabo & Short, 2000).
2.9 Prevention of HPV and treatment of cervical cancer

2.9.1 HPV Vaccine

The two available HPV vaccines both contain Virus-Like Particles (VLPs) made using recombinant vaccine technology. HPV vaccines are prophylactic meant to prevent initial HPV infection. They are therefore not therapeutic to clear an existing HPV infection. When given as a 3-dose series, HPV vaccines elicit antibody titres many times higher than those observed in natural infection (Villa et al., 2005; Harper et al., 2006; USA FDA, 2006). So far two vaccines have been licensed in Kenya: Cervarix® (GlaxoSmithKline) is a bivalent VLP HPV vaccine against types 16 and 18 is effective for more than four years and Gardasil® (bioCSL/Merck & Co Inc.) is a quadrivalent VLP HPV vaccine against types 16, 18, 6 and 11 is effective for 7-8 years (Cornelis et al., 2003; Mcneil, 2006; Harper, 2009). Both vaccines have demonstrated not only safety and immunogenicity but also more than 90% efficacy for the prevention of cervical neoplastic lesions as well as vaginal, vulval and genital warts for Gardasil (Castellsagué, 2008). HPV vaccines are targeted at girls and women of age 9-26 years because the vaccine only works if given before infection occurs (Cornelis et al., 2003; Mcneil, 2006; Harper, 2009). The risks of HPV vaccine are minimal and similar to other vaccines. The most common reported side effects are: redness and soreness where the shot is given, fever, and headaches.

The quadrivalent vaccine has also been approved by the FDA for males 9-26 years of age for the prevention of genital warts (caused by HPV 6 and 11) and in both males and females 9-26 years of age for the prevention of precancerous anal lesions and anal cancer associated with the vaccine HPV types. However, in many countries the debate continues as to whether or not to offer the vaccine to boys in the framework of government-funded national immunization programs as is done for girls (Villa, 2011).
2.9.2 Treatment of cervical cancer

It entails accurate diagnosis of pre-cancer or cancer cells followed by appropriate treatment and follow up of the patient, and effective rehabilitation and palliative care for advanced cancer patients (WHO, 2006). Alliance for Cervical Cancer Prevention (ACCP) (2007) found either HPV DNA testing or VIA, followed by cryotherapy to treat pre-cancerous lesions during the same visit, to be the most efficient and effective strategy for the secondary prevention of the cancer in LMICs. Cryotherapy has been found by them to be a highly safe procedure with high cure rates. It has also been found to protect HPV positive women from the future development of cervical cancer.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

The study was a laboratory based cross-sectional survey.

3.2 Study site

The study was conducted at Special Treatment Centre (STC) in Nairobi County. STC is located off River Road behind Kampala Coach Bus terminus right in the Central Business District (CBD) of the City. The facility has been known to be a referral Hospital for STIs and it approximately serves less than 400 commercial sex workers (CSWs) and other vulnerable subjects per day. The clinic offers a range of STI care and treatment; provides free condoms, Voluntary Counselling and Testing unit (VCT), cervical screening, family planning and other related services.

3.3 Study populations

The study population comprised of all men and women attending Special Treatment Centre (STC) in Nairobi County.

3.3.1 Inclusion criteria

- Men and women aged between 18-49 years.
- Men and women who consented to the study.

3.3.2 Exclusion criteria

- Men and women below the age of 18 and those above 49.
- Men and women who failed to consent to the study.
3.4 Sampling

3.4.1 Sample size determination

The minimum sample size for the study was calculated using the formula developed by Cochran, (1977). The proportion of women who are at risk of acquiring HPV in Kenya is 17% according to Yamada et al., 2008. Therefore, this value was taken to be the assumed prevalence of HPV.

\[ N = \frac{Z^2P(1-P)}{d^2} \]

Where;

\( N \) = minimum sample size required

\( Z = 1.96 \) standard error.

\( P = \) assumed prevalence (17% Yamada et al., 2008).

\( q = 1 - P \)

\( d = \) degree of accuracy desired, 0.05(95% Confidence Interval)

\[ N = 1.96^2 \times 0.17(1-0.17) \div 0.05^2 = 217 \text{ samples.} \]

3.4.2 Sampling procedure

The participants were sampled using simple random sampling. Potential respondents were approached individually in the facility and those willing to participate were invited for an interview. Written informed consents (Appendix I) were obtained to make sure that they understood everything about the study by a trained interviewer and
demographic information and factors associated with HPV infection was collected using a structured questionnaire (Appendix II).

3.5 Methodology

3.5.1 Questionnaire administration

A structured questionnaire (Appendix II) was translated into Swahili language. Structured questionnaires were distributed among the participants where information about demographic characteristics and factors such as smoking habits, alcohol uptake, contraceptive usage and parity; sexually transmitted diseases history were collected using a structured questionnaire by trained health care providers. The interviews were anonymous for confidentiality assurance to the participants and the participants had freedom to withdraw from the study at any convenient time.

3.5.2 Collection of urine samples

After completing the questionnaires (Appendix II), the participants were advised to provide approximately 10ml mid-stream self-collected urine samples. The samples were collected in 15 ml sterile falcon tubes containing 0.8 ml boric acid to prevent the growth of microorganisms such as Chlamydia Trachomatis. Labelling of the collected urine samples were matched with respective questionnaires to avoid mix up then transported to Kenya Medical Research Institute (KEMRI) in a cold chain every day and stored at -20°C before processing. All the questionnaires and samples were coded for anonymity purposes.

3.5.3 Extraction of HPV DNA

Each urine sample was subjected to centrifugation 5000 Revolutions per Minute (RPM) for 10 minutes and the pellet was suspended in 0.5 ml Phosphate Buffered Saline (PBS).
HPV DNA was extracted as outlined in the Qiagen QIAamp DNA mini kit and QIAamp DNA blood mini kit Handbook (2012) (Qiagen, Valencia, CA, USA). Briefly, 200 µL aliquots of urine sample was digested using 20µL proteinase K followed by 200µL lysis buffer then incubated at 56°C for 15 minutes finally DNA was eluted with 50 µL RNase free water. Extracted DNA was stored at -20°C until use.

