

**HUMAN PAPILLOMAVIRUS GENOTYPES IN WOMEN LIVING
WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION
ATTENDING ANTI-RETROVIRAL THERAPY CLINIC AT THE
KORLE-BU TEACHING HOSPITAL, ACCRA**

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**This thesis is submitted to the University of Ghana, Legon in partial fulfilment of
the requirement for the award of MPhil Microbiology degree.**



DECLARATION

This is to certify that this thesis is the result of research undertaken by Kathleen Korkor Glover towards the award of the Masters of Philosophy in Microbiology in the Department of Microbiology, University of Ghana Medical School.

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ABSTRACT

BACKGROUND: Annual global estimates show that 274,000 out of 490,000 women, newly diagnosed with invasive cervical cancer caused by oncogenic types of human papillomavirus (HPV), die. High risk HPV genotypes (hrHPV) cause the cancers whilst the low risk genotypes cause benign lesions (warts). HIV infection increases the prevalence and the persistence of hrHPV infection and human immunodeficiency virus (HIV) infected women are eight times more likely of developing invasive cervical cancer as compared to HIV negative women. There is currently paucity of data on the prevalent HPV genotypes that affect HIV infected women in Ghana and West Africa.

AIM: The aim of this study was to determine the genotype distribution of HPV infection in women living with HIV.

METHODOLOGY: This was a cross sectional study. A structured questionnaire was used to obtain demographic information and the likelihood of HPV infection from women of ages 18 years to 65 years attending Antiretroviral Therapy (ART) clinic at Korle-Bu Teaching Hospital. Papanicolau (Pap) smears were done on all women and examined by a cytologist. HPV DNA was extracted from their cervical cells and genotyped using a nested multiplex polymerase chain reaction. Recent CD4⁺ count of these women were obtained from their folders.

RESULTS: Hundred participants (mean age, 40.21 ± 8.44 years) took part in the study. Seventeen HPV genotypes (12 high risk and 5 low risk) were detected by PCR. Overall HPV prevalence was 61.4% of which the hrHPV genotype prevalence rate was 54%. hrHPV 35 (12%) and HPV 42 (12%) were the most prevalent. Genotypes 16 (2%), 39 (2%), 59 (2%) and 56 (1%) were the least prevalent. There were 41% single HPV infections and a total of 27% multiple HPV infections. Of the multiple infections, double infections (14%) were the highest. For the multiple infections HPV genotypes 6, 11, 35,

68, 42, 45, 51, in descending order, were the most prevalent. For single infections, genotypes 35, 42, 44, 52 and 45, in descending order, were the most prevalent. Smoking was not a risk factor in this study. Majority of the women (84%) reported negative for intraepithelial lesion or malignancy (NILM). Squamous cell lesions detected were low-grade squamous intraepithelial lesion (LSIL) [3%], high-grade squamous intraepithelial lesion (HSIL) [2%], atypical squamous cells of undetermined significance (ASCUS) [1%] and atypical squamous cells of undetermined significance - cannot exclude high grade (ASC-H) [1%]. A total of six high risk and two low risk genotypes were detected in the women who had squamous cell lesions. hrHPV genotypes 35, 45, 52 and 56 as well as 42 (low risk) were detected in (HSIL), hrHPV 35 and 44 (low risk) were detected in LSIL, hrHPV45 was detected in ASC-H, genotypes 42 and 44 (low risk) and hrHPVs 51 and 66 were detected in ASCUS. Genital infections associated with HPV genotypes were bacteria vaginosis (genotypes 6, 11, 42, 43, 44 and hrHPV 16, 18, 35, 66, 68), vaginitis (hrHPV 52), atrophic vaginitis (hrHPV 39) and candidiasis (hrHPV 45). Bacteria vaginosis was the most prevalent infection (16%). Most of the women (64%) had their CD⁺4 count > 350 cells/mm³

CONCLUSION: HPV genotypes in Ghanaian women living with HIV were determined. A total HPV prevalence of 61.4% was observed. hrHPV 35 (12%) and HPV 42 (12%) were the most prevalent genotypes. HPV genotypes were detected in all the squamous lesions; LSIL (3%), HSIL (2%), ASCUS (1%) and ASC-H (1%), detected. Multiple hrHPV genotypes 35, 45, 52 and 56 were detected in HSIL and 42 and 44 (all low risk) and hrHPV66 were detected in ASCUS.

DEDICATION

I dedicate this work to the almighty God who has made me what I am today. It wasn't by might but by His grace. I also dedicate this work to my late dad Mr Stephen Tetteh Glover who am sure will be so proud of my achievement. Also to my sweet mom Mrs Kathleen Glover and my brother Dr Harry Glover and my sisters Mrs Tetteh and Mrs Adwoa Amankwah who has been there for me through this successful journey.



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LIST OF ABBREVIATIONS

μ	Micro
bp	Base pair
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dDTTP	Deoxythymidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPX	Distrene, Plasticiser, Xylene
EDTA	Ethylenediaminetetra acetic acid
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hr	High risk
lr	Low risk
MgCl₂	Magnesium chloride
Nm	Nanometers
ORF	Open reading frame
Pap	Papanicolau
PCR	Polymerase Chain reaction
rpm	Revolutions per minute
SD	Standard deviation
SPSS	Statistical Package for Social Sciences
TAE	Tris acetate EDTA

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Worldwide, cervical cancer is the second most common cancer in women in developing countries (WHO, 2002; Snijders *et al.*, 2006). Cervical cancer also is the most common female malignancy in sub-Saharan Africa (Walboomers *et al.*, 1999). Human papillomavirus (HPV) is the leading cause of cervical intraepithelial neoplasia and cervical cancer. Studies conducted have estimated that approximately half of 490,000 women diagnosed of invasive cancer induced by oncogenic types of human papillomavirus (HPV) die (Maxwell *et al.*, 2001; Drain *et al.*, 2002; Schiffman, 2003).

HIV/AIDS and HPV which are both sexually transmitted have attracted a lot global attention because of the high mortality and morbidity recorded in individuals affected. Most of these cases are as a result of unprotected sex or in some cases multiple sexually partners in women of reproductive ages as they can be exposed to various oncogenic HPV genotypes. Both viruses can also be transmitted to a child during birth by an infected mother.