3.5.4 Human Papillomavirus DNA amplification

To increase sensitivity of HPV detection, nested PCR was performed using MY09 (5'-CGT CCA AAA GGA AACTGA TC -3') and MY11 (5'- GCA CAG GGA CAT AAC AAT GG -3') as outer and GP5+ (5'- GAA AAATAA ACT GTA AAT CAT ATT C -3') and GP6+ (5'- TTT GTT ACT GTG GTA GAT ACT AC -3') as inner primers. For first PCR, each 25 µL reaction contained 1× PCR standard buffer; 3.5mM MgCl2; 200 µmol/L dNTPS; 0.025 units Standard Taq polymerase (New England Biolabs); 500nM MY09; 500nM MY11 and 5 µL of the template. For second PCR, 5 µL of the MY09/11 product was used as a template for the nested PCR amplification with GP5+/GP6+ primers. Each 25 µL reaction contained 1 × PCR standard buffer; 3.5mM MgCl2; 200 µmol/L dNTPS; 0.025 units Standard Taq polymerase; 500nM GP5+; 500nM GP6+ and 5 µL of the MY09-MY11 product was used as a template. For every PCR reaction, a negative control (H2O) and a positive control (sample that amplified before) was always ran to control for possible contamination and accuracy. The samples were amplified using ABI Gene Amp PCR System 9700. The following amplification profile was used for primary PCR: 95°C initial activation for 30 seconds, 95°C denaturation for 30 seconds, 48°C annealing for 30 seconds, and 72°C extension for 1 min for 40 cycles; followed by a 10 minutes final extension at 72°C; and a hold step at 4°C while for secondary PCR: 95°C initial activation for 30 seconds, 95°C denaturation for 30 seconds, 43°C annealing for 30 seconds, and 72°C extension for 1 min for 40 cycles; followed by a 10 minutes final extension at 72°C; and a hold step at 4°C. After PCR, all specimens were analyzed for HPV positivity using gel electrophoresis. Briefly, 5 µL
PCR product was mixed up and down with 2 µL 2×DNA loading dye and loaded onto a 2% agarose gel and ran in 1×TAE buffer at 100 V for 45 min at room temperature until tracking dye had moved approximately 6cm. DNA bands was visualized by ultraviolet (UV) trans-illuminator, and visualization of approximately 150 bp product was considered positive for HPV DNA in the sample collected.

3.5.5 HPV DNA Purification

All PCR products that had amplified for HPV DNA were purified using QIAquick Purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Briefly, 5 volumes of buffer PB was added to 1 volume of PCR reaction and mixed. Followed by the addition of 10 microlitres of 3M Sodium acetate (PH 5.0) and mixed. In addition, washing was done by adding 750 µl buffer PE to the QIAquick column and then centrifuged for 30 seconds. Finally DNA was eluted by adding 50 microlitres of Rnase free water PH (7.0) and the column centrifuged at 13000 RPM for 1 minute.

3.5.6 Sequencing and Phylogenetic analysis

Purified PCR products (20 µl) were transferred from 1.5 ml micro-centrifuge tubes into sterile PCR reaction tubes where they were sealed and shipped together with 20 µl of HPV sequencing primers (GP5+/GP6+). Big dye terminator method of sequencing was used and it was outsourced at Macrogen Netherlands in Europe. Briefly, 1 µl of the nested PCR products was transferred from the PCR tube with a calibrated glass rod into a reaction mixture containing 1µl of HPV sequencing primers, 1 µl of Big Dye Terminator sequencing standard kit, 3.5µl 5X buffer and 14.5µl of Rnase free water to make a total volume of 20 µl. This reaction mixture was subjected to 20 enzymatic primer extension/termination reaction cycles according to the protocol supplied by the manufacturer.
Raw nucleotide sequences were edited using BioEdit sequence alignment editor software version 5.0.9. The alignment was also edited using SEAVIEW software. Haplotypes or clusters were then deduced for phylogenetic analysis. Newly sequenced (query sequences) were aligned along with reference sequences that were obtained through BLAST analysis at NCBI [http://blast.ncbi.nlm.gov/blast]. The accession numbers for the reference HPV nucleotide sequences are as follows: HPV-85 [GenBank:AF131950.1]; HPV-54 [GenBank:KF436895.1]; HPV-16 African isolate (Mali) [GenBank:JN617905.1]; HPV-83 Europe isolate (Cyprus)[GenBank:AJ617544.1]; HPV-11 Asia isolate (Iran) [GenBank:GU344763.1]; HPV-58 [GenBank:JN383597.1]; HPV-70 African isolate (Congo) [GenBank:JN617895.1]; HPV-6 African isolate (Congo) [GenBank:JN617897.1]; HPV-66 African isolate (Congo)[GenBank:JN617899.1] and HPV-81 African isolate (Congo)[GenBank:JN617887.1]. The file was exported to MEGA 6 software where alignment of the sequences was done using CLUSTALW. The sequences were joined using Neighbour Joining Software and the phylogenetic tree was constructed using MEGA 6 and viewed using TREE view Software. Bootstrap values were runned at 1000 replications and branches with bootstrap replicates less than 50% were collapsed.

3.6 Data management

Data from structured questionnaires and molecular work were coded and entered into Excel spread sheet as they came from the field everyday by the principal investigator. A back up of these data was done regularly to avoid any loss using flash disks and compact disks. Data cleaning and validation were performed to achieve a clean set that was then exported as a Statistical Package for Social Sciences (SPSS) file. The records were locked up in cabinet and could only be accessed by the Principal Investigator since the respondents were assured that information provided would be kept confidential.
3.7 Data analysis

Analyses were performed using SPSS version 19.0. Descriptive statistics were used in analysis to give proportions and frequencies. Bivariate analysis, chi square ($\chi^2$) test was used to determine the association between HPV infection and socio demographic characteristics and risk factors associated with HPV and p value ≤0.05 was considered significant.

3.8 Ethical considerations

Prior to the commencement of the study, approval was granted by Kenya Medical Research Institute National Review Board (SSC NO. 2442) and Nairobi County Department of Health Services. The purpose of the protocol was explained to the participants and each one of them was free to ask questions about the study to make sure that they understand everything prior to the study. This was then followed by the signing of written informed consents by the respondents. Moreover, the participants were informed that the study was not for profit or commercial purposes and they had the option of withdrawing from the study at any time without victimization. Unique codes were assigned to the questionnaires and urine samples for anonymity and the records were kept under lock and key in the cabinet and could only be accessed by the principal investigator for confidentiality. Lastly, the participants were promised that they would get their results after the study was over. The results were handed over to the management of STC and patients who tested positive for HPV DNA were advised to go for further tests at Kenyatta National Hospital or any other health facility.
CHAPTER FOUR

RESULTS

4.1 Socio-demographic and behavioural characteristics of the respondents

A total of 222 adults aged 18-49 years with a Mean age of 32±SD=1.2 years were recruited. Forty five (20.3%) were males (mean age 34±SD=2.73 years) while 177 (79.7%) were females (mean age 31±SD=1.33). Slightly less than half of males, 33.3% (15/45) were aged 40-49 years while 33.3% (59/177) females were aged 30-39 years. More than half of both males 66.7% (30/45) and females 54.2% (96/177) were married. Majority of males 48.9% (n=22) and females 55.4% (n=98) were childless. Most males 95.6% (43/45) and females 98.3% (174/177) were Christians. Majority of females 33.6% (56/177) had tertiary education. Less than half 33.3% (15/45) of males were formal employees while most females were self-employed, 40.7% (72/177). Slightly half 82.2% (37/45) of males lived in permanent houses whereas more than half of females 71.2% (126/177) also lived in permanent houses (Table 4.1).

Most males 100% (n=45) and females 98.3% (n=174) were sexually active. Slightly half of males, 48.9% (n=22) had sex before18 years while majority of females 56.5% (n=100) were aged 19-24 years. Eighty six point seven percent (n=39) and 73.4% (n=130) males and females had two or more sex partners respectively. Most males 35.6% (n=16) sometimes used condom during sex while most females 44.1% (n=78) never used condom during coitus. More than half males and females reported to have not used contraceptives at 95.6% (n=43) and 58.2% (n=103) respectively. Most males 82.2% (n=37) and females 98.3% (n=174) were not smoking. However, a large proportion 55.6% (n=25) of males were drinking alcohol while most females, 83.6% (n=148) were not taking alcohol (Table 4.2).
Table 4.1: Socio-demographic characteristics of the respondents

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<th>Male Percent (%)</th>
<th>Female Frequency (n=177)</th>
<th>Female Percent (%)</th>
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Table 4.2: Behavioural characteristics of the respondents

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<td>Frequency</td>
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<td>83.6</td>
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4.3 Association between HPV and socio-demographic characteristics of the respondents.

Majority, 40% (4/10) of males who tested positive for HPV DNA were aged 40-49 years while 37.9% (22/58) of females were in the 30-39 years age bracket. Most males 70% (7/10) were married, whereas majority of females who were single and married were the same (each 46.6% (27/58). Nearly all males and females who tested positive for HPV 90% (9/10) and 98.3% (57/58) were Christians respectively. Majority of males who had tertiary and secondary education stood at 40% (4/10) each whereas females who had secondary education formed the highest proportion at 39.7% (23/58). Most males and females were self-employed, 50% (5/10) and 37.9% (22/58) respectively. More than half of males 80% (8/10) and females 67.2% (39/58) lived in permanent houses. There was no association between HPV infection and age (P=0.807), marital status (P=0.22), religion (P=0.983), level of education (P=0.127), occupation (P=0.463) and housing standard (P=0.720) (Table 4.3).
Table 4.3: Association between HPV and socio-demographics of the respondents

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<tr>
<td>Agric. Labourer</td>
<td>1</td>
<td>10</td>
<td>2.9</td>
</tr>
<tr>
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<td>1</td>
<td>10</td>
<td>14.0</td>
</tr>
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<td>Students</td>
<td>1</td>
<td>10</td>
<td>8.6</td>
</tr>
<tr>
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<td>0</td>
<td>2.9</td>
</tr>
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<td>50</td>
<td>28.6</td>
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<td>Housing</td>
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<tr>
<td>Temporary</td>
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<td>0</td>
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<tr>
<td>Semi-permanent</td>
<td>2</td>
<td>20</td>
<td>8.6</td>
</tr>
<tr>
<td>Permanent</td>
<td>8</td>
<td>80</td>
<td>82.9</td>
</tr>
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</table>
4.4 HPV DNA

Of the 222 samples collected, 68 (30.6%) were positive for HPV DNA by PCR. Ten out of 45 (22.2%) and 58/177 (32.8%) were positive for HPV DNA among males and females respectively. Gel image of approximately 150bp product was sufficient for evidence of HPV DNA in the sample (Figure 4.1). Lanes 2, 4, 7, 15 and 16 shows PCR products that were positive for HPV DNA while lanes 3, 5, 6, 8, 10, 11, 12, 13, 14 and 17 were negative for HPV DNA. Lane 1 shows the molecular marker at 150bp.

![Gel electrophoresis image](image)

Figure 4.1: HPV DNA amplification with GP5+/GP6+ primers from urine samples collected from men and women attending Special Treatment Centre in Nairobi County.

4.5 Distribution and frequency of HPV genotypes among the participants

Out of the 68 positive samples (10 males and 58 females), 44 (4 males and 40 females) were successfully identified through BLAST analysis. Twenty four sequences were either short or of poor quality therefore could not achieve any similarity when aligned
together with reference sequences. HPV-6 (75%) was the most prevalent type followed by HPV-58 (25%) among males while HPV type 6 (27.5%) was the most prevalent type among females followed by type 81 (25%), type 83 (10%), type 16 (10%), type 11 (7.5%), type 66 (7.5%), type 70 (7.5%) type 54 (2.5%) and type 85 (2.5%) (Table 4.5).

Table 4.5: Distribution of high and low risk HPV types in urine from the respondents

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>HPV genotypes</th>
<th>Gender</th>
<th>Number</th>
<th>(%)</th>
<th>Number</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
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<td>Low Risk</td>
<td>6</td>
<td>Male</td>
<td>3</td>
<td>75</td>
<td>11</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Male</td>
<td>3</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>Male</td>
<td>10</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>Male</td>
<td>1</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>Male</td>
<td>4</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>Male</td>
<td>3</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Female</td>
<td>4</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>Female</td>
<td>1</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>Female</td>
<td>3</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>Female</td>
<td>1</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>4</td>
<td>100</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>

4.6 Factors (behaviour) associated with HPV infection among the respondents

Age was not significantly associated with HPV infection in men (P=0.927) and women (P=0.807). Majority 40% (4/10) of men who tested positive for HPV DNA were in the 40-49 years age bracket whereas women who formed the highest proportion at 37.9% (22/58) were aged between 30-39 years.