Previously women living with HIV/AIDS in settings like Ghana had very limited access to antiretroviral therapy and consequently had a very limited survival period after being diagnosed with HIV. However presently, antiretroviral therapy has become accessible to all individuals who are infected with HIV. This has enabled these women to live longer thereby enabling pre-invasive lesions to manifest before progressing to invasive carcinoma. Cervical screening is the key to detection of pre-invasive lesions which can be easily treated. However in health management of HIV infected women in resource

constrained environments, cervical screening does not form part of the free service. Thus those who cannot afford the cervical screening, which is usually a pap smear, live with these pre-invasive lesions for many years. Unfortunately by the time it is detected it would have progressed to the invasive state and it may be too late to save the woman.

1.2 HISTORICAL PERSPECTIVE OF HPV

Harald zur hausen cited in his paper of “Papillomaviruses in the causation of human cancers- a brief historical perspective” that in 1842, an Italian physician became curious in the increase of the number of death cases as a result of cervical cancer that were being recorded in married women, widows and prostitutes living in Verona during the period 1760-1839 after he reviewed their death certificates (Rigoni-Stern, 1842). Interestingly death as a result of cervical cancers was not common so in virgins and nuns. This led to the conclusion that whatever was responsible for the cervical cancer that was killing these married women, widows and prostitutes and not virgins and nuns was sexually transmitted.

Harald cited that *Herpes simplex* virus type 2 was initially thought to be the causative agent for cervical cancer (Rawls *et al.*, 1968; Naib *et al.*, 1969; Nahmias *et al.*, 1970). However a study done in former Czechoslovakia disapproved this initial result (Vonka *et al.*, 1984a, 1984b). It was not until the early 1970s that studies on the possible role of HPV’s role in cancer was started. Determination of HPV causation of cervical cancer started in 1972 when it was reported in the anecdotal reports in the medical literature of rare malignant conversion of genital warts (*Condylomata acuminata*) into squamous cell carcinomas (zur Hausen, 1977).

1.3 THE HUMAN PAPILOMAVIRUS (HPV)

The human papillomavirus (HPV) is a naked double-stranded DNA virus that belongs to the Papillomaviridae family (Markowitz *et al.*, 2007). Its capsid is icosahedral and composed of 72 capsomers surrounding a circular DNA genome of ~7900 base pairs (Moody *et al.*, 2010).

1.3.1 Genomic structure

The genome of the human papillomavirus (Fig.1) has three main regions namely:

- i. An upstream regulatory region (URR)
- ii. An early region, which contains open reading frames (ORFs; e.g. E1, E2, E4, E5, E6 and E7)
- iii. A late region, which codes for the L1 and L2 capsid proteins.

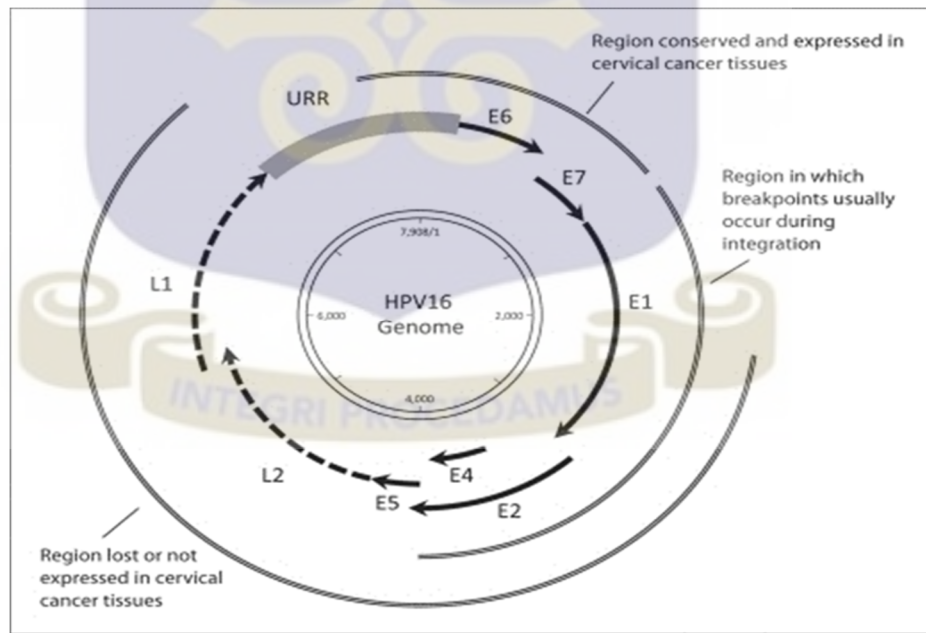


Fig.1: A schematic diagram of a representative HPV 16 genome which is an example of a high risk HPV genotype (Adapted from Robert *et al.*, 2009)

All the three main regions in the HPV genome are separated by two polyadenylation (pA) regions. The upstream regulatory region (URR) [Fig. 1] contains sequences that control viral replication leading to the formation of viral DNA and transcription which leads to the formation of messenger RNA (mRNA) (Robert *et al.*, 2009).

The early region has several functions namely *trans*-activation of transcription, transformation, replication, and viral adaptation to different cellular environment. The early region (Fig. 1) occupies half of the viral genome. It encodes six common open reading frames (E1, E2, E4, E5, E6 and E7) which code for non-structural viral regulatory proteins (Katinka and Yaniv, 1982). E1 and E2 are involved in viral DNA replication and the regulation of early transcription. E4 is expressed in a productive infection associates with cyokeratin filament collapse. E5, E6, and E7 are the main viral oncogenes and their expression results in cell immortalization and transformation (Zhi, 2006). E6 and E7 are two main viral oncoproteins that inactivate the two cellular tumor suppressor proteins, p53 and pRb, thereby causing a tumour to grow uncontrollably (Zhi, 2006).

The late region of all papillomavirus covers almost 40% of the viral genomes. The late region forms the structure of the virus that enhances viral DNA packaging and maturation (Robert *et al.*, 2009). It encodes L1 and L2 Open Reading Frames which translate the two capsid proteins namely major (L1) and a minor (L2) capsid protein (Zhi, 2006). A segment of the late region which contains about 850 bp has no protein-coding function, but bears the origin of replication as well as multiple transcription factor binding sites that are important in regulation of RNA polymerase II-initiated transcription from viral early as well as late promoters (Bernard, 2002).

All human papillomavirus genotypes have two main promoters namely P1 and P3. The P1 promoter is located at the 5' of the E6 gene while the P3 promoter is located within the E7 gene (Fig. 2). There is a third promoter P4 located upstream of the E8 open reading frame region. HPV undergoes alternative splicing which enable the virus to access various reading. The human papillomavirus forms bi or poly- cistronic mRNA which enables it to encode for more viral proteins. At the genomic level what differentiates the high risk HPV genotypes from the low risk HPV genotypes is the presence of an E6 intragenic splices from one splice donor to one or two alternative splice acceptors (Louise *et al.*, 2010).

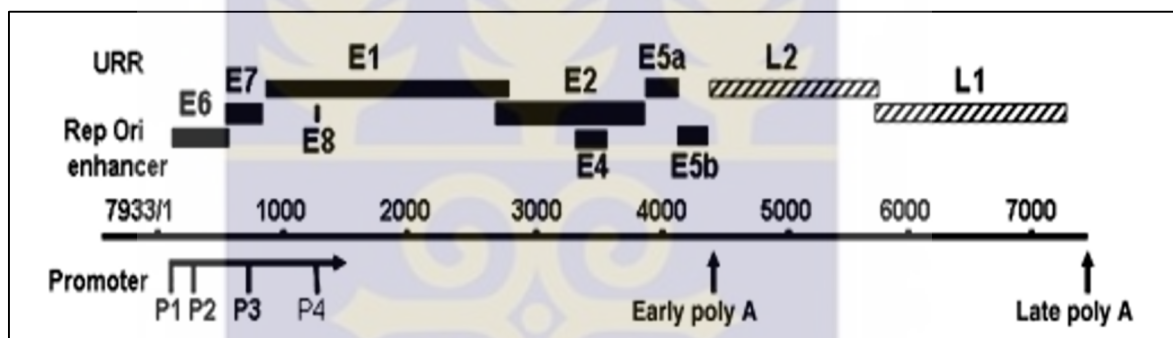


Fig. 2: Genomic organization of HPV-11. The genome contains a non-coding upstream regulatory region (URR), also called the long control region (LCR), an Early (E) region and a late (L) region. The open reading frames (ORF) are denoted as boxes. The three major promoters (P1, P2, P3), a minor promoter (P4), and two polyadenylation sites are indicated. (Adapted from Louise *et al.*, 2010)

1.3.2 Genotype and distribution

Presently more than 200 types different HPV types have being identified out of which 40 are known to infect the female anogenital tract (zur Hausen, 1999).According to the severity of the disease that occur, HPV's are classified as: low-risk virus that produce benign lesions (primarily types 6 and 11 in condylomatose injuries together with types 40, 42, 43, 44, 54, 61, 70, 72, and 81) and high risk viruses that cause precancerous lesions

and cervical cancer (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) (Munoz *et al.*, 2003).

The clinically most important high risk HPVs, HPV 16 and HPV 18, are found in 50–65% and 7–20% of cases of cervical cancer, respectively (Li *et al.*, 2010). This is similar to what Munoz *et al.* (2003) reported (Fig. 3) where HPV 16 and 18 accounted for 70% of the cervical cancer cases worldwide. In addition to cervical carcinoma high risk HPV types, the most frequent being HPV-16, play the leading etiological role in the development of anal cancer and a substantial proportion of vaginal, penile, vulvar and Oropharyngeal (mainly tonsillar) cancers (Hobbs *et al.*, 2006; Smith *et al.*, 2007; Backes *et al.*, 2009; De Vuyst *et al.*, 2009).

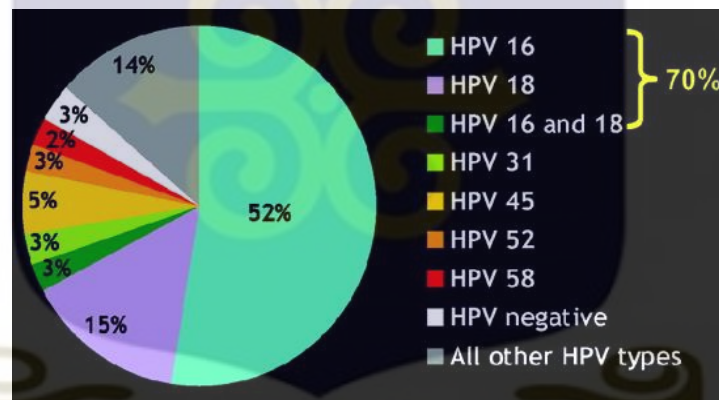


Fig 3: HPV types that cause squamous cell cervical cancer worldwide. Adapted from Munoz *et al.* (2003).

1.3.3 Transmission

HPV is sexually transmitted. The use of condom seems to be less completely protective against acquiring HPV (Manhart, 2002). Like other sexually transmitted infections (STIs), the risk for acquiring HPV is increased for individuals with a higher number of sexual

partners, other STIs and a younger age at initiation of sexual activity (Ley *et al.*, 1991; Moscicki *et al.*, 2001).

1.3.4 Pathogenesis

HPV has a simple and small genome size of 55 nm in diameter (Brook *et al.*, 2007). Thus it is unable to code its own DNA polymerase and additional proteins that are required for its DNA replication. Hence it depends on the host cell to undergo its replication. HPV has affinity for epithelial surface targeting parabasal and basal cells because these cells are constantly dividing.

The virus gains access to these cells often through mild trauma (Burd, 2003). The mechanism of action of the two main oncogene E6 and E7 is one of the bases for distinguishing HPV into the high risk group and the low risk group. In formation of warts which are benign lesions which are usually caused by the low risk HPV genotypes, the HPV DNA is located in the outer part of the chromosome of the host cell's nucleus (Gómez and Santos, 2007). However in the cancer which are usually caused by the high risk genotypes, the HPV DNA is integrated in the host cell's genome (Gómez and Santos, 2007). Integration of the viral DNA results in loss of expression of the E2 region which is part of the HPV genome. The E2 region is responsible for regulating the expression of the E6 and E7 genes. Loss of the E2 region results in over expression of the E6 and E7 genes (Yoshinouchi *et al.*, 1999). E6 and E7 result in cancer formation by inactivating two tumour suppression proteins p35 and Retinoblastoma (Rb) (Gómez and Santos, 2007). P53 initiates DNA repair when there is damage or induces apoptosis (programmed cell death). The Rb gene monitors the damaged cell from entering the cell cycle. These two tumour suppressor genes namely p35 and Rb gene are both inactivated the E6 protein and E7 protein respectively (Gómez and Santos, 2007). In low risk HPV infections the two

oncogenes E6 and E7 genes do not bind to the two main tumour suppressor genes (Gómez and Santos, 2007). The inactivation of p53 and Rb results in an increase in the accumulation of cells with damaged DNA which leads to genomic instability resulting in the inability to repair secondary mutations in the host cell DNA (von Knebel Doeberitz, 2002). This results in the formation of cancerous cells (Park, 1995).