Whether one was sexually active among men and women (P=0.223) was not related to the risk of HPV infection. However, all men and women reported to have been sexually
active. Age at first sexual debut was not associated with HPV infection in men (P=0.065) and women (P=0.130). However, majority of men 60% (6/10) had sexual contact before 18 years of age while majority of women 50% (29/58) were aged 19-24 years. The number of sexual partners was not associated with HPV infection in men (P=0.725) and women (P=0.573). However, majority of men, 90% (9/10) and women 77.6% (45/58) reported to have had two or more than two sexual partners. Condom use was not significantly associated with the risk of HPV infection in men (P=0.866) and women (P=0.099). However, majority of men reported to have never used any protection during sex, 40% (4/10) while 39.7% (23/58) of women reported to have used protection sometimes during coitus formed the highest proportion. Moreover, sex frequency in a day was not significantly associated with HPV infection in men (P=0.58) and women (P=0.492). However, majority of men and women 50% (5/10), 55.2% (32/58) reported to have had coitus once in a day respectively. Genital hygiene was also not significantly associated with HPV infection in men (P=0.660) and women (P=0.246). However, those who reported to have had genital hygiene formed the highest proportion among men and women representing 80% (8/38) and 89.7% (52/150) respectively (Table 4.6).
Table 4.6: Sexual behaviour associated with HPV infection among the respondents

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male (n=45)</th>
<th>Female (n=177)</th>
<th>p-value</th>
<th>Male (n=45)</th>
<th>Female (n=177)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>Age in yrs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18-24</td>
<td>1</td>
<td>10</td>
<td>6</td>
<td>14</td>
<td>24.1</td>
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<td>25-29</td>
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<td>14</td>
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<td>30-39</td>
<td>3</td>
<td>30</td>
<td>10</td>
<td>22</td>
<td>37.9</td>
<td>37</td>
</tr>
<tr>
<td>40-49</td>
<td>4</td>
<td>40</td>
<td>11</td>
<td>8</td>
<td>13.8</td>
<td>21</td>
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<tr>
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</tr>
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<tr>
<td>&lt;18 years</td>
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<td>60</td>
<td>16</td>
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<td>23</td>
<td>39.7</td>
</tr>
<tr>
<td>19-24 years</td>
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<td>20</td>
<td>18</td>
<td>51.4</td>
<td>29</td>
<td>29</td>
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<tr>
<td>&gt;25 years</td>
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<td>20</td>
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<td>2.9</td>
<td>6</td>
<td>60</td>
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<td>Past sex partners</td>
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<td>One</td>
<td>1</td>
<td>10</td>
<td>5</td>
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<td>13</td>
<td>28.3</td>
</tr>
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<td>2 or more</td>
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<td>90</td>
<td>30</td>
<td>85.7</td>
<td>45</td>
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<td>40</td>
<td>10</td>
<td>28.6</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>Rarely</td>
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<td>6</td>
<td>17.1</td>
<td>7</td>
<td>12.1</td>
</tr>
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<td>Sometimes</td>
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<td>30</td>
<td>13</td>
<td>37.1</td>
<td>23</td>
<td>39.7</td>
</tr>
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<td>10</td>
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<td>17.1</td>
<td>10</td>
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</tr>
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<td>18</td>
<td>51.4</td>
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<td>55.2</td>
</tr>
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<td>9</td>
<td>25.7</td>
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<td>5</td>
<td>14.3</td>
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<td>30</td>
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<td>20</td>
<td>5</td>
<td>14.3</td>
<td>6</td>
<td>10.3</td>
</tr>
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</table>
4.7 Reproductive factors associated with HPV infection

The number of children was not significantly associated with HPV infection in men and women (P=0.098; P=0.634) respectively. For instance, majority of either population had no children, 40% (4/22) and 50% (29/98) were men and women respectively. Contraceptives usage was not significantly related to HPV positivity in men and women (P=0.439; P=0.807). For instance, majority of men and women 100% (n=10) and 56.9% (33/58) reported to have not used contraceptives before respectively. History of STIs was significantly associated with HPV infection among women only ($\chi^2=9.894; P=0.002; df=1$). Most men 90% (9/10) and women 63.8% (37/58) reported to have had history of STIs before. Cervical screening (P=0.231) and HPV vaccination (P=0.602) was not significantly associated with HPV positivity among women. For instance, only 31% (18/58) of women reported to have had cervical cancer screening before according to self-reported history whereas 69% (40/58) had never been screened before. Only one female (1/58) reported to have been vaccinated against HPV representing 1.7%. Lastly, smoking habits was not significantly associated with HPV DNA infection among men (P=0.835) and women (P=0.983). However, majority of males and females 80% (8/10) and 98.3% (57/58) reported to have been not smoking respectively. Alcohol use was also not associated with HPV infection among men (P=0.297) and women (P=0.130). However, 70% (7/10) and 22.4% (13/58) men and women reported to have been drinking respectively (Table 4.7).
Table 4.7: Reproductive factors associated with HPV DNA infection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male (n=45)</th>
<th>Female (n=177)</th>
<th>p-value</th>
<th>Male (n=45)</th>
<th>Female (n=177)</th>
<th>p-value</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>4</td>
<td>20</td>
<td>51.4</td>
<td>29</td>
<td>69</td>
<td>58</td>
</tr>
<tr>
<td>One</td>
<td>4</td>
<td>6</td>
<td>17.1</td>
<td>18</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>Two</td>
<td>0</td>
<td>8</td>
<td>22.9</td>
<td>7</td>
<td>12.1</td>
<td>13</td>
</tr>
<tr>
<td>Three</td>
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<td>3</td>
<td>8.6</td>
<td>2</td>
<td>3.4</td>
<td>7</td>
</tr>
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<td>2</td>
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</tr>
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<td>-</td>
<td>-</td>
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<td>31</td>
<td>27</td>
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<td>40</td>
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<td></td>
<td></td>
</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1.7</td>
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</tr>
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<td>Smoking</td>
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<td>1.7</td>
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<td>8</td>
<td>29</td>
<td>82.9</td>
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<tr>
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<td>51.4</td>
<td>13</td>
<td>22.4</td>
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</tr>
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<td>3</td>
<td>17</td>
<td>48.6</td>
<td>45</td>
<td>77.6</td>
<td>103</td>
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</tbody>
</table>

* Significant p≤0.05
4.8 Phylogenetic analysis.

The nucleotide sequences that were determined in the present study were deposited in the Gene Bank and these are their accession numbers: [GenBank: KR674038-KR674081].

A total of 10 Haplotypes were determined using SEAVIEW EDIT 4 as shown in Figure 4.2. This method was able to classify different query sequences into clusters. For instance, Haplotype 1 clustered with HPV type 6 with bootstrap value of 99%, Haplotype 2 clustered with HPV type 11 with bootstrap value of 91%, Haplotype 3 clustered with HPV type 16 with bootstrap value of 79%, Haplotype 4 clustered with HPV type 58 with bootstrap value of 98%, Haplotype 5 clustered with HPV 85 with bootstrap value of 99%, Haplotype 6 clustered with HPV 83 with bootstrap value of 99%, Haplotype 7 clustered with HPV 81 with bootstrap value of 97%, Haplotype type 8 clustered with HPV 70 with bootstrap value of 98%, Haplotype 9 clustered with HPV 54 with bootstrap value of 96% and lastly Haplotype 10 clustered with HPV 66 with bootstrap value of 76%. These are detailed in figure 4.2.
Figure 4.2: HPV Haplotypes.
Figure 4.3: Phylogenetic analysis of L1 nucleotide sequences amplified by GP5+/GP6+ primers in HPV isolates from the respondents.
CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Introduction

Few studies have been conducted on the prevalence of HPV in sub-Saharan Africa including Kenya. Most of the data that are available are from HPV studies in women (Brinkman et al., 2002; Gichangi et al., 2003; Hawes et al., 2003; Moscicki et al., 2004). However, data on infections in men are very limited (Van der Snoek et al., 2003). It was also difficult to compare the present study with previous studies as it relied only on urine sampling. Most HPV studies that have been done in Kenya used cervical smears and HPV testing as an adjunct therefore this study is the first one to use urine samples.