1.3.5 Epidemiology

Most studies done on cervical cancer have indicated high risk HPV genotypes to be the main cause globally. However certain prevailing factors enable the acquisition and the progression of HPV that will cause the pre-invasive lesion caused by these HPV genotypes to become invasive. These factors are as follows;

1.3.5.1 Genetic heritability

Inheriting certain genes within a family which may be as a result of mutations increases an individual's susceptibility to HPV infection, ability to clear HPV infection, and time to development of disease (Gómez and Santos 2007).

1.3.5.2 Number of sexual partner

HPV which has been known to be the main cause of cervical cancer is sexually transmitted. Thus having unprotected sex with multiple partners increases one's susceptibility to various high risk HPV genotypes that can lead to cervical cancer (Peyton *et al.*, 2001).

1.3.5.3 Age of first sexual contact

Cervical cancer occur at the region between the endocervix and the ectocervix which known the transformational zone. This region is prone to metaplastic changes that make it susceptible to HPV infection which can lead to cervical cancer (Gómez and Santos 2007)).

Metaplastic change is more common during puberty and first pregnancy and declines after menopause. Thus when a woman engages in sex at a younger age especially during her puberty stage, she becomes more prone to HPV induced cervical cancer. Some studies have reported a high HPV prevalence in the young age groups after their first sexual encounter. The HPV prevalence reaches its peak in young adults (18 to 30 years of age) and declines at older ages (Burk *et al.*, 1996).

1.3.5.4 Chlamydia infection

This is a bacterium that is sexually transmitted and one of the species *Chlamydia trachomatis* increases the persistence of high-risk HPV which leads to cervical cancer but how it does this remains unclear (Samoff *et al.*, 2005).

1.3.5.5 Use of oral contraceptives

The upstream regulatory region (URR) 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) (Gómez and Santos 2007). Long-term use of oral contraceptives is a significant-risk factor for high-grade cervical disease according to some studies (WHO, 1993; Adam *et al.*, 2000).

1.3.5.6 Smoking

Smoking damages the Langerhans cells found in the lining of the cervix (Cancer Research UK, nd). Langerhans cells (LC) are unique dendritic cell subsets that are located in mucosal stratified squamous epithelium and skin epidermis. Their location is ideally suited for their function as antigen presenting cells that capture invading viruses and induce anti-viral immunity (Cunningham *et al.*, 2008). Smoking acts immunosuppressively on the cervix by decreasing the population of Langerhans cell and this may increase ones chances of being

infected with HPV (Schiffman et al., 1987; Barton *et al.*, 1988). Schiffman *et al* (1987) reported in their publication that an “Elevated concentrations of nicotine and cotinine were found in the cervical mucus of smokers and may transform HPV infected tissue”.

1.3.5.7 Nutritional factors

Patients who are deficient in vitamin A, B carotene, vitamin C, and folic acid have been reported to have an increased chance of acquired infections caused by HPV (Schneider *et al.*, 1989).

1.3.5.8 Weakened immune system

The primary immune response to HPV infection is cell mediated; therefore, conditions that impair cell mediated immunity such as renal transplantation or HIV disease, increase the risk of acquisition and progression of HPV (Ho *et al.*, 1994; Sun *et al.*, 1997). Many studies worldwide have shown a higher prevalence of cervical cancer among HIV-positive women than among HIV-negative women and this is due to the fact that they lose their cell mediated immunity when infected with HIV (Lesley, 2011).

1.4 NORMAL VAGINAL MICROBIOTA

The microbiota found in the human vaginal region plays a pivotal role in the maintenance of the health of a woman, her partner, and a new born. Advances in molecular techniques have made it possible to examine the composition and dynamics of organisms present in the vaginal region. In addition, their association with a number of STI's have been studied.

Lactobacilli are the predominant vaginal microbiota in the lower genital tract in women of reproductive age (Nikolaitchouk, 2009). Lactobacilli are, facultative and obligate anaerobic, catalase-negative, gram-positive, non-spore-forming, rod shaped bacteria. The

presence of these bacteria is a prerequisite for a healthy vaginal condition. Vaginal microbiota play a crucial protective role in women's health, and the host biochemical and immunological response to the microbiota can serve as an indicator of the health indicators of the vaginal environment (Ling *et al.*, 2010). A healthy microbiota prevents or hinders many urogenital infections such as yeast and human immunodeficiency virus (HIV) (Gupta *et al.*, 1998; Cherpes *et al.*, 2003; Wiesenfeld *et al.*, 2003; Watts *et al.*, 2005; Lai *et al.*, 2009).

An abnormal microbiota, such as that found in bacterial vaginosis (BV) is associated with an increased risk of upper genital tract health issues, miscarriage (Leitich *et al.*, 2003), recurrent abortion, preterm delivery (Gillet *et al.*, 2011) and sexually transmitted infections such as HIV and HPV infection which result in cervical cancer (Thomas *et al.*, 2004; Spear *et al.*, 2008).

1.5 HPV AND CERVICAL CANCER

There is increasing evidence linking human papillomavirus (HPV) with cervical intraepithelial neoplasia (CIN) and carcinoma (Reeves *et al.*, 1989; Vermund *et al.*, 1991). Human papilloma virus (HPV) affects the skin and moist membranes that form a lining of parts of the human body, including the lining of the mouth and throat, the vulva, the cervix, the vagina and the anus (NHS Wale Encyclopaedia, 2013). When HPV for instance, affects the cervix (which is a narrow, outer end of the uterus), it causes the cells in the lining to change and grow into mass of cells known as a tumour. This condition develops over time and become cancerous.

The prevalence of HPV infection varies among different geographic areas and populations, being higher in low-resource countries and most common among women younger than 25

years old (de Sanjosé *et al.*, 2007). Of over 100 different types of HPV known to affect the human body, 30 types are said to affect the genitals which includes the vulva, vagina, cervix, penis and scrotum. Of these, 13 types or strains are considered high risk because exposure to them leads to cervical cancer (Munoz *et al.*, 2003; Clifford *et al.*, 2005a).

The prevalence of genital HPV is known to be directly related to the number of lifetime sexual partners, recent change in sexual partners, marital status, illiteracy, oral contraceptive use, alcoholism, smoking, hormonal and dietary factors, and immunosuppression (Melamed *et al.*, 1969; Alloub *et al.*, 1989; Thomas *et al.*, 2004). Thus when infected by a high risk HPV genotype, that individual is more likely to have precancerous lesions or cervical cancer (Cancer Research UK, nd). Plummer *et al.* (2003) reported that smoking increases the risk of cervical cancer among HPV positive women.

1.6 CLINICAL AND DIAGNOSTIC PROFILE OF CERVICAL CANCER

There are two main cervical abnormalities that can lead to cervical cancer namely; squamous cell carcinoma and adenocarcinoma (American Cancer Society, 2006; Saraiya *et al.*, 2007). However majority of the reported cervical cancer case usually arises from squamous cell carcinomas which occur at the transformational zone, also known as the squamo-columnar junction (American Cancer Society, 2006; Saraiya *et al.*, 2007).

Adenocarcinomas develops from the mucus-producing cells of the endocervix (Abeloff *et al.*, 2004). Cervical cancer can be detected in the early stages where preinvasive lesions are detected when a Papanicolaou (pap) smear is taken and these lesions are confined to the cervix. In the advanced stage the abnormal cancer cells metastasize beyond the cervix and may involve the vagina, uterus, rectum lung and brain. Figure 4 shows the stages

cervical cancer from the initial stage where it is confined to the cervix to the advanced stages where the cancerous cells metastasize the cervix.

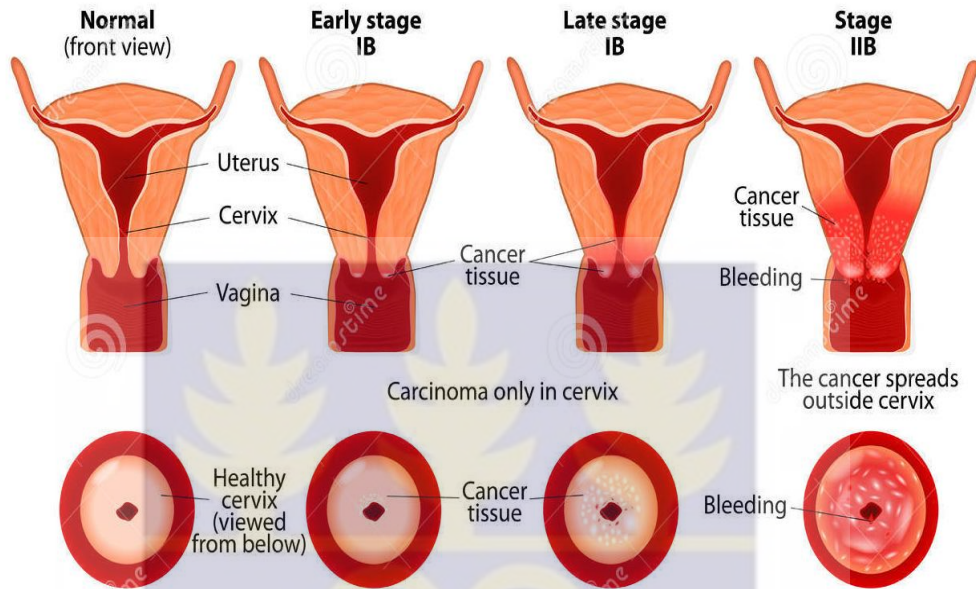


Fig4: Stages of cervical cancer. Source: <http://thumbs.dreamstime.com/z/cervical-cancer-carcinoma-cervix-malignant-neoplasm-arising-cells-uteri-vaginal-bleeding-vector-diagram-35899452.jpg> Accessed on 23/06/14

1.7 CERVICAL CANCER VACCINATION

There are currently two vaccines namely Cervarix and Gardasil for HPV that are being marketed internationally as preventive vaccines, Gardasil provides protection from HPV types 6, 11, 16 and 18 and is manufactured by Merck (Lesley, 2011). Cervarix provides protection from only HPV types 16 and 18 and is manufactured by GlaxoSmithKline (GSK). Neither vaccine contains live biological products or viral DNA, so they are non-infectious (Lesley, 2011). Gardasil has also shown a modest impact on other HPV strains such as types 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, indicating that Gardasil may be able to prevent more cases of cervical cancer, and offer wider protection than originally

estimated (Brown *et al.*, 2009). The mechanisms by which these vaccines induce protection have not been fully defined, but involve the development of cellular immunity (WHO/ICO, 2009).

Both vaccines are intended to be administered to females before the onset of sexual activity or before they have potentially been exposed to HPV. In most countries, recommendations state that girls of the ages 10 and 14 years should be vaccinated. Gardasil is given at baseline and is repeated at two months and six months (Lesley, 2011). Cervarix is given at baseline and again at one month and six months. Alternative schedules are being explored for both vaccines (WHO/ICO, 2009).

The duration of protection is not yet known, but there is evidence of protection for at least six years after vaccination for both vaccines. Studies of both vaccines are currently evaluating longer-term efficacy (WHO, 2007). Currently, the safety and efficacy of HPV vaccines are now being evaluated in parts of Africa and in populations with high HIV prevalence such as Khayelitsha in South Africa but results are not yet available (WHO, 2007).

1.7.1 Dosage and administration

Gardasil should be administered intramuscularly in the deltoid region of the upper arm or in the higher anterolateral area of the thigh as a 0.5-mL dose at the following schedule: 0, 2 months, 6 months.

Immunosuppression due to disease conditions such as HIV/AIDS or immunosuppressive therapies, including irradiation, antimetabolites, alkylating agents, cytotoxic drugs, and

corticosteroids reduces the immune responses to Gardasil (Merck Sharp and Dohme Corp, 2006.).

1.8 PROBLEM STATEMENT

HIV infected women in resource constrained environments are at heightened risk for the development and progression of HPV-induced cervical precursors to invasive cancer due to their immunocompromised state.

HPV genotypes 16 and 18 which are all high risk types have been the most common cause of cervical cancer worldwide. Currently there are two vaccines, namely Cervarix and Gardasil, that are being used to vaccinate girls between the ages of 10 and 14 years before they become sexually active (Lesley, 2011). Denny *et al.* (2013) reported genotypes 16, 18, 45, and 35 as the most common HPV types in sub-Saharan African women with invasive cervical carcinoma and that HPV infections were more common in HIV-positive women. All these genotypes were high risk HPV. Due to their susceptibility to opportunistic infections it could be possible that aside the high risk types which are the most common cause of cervical cancer, low risk HPV genotypes could also cause cervical cancer in these HIV infected women.

1.9 JUSTIFICATION

HIV infection suppresses the immune system of an individual making them susceptible to opportunistic infections. Thus organisms which under normal condition will not cause severe diseases conditions tends to do so in HIV infected individuals. Most of HPV induced cervical cancer cases are caused by high risk HPV genotypes. However in HIV women, low risk HPV could be found to be carcinogenic. Thus if such a situation is

detected at the end of the study, new HPV induced cervical treatment program will be designed to manage such cases low risk HPV cases.