5.1.2 Socio-demographic characteristics of the respondents

In a Kenyan study conducted in Thika District among women revealed that majority (18.1%) of the participants: were in 30-34 years age bracket, 66.9% were single while 37.1% had secondary education (Ngugi et al., 2011). These findings are not in agreement with the present study as the highest proportion (33.3%) of women participants were in the 30-39 years age group, 54.2% were married whereas majority (33.6%) had tertiary education. However, in terms of occupation there was an agreement between Ngugi et al. (2011) (39.4%) and the present study as majority (40.7%) of the women participants were self-employed.

The present study established that most of the females participants were aged 18-49 years and majority (54.2%) of them were married. These findings are in agreement with a study carried out at a Reproductive Clinic in Kenyatta National Hospital (KNH)
among women aged 18-50 years, majority (79.7%) of who were married (Omire et al., 2014).

5.1.3 Prevalence of HPV and HPV types among respondents

The results of this study show an overall high prevalence of 30.6% in urine samples at a point in time (68/222). However, the prevalence in men (22.2%) and women (32.8%) was high. These findings are in discrepancy with a study by Yamada et al. (2008) that was carried out in Riruta Health Centre in Nairobi among sexually active women and found an overall HPV prevalence of 27%. However, among HIV positive women, 49% were HPV positive while 17% of HIV negative women were HPV positive.

The present study found out the most prevalent LR HPV type detected in females was HPV type 6 (27.5%) followed by HR HPV-16 (10%). However, this result was not in agreement with Ngugi et al. (2011) who demonstrated HR-HPV 16 to be the most prevalent (4.6%) type in Thika District, Kenya. Furthermore, in a study that was undertaken at KNH Reproductive Health Clinic also demonstrated that HPV 16 was the most prevalent type (22.1%) (Unpublished data).

In another study, Johnson et al. (2012) found that the most prevalent HPV type among women was HR-HPV 16 (4.2%) followed by LR HPV 6 (3.1%). However, in men the most predominant type detected was HR-HPV 16 (2.3%) followed by HR-HPV 51 (1.5%) and LR-HPV 11 (1.2%). These study findings are not in agreement with the present study that demonstrated that LR-HPV type 6 (27.5%) was frequently detected, followed by 81 (25%), 83 (10%), 11 (7.5%), 70 (7.5%), HR-HPV-16 (10%) and 66 (7.5%) among women whereas in men LR type HPV 6 (75%) and HR-HPV 58 (25%) were detected. This is due to the fact that the study populations were from different regions. In addition, 3.4% of women and 2.6% of men had multiple HR-HPV infection in the former study as compared to the present study that found no coinfection or multiples infection in samples. The similarity between the two studies is that both
genders were sampled who were sexually experienced. Moreover, the present study demonstrated that LR-HPV type 6 (27.5%) was frequently detected, followed by 81 (25%), 83 (10%), 11 (7.5%), 70 (7.5%), HR-HPV-16 (10%) and 66 (7.5%) and among women. These study findings are not in tandem with Muchiri et al. (2012) showed that HPV types detected 16, 56, 53, 35, and 39 were detected with the highest proportion.

The present study is not in accord with a study by De Vuyst et al. (2003) that was conducted in family planning centre in Nairobi among women. This study found a high HPV prevalence of 44.3% as compared to the present study the reported a prevalence of 32.8% among women. The former study also found that the most common HPV types were HR types HPV 52 (17.9%), HPV 16 (14.7%), HPV 35 (11.6%), and HPV 66 (9.0%). An uncharacterized type (type X) was found in 15.8% of the HPV-positive samples. These findings are not in agreement with the present study that found that LR-HPV type 6 (27.5%) was frequently detected, followed by 81 (25%), 83 (10%), 11 (7.5%), 70 (7.5%), HR-HPV-16 (10%) and 66 (7.5%) among women.

5.1.4 Factors associated with HPV infections

The present study found that having history of STIs was a significant factor for HPV infection (P=0.002) among women at bivariate level. This result was however not in agreement with a study that was conducted in Tigoni Kenya by Muchiri et al. (2012) that demonstrated that contraceptive use (P<0.0001), age and HIV status were significant factors associated with HPV infection.

Johnson et al. (2012) reported that in bivariate analysis, the prevalence of HR-HPV was strongly associated with sexual behaviour in men and women. However, in the present study, HPV infection was significantly associated with history of STIs (P=0.002) among women in bivariate analysis. In the former study, HR-HPV was significantly associated with younger age, being single, increasing numbers of new sexual partners in the last year and concurrency between the most recent sexual partners in the last year among
women in multivariate analysis while in men, HR-HPV remained significantly associated with younger age at first sex, increasing numbers of sexual partners without a condom in the last year and the number of new sexual partners in the last year. These results are not in tandem with the present study as none of the variable was significantly associated with HPV infection in multivariate analysis.