Also Pap smears women which will help early detection and management of HPV induced preinvasive cervical lesion before they progress to cancer is not part of the free patient health management This study may help to the inclusion of Pap smears in their health management. Globally, HPV strains 16 and 18 are known to be the most common strains responsible for majority of cervical cancers. Consequently, two vaccines (namely Cervarix and Gardasil) were made available for protection against HPV, based on these two strains. However, because of regional and sub-regional variations in HPV, cumulative reports have suggested that these vaccines may be less effective in sub-Saharan Africa (Mayaud *et al.* 2003; Blossom *et al.*, 2007). In support of this notion are some studies from sub-Saharan Africa that reported increased prevalence of other strains of HPV (such as, HPV 31, 35 and 58) and lower prevalence of HPV 16 and 18 as responsible for new cases of cervical cancer in the region. Given the high prevalence of both HIV and HPV infection in West Africa, it is necessary to characterize the distribution of HPV genome strains in circulation in this region. This activity and the resulting data sets will not only increase our knowledge and understanding of the scope of the disease, it will also assist in developing strategies for prevention and control, including the use of available resources to determine prevalent strains as targets for effective treatment. HPV vaccination and cervical screening are ways of reducing the prevalence of HPV induced cervical cancer. However in developing countries like Ghana regular cervical screening is not possible due to financial constraint. Also the HPV vaccine is not covered by the national health insurance scheme. There is currently no published work on the prevalent HPV genotypes circulating in HIV infected women. This study will provide baseline data on the currently circulating HPV genotypes in HIV women.

1.10 AIM

To determine HPV genotypes in Ghanaian women living with HIV at the Korle-Bu Teaching Hospital, Accra in view of their immune suppression

1.11 SPECIFIC OBJECTIVES

1. To assess the severity of cervical neoplasia among HIV infected women.
2. To determine risk of HPV and multiple HPV infections with respect to the severity of cervical neoplasia among HIV infected women.
3. To determine the prevalent and circulating HPV genotypes among women living with HIV.



CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 CANCERS

Cancers are a broad term for a class of disease characterized by abnormal cells that grow and invade healthy cells in the human body (American Cancer Society, 2014). Cancerous cells are initially normal cell within the body. However certain internal factors (genetic mutations, hormones, immune conditions, and mutations that occur from metabolism) and external factors (smoking, micro-organisms, chemicals, and radiation) predisposes the cells of the body to grow and divide abnormally leading to the formation of tumours which lead to the destruction of the organ affected (American Cancer Society, 2014). Not all the damaged cells however develop into the cancer state.

Some of these abnormal cells are destroyed by the immune system however when certain conditions such as drug therapy or diseases such as diabetes or HIV/AIDS weakens the immune system, these abnormal cell remain in the body and eventually result in cancer. Also if the number of abnormal cells produced is too high for the immune system to destroy cancer may develop. Cancer sometimes results in death of the individual when metastasis of the cancerous cell occurs to other parts of the body from the affected organ. Examples of the types of cancers reported are cancers of the breast, ovaries, colon, rectum, cervix, oral cavity and pharynx, prostate and lung (American Cancer Society, 2014).

Cervical cancer is the third most commonly diagnosed cancer and the fourth most common cancer related death in women globally (Ahmedin *et al.*, 2011).

2.2 BURDEN OF HPV IN WOMEN

Human papillomavirus (HPV) is the most common sexually transmitted infection and at least 50% of sexually active people will get HPV at some time in their lives (Bernard *et al.*, 2010). Infection with HPV can lead to serious diseases such as genital warts, cervical and other cancers. Though majority of the virus can be cleared within 2 years of being infected by the human immune system, some infections persist in infected cells and for people with a compromised immune system this leads to a rapid transformation of the infected cells to precancerous and cancerous lesions (Sonnex *et al.*, 2013). In particular, HPV affects those with advanced HIV infection and AIDS due to impairment of their cells. Cervical cancer has been reported to be the most common cancer affecting sub-Saharan African women with 75,000 new reported resulting in 50,000 deaths each year (Ferlay *et al.*, 2010).

In addition, a number of reports have shown that HIV-infected women are at a higher risk of infection with HPV (Palefsky *et al.*, 1999; Luque *et al.*, 2006). Though the introduction of antiretroviral therapy and increased access to HIV care in low income countries have led to improvement of clinical outcomes and life expectancy of people infected with HIV, there have not been similar improvement in cervical cancer cases and deaths.

It is estimated that 80% of women at some time during their lives are affected by hrHPV (Bovicelli *et al.*, 2000). However, most of these infections are sub-clinical or transitional and a spontaneous clearance of the infection occurs after 12-30 months in 70-90% of infected women (Alba *et al.*, 2009; Bovicelli *et al.*, 2000). For both hrHPV and low risk viruses, there are reports that the infection lasts for about 4-5 months (Alba *et al.*, 2009). Three main features of infection can result from HPV infection, namely: clinical, sub-clinical and latent form.

The prevalence of sub-clinical infection in sexually active younger women is about 40% and reduces in the older age groups (from 30 years-old) (Alba *et al.*, 2009). HPV genotypes 6-11 which are classified as low risk HPV are usually the cause of the clinical forms of HPV infection, usually a benign form. The sub-clinical forms are caused by high-risk oncogenic HPV types namely 16 and 18. The infection presents as benign lesions which can become malignant (Alba *et al.*, 2009).

A latent infection occurs when the HPV genome remains on the host cell at a relatively low copy number of approximately 100 genomes per cell. However, when there are more than 100 genomes per cell the likelihood of a productive HPV infection developing is very high (Mahalakshmi *et al.*, 2013).

An increased prevalence of hrHPV types 16, 18, 31, 33, 35, 45, 51, 52, 58, 59 is found in Africa and Latin America (Alba *et al.*, 2009). The most common HPV type in the world is HPV16, exceptions are noted in Indonesia and Algeria, where HPV 18 is more common. Whereas types 33, 39, and 59 are concentrated in Central and South America, in West Africa, there is a high frequency of HPV 45 oncogenic genotype accounting for majority of cases of cervical cancer in the region (Alba *et al.*, 2009). Figure 5 illustrates the global distribution of cervical cancer globally with Ghana located in Africa having a prevalence of greater than or equal to 41.7%.