5.1.5 Phylogenetic divergence of HPV strains identified

Based on the phylogenetic analysis, all HPV Types were of the genus Alpha papillomavirus. The phylogenetic tree was able to cluster the HPV types based on the genotypes and oncogenic potential and pathogenicity. These results are in agreement with a study that was conducted at Kenyatta National Hospital Reproductive Health Clinic (Unpublished data). However, the present study identified 10 Haplotypes of HPV (Haplotype 1 clustered with HPV type 6 with bootstrap value of 99%, Haplotype 2 clustered with HPV type 11 with bootstrap value of 91%, Haplotype 3 clustered with HPV type 16 with bootstrap value of 79%, Haplotype 4 clustered with HPV type 58 with bootstrap value of 98%, Haplotype 5 clustered with HPV 85 with bootstrap value of 99%, Haplotype 6 clustered with HPV 83 with bootstrap value of 99%, Haplotype 7 clustered with HPV 81 with bootstrap value of 97%, Haplotype type 8 clustered with HPV 70 with bootstrap value of 98%, Haplotype 9 clustered with HPV 54 with bootstrap value of 96% and lastly Haplotype 10 clustered with HPV 66 with bootstrap value of 76%) whereas the former study identified 15 Haplotypes, Haplotype 1 and 2 clustered with HPV type 16, Haplotype 3 clustered with HPV type 31 with bootstrap value of 97%, Haplotype 4 clustered with HPV type 33 with bootstrap value 95%, Haplotype 5 clustered with HPV type 35 with bootstrap value of 99%. Furthermore, HPV 16 was the most prevalent (4.6%) with 15 samples being identified to have HPV 16 followed by type 73(14.7%), Type 6 (13.2%), Type 81 (7.4%), Type 52 (4.4%), Type 35 (4.4%), Type 33 (2.9%), Type 26 (2.9%), Type 66 (2.9%), Type 83 (2.9%), Type 102 (2.9%), Type 11 (1.5%), Type 31 (1.5%), Type 51 (1.5%).
The study was carried out within limitations: the prevalence and risk factors for HPV among patients attending STC was assessed at particular point in time regardless of what may have preceded; in addition, urine doesn’t directly come from the original sites of HPV infection site. Cervical cells are shed through the genital tract but the challenge is that the samples were not taken directly from the transformation zone of the cervix as the study exclusively relied on urine.

5.2 Conclusion

From the findings of this study the following conclusions can be made:

1. Socio-demographic characteristics were not associated with HPV infection.
2. High and low risk HPV types were detected in urine of sexually active men (22.2%) and women (32.8%). Therefore, this prevalence is high and there is need to increase surveillance of HPV infection.
3. There was significant association between history of sexually transmitted infections and HPV infection among women at bivariate analysis. However, none of the factors was found to independently contribute to HPV infection.
4. The phylogenetic analysis of the HPV variants clustered HPV genotypes based on their oncogenic potential as well as their taxonomy. Results show that HPV 6 is more prevalent in this population as compared to other geographical areas where HPV 16 is more prevalent.

This study concludes that there is difference in the genetic variability of HPV among men and women.
5.3 Recommendations

This study recommends the following:

1. More studies on risk factors associated with HPV infection need to be carried out in the entire country. The result can be used to measure the entire HPV prevalence and the factors that contribute independently to HPV infection. This will be valuable information for raising awareness to the public on the risk factors associated with HPV infection.

2. The current vaccines that are available in our country are manufactured using Virus Like Particles (VLPs) of HPV 16 and 18 for Cervarix while HPV 16, 18, 6 and 11 for Gardasil. Therefore there is need to develop effective region specific HPV vaccines based on the observed distribution of HPV types in Kenya. According to this study HPV 35, 66, 70 and 85 were detected and yet they are not included in the vaccines available commercially.

3. Data from this study has provided evidence that HPV can be detected in urine from men and women. This new insight may contribute to the ongoing debate on HPV vaccination among men due to the increasing types of cancers that may be linked to HPV transmitted by men. Men are reservoirs of HPV thus there is need to consider rolling out vaccination programs for both genders.

4. There is need for consensus on the current available methods for detecting and genotyping HPV for effective surveillance of HPV. It is important to consider HPV testing in urine as this may increase screening coverage as it by passes medical examination as compared to other tests.

5. Future studies should establish if vaccination of women will protect their sexual partners from HPV transmission.
REFERENCES


Hubbard, R.A. (2003). Human papillomavirus testing methods. *Archives of Pathology and Laboratory Medicine, 127*(8), 940-945.


APPENDICES

Appendix 1: Informed Consenting Form

Introduction

Hallo. I am Langat Hillary, from Jomo Kenyatta University of Agriculture and Technology. This information form seeks informed consent for your participation in a study that seeks to determine the genotypic diversity and factors associated with Human papillomavirus in urine from patients attending Special Treatment Centre (STC) in Nairobi County, Kenya.

Purpose

1. To determine the socio-demographic and behavioural characteristics of men and women attending Special Treatment Centre (STC) in Nairobi County.
2. To determine the genetic diversity of HPV types in urine specimens from men and women attending Special Treatment Centre (STC) in Nairobi County.
3. To determine the factors associated with HPV DNA infection among men and women attending Special Treatment Centre (STC) in Nairobi County.

List of investigators

<table>
<thead>
<tr>
<th>Name</th>
<th>Role</th>
<th>Institution/Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hillary Langat</td>
<td>Principal Investigator</td>
<td>JKUAT/ITROMID</td>
</tr>
<tr>
<td>Dr. Janeth Kombich</td>
<td>Co-investigator</td>
<td>KEMRI</td>
</tr>
<tr>
<td>Dr. Juliette Ongus</td>
<td>Co-investigator</td>
<td>JKUAT</td>
</tr>
</tbody>
</table>
Dr. Lwembe Raphael Co-investigator KEMRI

**Procedure**

If you agree to participate in this study, you will receive an identification number. A trained interviewer will ask you several questions on risk factors associated with cervical cancer. The questions will be about sexual behaviour, socio-demographic characteristics, genital hygiene, reproductive history, smoking habits and alcohol uptake.

**Risks/ Discomforts**

This study does not have any physical risks though there could be a minor invasion to your privacy when sensitive questions are being asked.

**Benefits**

You will benefit from the study by knowing your HPV status and learning more on the risk factors associated with cervical cancer caused by HPV and the information you give will guide the understanding of the burden of HPV infections and it will also be useful to clinicians for diagnosis and prevention of STIs.

**Alternatives to participation**

If you decide not to take part in this study no one will force you to, so you will be free to make your own decision. You can also choose to take part in any other studies in future.

**Confidentiality**

Any information you provide during the study will be kept strictly confidential. Your name will not appear on any study document and instead, you will be given an identification number.
Voluntariness

Your participation in this study, which will be in the form of an interview, is completely voluntary. You are free to choose whether or not to participate in this study. You are also free to withdraw from the study at any time you wish to do so.

Who to contact

You are encouraged to ask any questions to clarify any issues at any time during your participation in the study. If you need more information on the study, here are the contacts of persons coordinating the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>mobile phone number</th>
<th>email address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr. Hillary Langat</td>
<td>0720562200</td>
<td><a href="mailto:mchlangat09@gmail.com">mchlangat09@gmail.com</a></td>
</tr>
<tr>
<td>Dr. J. Kombich</td>
<td>0721573959</td>
<td><a href="mailto:jkombich@gmail.com">jkombich@gmail.com</a></td>
</tr>
<tr>
<td>Dr. J. R. Ongus</td>
<td>0722339682</td>
<td><a href="mailto:julietteongus@yahoo.co.uk">julietteongus@yahoo.co.uk</a></td>
</tr>
<tr>
<td>Dr. R. Lwembe</td>
<td>0718876976</td>
<td><a href="mailto:rlwembe@yahoo.com">rlwembe@yahoo.com</a></td>
</tr>
</tbody>
</table>

You can also contact Kemri ERC office on through the telephone that is 0202722541 or email ercadmin@kemri.org

Declaration

I have read and understood the study information. I have been given the opportunity to ask questions about the study. I understand that my taking part is voluntary; I can withdraw from the study at any time and I will not be asked any questions about why I no longer want to take part. I understand my personal details will be kept private. I
hereby consent to participate in the study as has been explained and as I have understood.

Participants’ name: ............................................................................................................

Participants’ signature: ........................................................................................................

Date: ....................................................................................................................................

Researcher's name: ..................................................................................................................

Researcher’s signature: ..........................................................................................................}

Date: .....................................................................................................................................
Appendix 2: Fomu ya Kuomba Ridhaa

Utangulizi

Hujambo, naitwa Langat Hillary, kutoka chuo Kikuu cha Jomo Kenyatta Kilimo na Teknolojia. Fomu hii ina habari inayoomba ridhaa yako ili ushiriki kwenye utafiti utakaofanywa ilikubaini virusi aina ya binadamu papilloma na Kuamua hatari zinazohusishwa kwenye mikojo ya wagonjwa ambao wanahudhuria kliniki ya zinaa (STC) ambalo liko katika kaunti ya Nairobi, Kenya.

Madhumuni au lengo

1. Kuamua mambo ya kijamii kidemografia na tabia kwa wanaoshiriki utafiti huu.
2. Kuchunguza virusi tofauti ya binadamu papilloma ndani ya mkojo kutoka wanaume na wanawake ambao wanahudhuria kliniki ya STC ya Kaunti ya Nairobi.

Orodha ya watafiti

<table>
<thead>
<tr>
<th>Jina</th>
<th>Jukumu</th>
<th>Taasisi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bw. Hillary Langat</td>
<td>mtafiti mkuu</td>
<td>JKUAT</td>
</tr>
<tr>
<td>Dr. Janeth Kombich</td>
<td>mtafiti mzaidizi</td>
<td>KEMRI</td>
</tr>
<tr>
<td>Dr. Juliette Ongus</td>
<td>mtafiti mzaidizi</td>
<td>JKUAT</td>
</tr>
<tr>
<td>Dr. Raphael Lwembe</td>
<td>mtafiti mzaidizi</td>
<td>KEMRI</td>
</tr>
</tbody>
</table>

Utaratibu
Ukikubali kushiriki kwenye utafiti huu utapata namba yako ya kitambulizi halafu mtu ambaye amehitimu atakuuliza maswali kuhusu hatari zinazohusishwa na saratani ya mfuko wa kizazi. Maswali yatakuwa kuhusu tabia ya kushiriki ngono, sifa ya mambo ya kijamii kidemografia, usafi kwa maeneo ya sehemu za siri, historia ya kutumia dawa ya kupanga uzazi, uvutaji sigara na unywaji pombe.

**Madhara au changamoto**

Utafiti huu hauna hatari zozote za kimwili, ila tu kutakuwa na uvamizi wa mambo madogo ya kibinafsi wakati wa kuulizwa maswali magumu.

**Manufaa**

Utanufaika kushiriki utafiti huu kwa kujifunza mengi kuhusu hatari zinazohusishwa na saratani ya mfuko wa kizazi inayoletwa na kirusi aina ya binadamu papilloma na pia habari utakayotupatia itasaidia ufahamu wa kirusi aina ya binadamu papilloma. Pia itakuwa muhimu kwa madktari kufanya matibabu na uzuiaji wa ugonjwa huu.

**Njia mbadala za kushiriki**

Ukiamua kutoshiriki utafiti huu hakuna mtu yeyote ambaye atakulazimisha kufanya hivyo, kwa hivyo utakuwa huru kufanya uamuzi wako wenyewe na pia utajichagulia kushiriki kwenye utafiti mwingine wa siku zijazo.

**Usiri**

Habari yoyote ile utakayotupatia wakati wa utafiti huu utawekwa kwa siri kikamilifu. Jina lako halitajulikana popote bali tu utapatiwa namba yako ya kitambulizi.

**Hiari**
Ushirikiano wako kwenye utafiti huu ambao utakuwa kwa njia ya mahojiano ya moja kwa moja ni kujitolea kwa hiari yako. Utakuwa huru kuchagua kama utashiriki au kutoshiriki utafiti huu pia, utakuwa huru kujiondoa kwenye utafiti huu wakati wowote utakaotaka.

**Nani wa kuwasiliana nayo.**

Unashauriwa kuuliza maswali yoyote ilikubaini maswala yote yanayoibuka wakati wa kushiriki kwenye utafiti. Kama utahitaji habari au mambo mengine kuhusu utafiti huu haya ndiyo majina ya wale ambao watahakikisha utafiti huu utafanyika bila tashwishi.

<table>
<thead>
<tr>
<th>Jina</th>
<th>Nambari za simu</th>
<th>Barua pepe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hillary Langat</td>
<td>0720562200</td>
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<td>0718876976</td>
<td><a href="mailto:rlwembe@yahoo.com">rlwembe@yahoo.com</a></td>
</tr>
</tbody>
</table>

Pia unaweza wasiliana na Ofisi ya taasisi ya utafiti KEMRI kupitia nambari ya simu ambayo ni 0202722541 au barua pepe ercadmin@kemri.org

**Mkataba**


**Jina la mshiriki:** ........................................................................................................................................

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Sahihi ya mshiriki: ........................................................................................................

Tarehe: ............................................................................................................................

Jina la mtafiti: ...................................................................................................................

Sahihi ya mtafiti: .............................................................................................................
Appendix 3: Questionnaire

Interview to determine the risk factors associated with Human Papillomavirus infection.

Good morning/afternoon, my name is Hillary Langat from Jkuat I am currently conducting a study to detect and type HPV in urine from patients attending a STIs in Nairobi County, Kenya. Please allow me to kindly ask you a few questions to make this study a success. The information you give will be kept strictly anonymous and confidential and will be used solely for the purposes of the study.

Identification

Respondent number/name……………………………………………………………………………………………………………………………

Date of interview …………………………………………………………………………………………………………………………………………

Socio-demographic characteristics

1. Age in years........................................................................................................................................................................
2. Gender : Male (1) Female (2)
3. Marital status : Married (1) Single (2) Separated (3) Widow(er) (4)
4. Religion : Christian (1) Muslim (2) Atheist (3)
5. Level of education: No formal education (1) Primary level (2) Secondary level (3) Post secondary education (4)
6. Occupation: Agricultural labourer (1) Formal employee (2) Unemployed (3) Student (4) Self employed (5) Others (specify)

..............................................................................................................................................................................