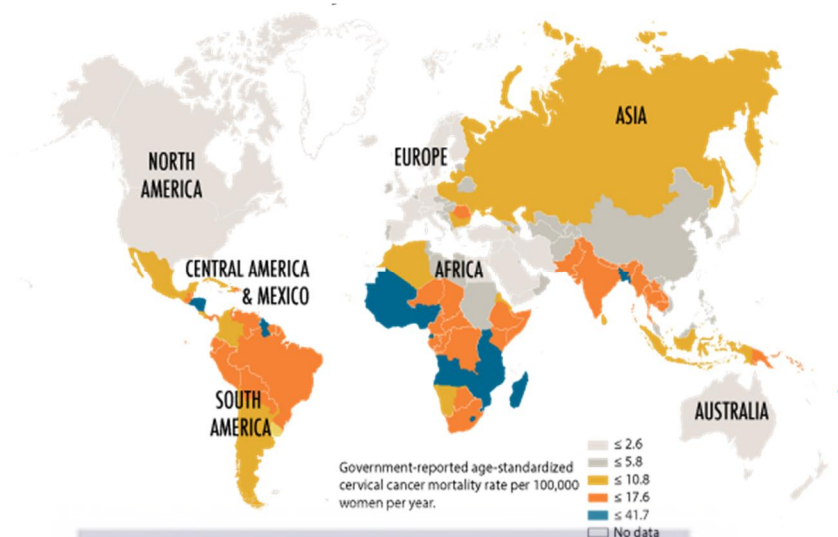


Fig. 5: Global distribution of HPV induced cervical cancer related mortality (Adapted from Crow, 2012)

2.3 HIV IN WOMEN

The human immunodeficiency virus (HIV) which is sexually transmitted has had a great impact all around the world due to the large number of people living with such infection and the frequent appearance of new cases (WHO; 2011). HIV is retrovirus which belong to the family lentivirus which are single stranded enveloped RNA viruses (Brook *et al.*, , 2007). HIV can be transmitted through body fluids like semen, breast milk and blood.

Globally, women account nearly half of the over 40 million people who are infected with the virus. Women in sub-Saharan Africa make up 57 per cent of adults with HIV. These women are with the ages 15 to 24 and their chances of being infected is three times higher than that of young men (WHO, 2004). In Ghana 225,478 persons have been reported to be infected with HIV of which 100,336 males and 125,141 females (NHARCON, 2013). The reason why women are noted to have a higher prevalence of HIV/AIDS than in men is because the male semen is known to be rich in lymphocytes which are targeted by HIV thus high viral load. Females who are the receivers during sexual intercourse tend to

harbour large volume of semen. The dendritic cells which are antigen presenting cell tries to eliminate the virus by presenting it to the body's immune system and in so doing spreads the virus throughout the body. (Kenya Youth Summit, 2009). Figure 6 is a line diagram which illustrates the high prevalence of HIV in women as compared to men in a study conducted within the period of 1985-2009.

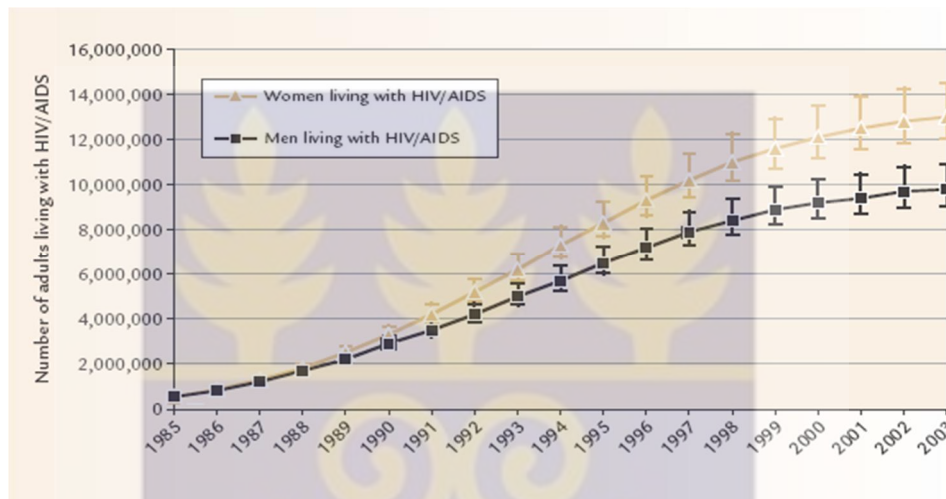


Fig 6: Estimated number of adult (15-49) women and men living with HIV/AIDS in sub-Saharan African over time (1985-2003), (Adapted from WHO estimates 2004. Women and HIV/AIDS: confronting crisis. UNAIDS)



2.4 THE EFFECT OF HIV ON HPV ACQUISITION

HIV affects the immune system CD4+ cells, thereby leading to a reduction in the body's response to infections (Feller, 2007). Some research findings have suggested that women living with HIV/AIDS have increased frequency and incidence of single and multiple infections caused by human papillomavirus (HPV) (Ferenczy *et al.*, 2003).

The natural history of HPV infection becomes altered, thereby leading to an increased risk of developing cervical cancer (CC) and contributing towards this type of cancer being the most frequently diagnosed in HIV-positive women (Clarke *et al.*, 2002). This relationship may be due to: higher HPV exposure in HIV-infected women, increased frequency of main risk factors involved in cervical cancer development or the role of HIV-related immunosuppression in favouring carcinogenesis (Chirenje, 2005). HIV infected women are eight times more likely of developing invasive cervical cancer than in HIV negative women (Clifford *et al.*, 2005b).

Many studies worldwide have shown a higher prevalence of cervical cancer among HIV-positive women than among HIV-negative women (Lesley, 2011). A more recent study conducted in South Africa showed a high prevalence and incidence of pre-cancerous cervical lesions in HIV-positive women. The study showed that women with lower CD4 counts were more likely to have abnormal Pap smears (Lesley, 2011).