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7. Housing: Temporary (1) Semi-permanent (2) Permanent (3)

Sexual behaviour

8. Are you currently sexually active or have you ever been? (Yes) (No)

9. What was your age at first sexual intercourse?

   Below 18 years (1) 19-24 years (2) Above 25 years (3)

10. How many partners have you had in the past? None (1) One (2) Two or more (3)

11. Do you use protection during sexual intercourse with your partner?

   Never (1) Rarely (2) Sometimes (3) Often (4)

Genital hygiene

12. Do you practice any genital hygiene after intercourse/bathing after sex?

   Yes (1) No (2)

Reproductive history

13. Do you have child/children? Yes (1) No (2)

14. If yes, how many.................................................................................................................................

15. Do you use contraceptives? Yes (1) No (2)

16. If yes, how often do you use? Regular (1) Rarely (2) Sometimes (3)
History of STDs

17. Have you ever been diagnosed with a sexually transmitted disease/infection?

Yes (1)                                            No (2)

18. If yes, which one below here

Gonorrhoea (1)                Syphilis (2)               HIV (3)                Chlamydia (4)

Others (specify) …………………………………………………………………………………

Cervical cytologic screening histories

19. Have you had cervical cytologic screening before?  Yes (1)                        No (2)

20. If yes, how times have you been screened? Never (1)       Once (2)        Twice or more (3)

21. If no, what is the reason for not getting screened?................................................................

22. Have you been vaccinated against human papillomavirus?     Yes (1)            No (2)

23. If yes, how many times? ……………………………………………………………………………

Smoking habits

24. Do you smoke?     Yes (1)                     No (2)

25. If yes, how often have you been smoking?

    Rarely (1)    Regularly (2)    Sometimes (3)
Alcohol uptake

26. Do you take alcohol?       Yes (1)       No (2)

27. If yes, for how long have you been drinking?.................................

Appendix 4: Kidadisi

Mahojiano ya moja kwa moja ili kubaini hatari zinazohusishwa na kirusi aina ya binadamu papilloma (HPV).


Utambulisho

Jina/Nambari ya kitambulizi .................................................................

Tarehe ya mahojiano................................................................................

Mambo ya kijamii kidemografia ya wakazi

1. Umri kwa miaka............................................................................................

2. Jinsia:                         Mume (1)                           Mke (2)

3. Hadhi ya ndoa:       Kwenye ndoa (1)   Kapera (2) Ametenga (3)   Ametaliki (4)
4. Dini: Mkristo (1) Mwislamu (2) Kipagani (3)

5. Kiwango cha elimu: Hajasoma (1) elimu ya msingi (2) elimu ya sekondari (3)
   Elimu ya sekondari kwenda juu (4)

6. Ajira: Anafanya kazi ya Kilimo (1) Anafanya kazi (2) Mwanafunzi (3) Hafanyi kazi (4) Amejajiri (5) Kazi zingine (elezea) ………………………………………

7. Makazi: Nyumba ya muda (1) Nyumba ya kutodumu (2) Nyumba ya kudumu (3)

   Tabia za kushiriki ngono

8. Kwa sasa, huwa unashiriki ngono au umewahishiriki ngono? (Ndio) (La)

9. Ulikuwa na miaka mingapi uliposhiriki ngono?
   Chini ya miaka 18 (1) Miaka 19-24 (2) Miaka 26 kwenda juu (3)

10. Umeshiriki ngono na watu wangapi? Hamna (1) Mmoja (2) wawili au Zaidi (3)

11. Unatumia mipira za kufanya mapenzi unaposhiriki ngono na mpenzi wako?
   La (1) Nadra (2) Wakati mwingine (3) Kila mara (4)

Usafi wa sehemu za siri

12. Je, unafanya usafi wa sehemu nyeti baada ya kushiriki ngono? Ndio (1) La (2)

Historia ya uzazi

13. Je, una mtoto/watoto? Ndio (1) La (2)
14. Kama jibu lako ni ndio, ni wangapi?.................................................................................................

15. Je, unatumia au umewahitumia dawa ya upangaji uzazi? Ndio (1) La (2)

16. Kama jibu lako ni ndio, je, Unatumia kivipi? Kila mara (1) Nadra (2) Wakati mwingine (3)

Historia ya magonjwa ya zinaa

17. Je, una historia ya kuambukizwa magonjwa aina ya zinaa? Ndio (1) La (2)

18. Kama jibu lako ni ndio, je, ni lipi hapa chini?

Kisonono (1) Kaswende (2) Ukimwi (3) Klamidia (4)
Mengine (elezea) ..........................................................

Uchunguzi wa historia ya kizazi cytologia

19. Umewahipata matibabu aina ya screening kutokana na saratani ya njia ya uzazi?
Ndio (1) La (2)

20. Kama jibu lako ni ndio, je, umefanyiwa mara ngapi?

Mara moja (1) Mara mbili (2) Zaidi ya mawili (3)

21. Kama ni la kwa nini haujafanyiwa uchunguzi aina ya screening?.................................

22. Je, umepata chanjo kutokana na kirusi aina ya binadamu papilloma? Ndio (1) La (2)

23. Kama ndio, umepata chanjo mara ngapi?........................................................................}

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Tabia ya uvutaji sigara

24. Je, unavuta sigara? Ndio (1) La (2)

25. Kama jibu lako ni ndio, je, ni mara ngapi? Nadra (1) Kila mara (2) Wakati mwingine (3)

Unywaji pombe

26. Je, unakunywa pombe? Ndio(1) La(2)

27. Kama jibu lako ni ndio, umekunywa pombe kwa miaka mingapi sasa?
Appendix 5: Ethical Review Committee Approval letter
Appendix 6: Scientific Steering Committee Approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 34454-00200, NAIROBI, Kenya
Tel (254) 020) 332 3648, 332 3650, 0722 36601, 0733 49055, Fax: (254) 020) 332 3650
E-mail: info@kenmi.org, cvr@kenmi.org, mmp@kenmi.org www.kenmi.org

ESACIFAC/SSC/101549

5th April, 2013

Hillary Langat
Thru:
Director, CVR
NAIROBI

REF: SSC No. 2442 (Revised) – Detection and characterization of Human Papilloma Virus in urine from men and women attending a sexually transmitted infections clinic at the City Council of Nairobi

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 200th meeting held on 5th March, 2013 and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised protocol to SSC within 2 weeks from the date of this letter i.e., 19th April, 2013.

We advise that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

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Appendix 7: Publication