HIV infection increases the prevalence and the persistence of high risk HPV infection and worsens its evolution leading more commonly to cervical high-grade squamous intraepithelial lesions (HSIL) and cervical cancer (Palefsky, 2003; Ahdieh *et al.*, 2001). The overall combined estimate of HPV16 and HPV18 prevalence among cervical cancer cases in sub-Saharan Africa is 69.2%, which is consistent with the worldwide estimate of 70%

(Louie *et al.*, 2009). However, about 50% of HIV-positive women with HPV 16 and/or 18 are co-infected with other HPV types, so it is unclear whether the other high risk types would be responsible for invasive disease in the absence of HPV 16 or 18 (De Vuyst *et al.*, 2008)

2.5 OVERVIEW OF CERVICAL CANCER IN GHANA AND IN AFRICA

Cervical Cancer is the leading cause of cancer related morbidity and death among females in Ghana and in other developing countries. There is overwhelming evidence to suggest that, majority of cases of cervical cancer is caused by infection with the high risk oncogenic types of Human Papillomavirus (Reports of National strategy for cancer control in Ghana, 2012 - 2016). As at 2009, estimates indicated that approximately 6.57 million Ghanaian women aged 15 years and older were at risk of developing cervical cancer. Current estimates indicate that of 3,038 women diagnosed every year, 2,006 die from the disease.

In Ghana, Domfeh *et al.* (2008) in their work on HPV using a sample size of 75 found a prevalence of 10.7%. They suggested that there could be a distinctive feature of polygamous populations where HPV transmission continues in the middle age and cervical cancer incidence was high. The sample size used in this study was too small (75) to give a true reflection of the HPV prevalence rate in the country. The study by Domfeh *et al.* (2008) focused on detecting the HPV DNA but didn't genotype the HPV to determine whether they were high risk HPV or low risk HPV which would informed them the chances of these consenting women developing cervical cancer or not. Also detection of any abnormalities in the cells taken from the transformational zone was not done in this the study. This would have informed them if these were prone to or were developing cervical abnormalities suggestive of cervical cancer. However the study indicated that illiteracy

aside multiple sexual partners was one of the risk factors for acquiring HPV infection and this was similar to the finding by Thomas *et al.* (2004).

Attoh *et al.* (2010) in their work reported genotypes 16, 18, 35, 39, 45, 52, 56 and 66 as the oncogenic types dominant in women with cervical cancer after genotyping formalin fixed paraffin embedded blocks of 50 patients with histologically proven cervical cancer. These were archival specimens obtained from the Pathology Department, Korle-Bu Teaching Hospital, Accra, between January and December 2003. However genotype 18 was the most prevalent in this study instead of genotype 16 which is normally encountered in most cervical carcinoma cases worldwide.

Denny *et al.* (2013) reported genotypes 16, 18, 35, and 45 as the most common HPV types in sub-Saharan African women with invasive cervical carcinoma and HPV infections were more common in HIV-positive women. Denny *et al.*, 2013 analysed paraffin embedded tissue blocks from women with diagnosed invasive cervical cancer which was similar to the work done by Attoh *et al.* (2010). Both studies didn't indicate the demographic of the women samples were used to ascertain the risk factors for HPV induced cervical cancer in these women. Also the use of formalin fixed paraffin embedded blocks of 50 patients with histologically proven cervical cancer used would not be ideal since in cases where the cervical sample was taken appropriately, certain HPV genotypes will be missed.

Groesbeck *et al.* (2006) detected a high prevalence of abnormal squamous cytology in Zambia. They also found out that 85.3% of the HIV infected women had high risk HPV types and reported that their findings were the highest reported in any population worldwide. The liquid based cytology (LBC) was used for the cervical smears collection. This method is known to yield better results than the route in Pap smears as cytology

reduces the unsatisfactory results that are mostly reported in pap smears which can result in false positive or false negative. However the though it is more expensive. The LBC method is much more expensive the Pap smear thus cannot be routinely used in hospitals.

In Nigeria, Akarolo-Anthony *et al.* (2013) reported a high prevalence of high risk HPV infection as well as multiple HPV in HIV positive women than in HIV negative. In decreasing order, HPV35, 56, 58, 59 and 45 were the most prevalent types of high risk HPV infection found among those who were HIV positive for single infection and for multiple infections the high risk genotypes detected were 16, 24-27, 38 and 39 (Akarolo-Anthony *et al.*, 2013). In their study their questionnaire didn't factor in any predisposing conditions such as sexual behaviour, sexual hygiene, diet, smoking, alcohol consumption, concurrent genital tract infections, other illnesses and sexual partner health behaviour that will increase the women acquisition of HPV infection leading to cervical cancer

2.6 CONTROL ISSUES

Women who are already sexually active should have regular cervical screening which will enables early detection of HPV induced preinvasive lesions before they progress to cervical carcinoma. Cervical screening exposes premalignant cervical lesion which are usually asymptomatic as compared to invasive cervical lesions which presents as post-coital bleeding. Due to the asymptomatic nature of these premalignant cervical lesions, cervical screening which can either a Pap smear or visual inspection of cervix with acetic acid (VIA) are the only way these lesions can be detected early before they progress to invasive cervical cancer in many hospital in Accra (Adadevoh and Forkouh, 1993; Sanghvi *et al.*, 2008; Adanu *et al.*, 2010). Screening for cervical cancer is practised more in developed countries than in developing countries (Fig.7).

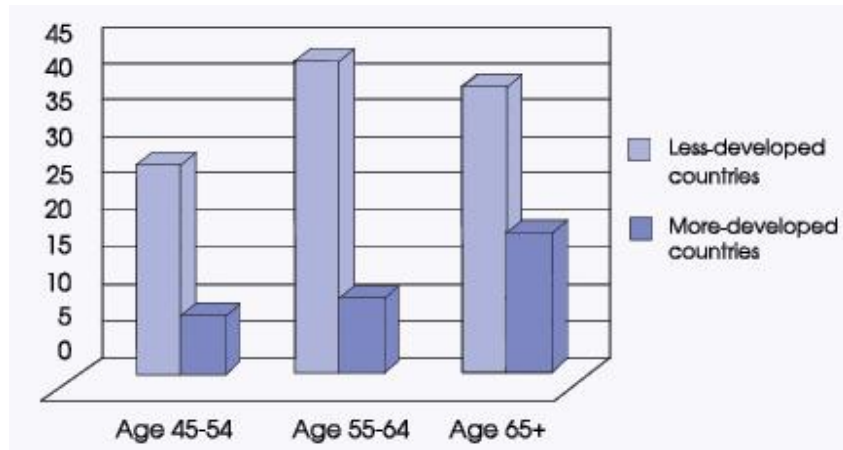


Fig 7: Age-specific cervical cancer mortality rates per 100,000 women. (Adapted from Cervical cancer mortality rates in both developed and developing countries, RHO Cervical Cancer, 1997-2012).

Adanu *et al* (2010) reported that the Ghana currently does not have a national wide screening programme. Most of the cervical screening that takes place in the country are described as opportunistic whereby the screening is requested for during general medical examination or in cases unrelated to cervical cancer. This thus account for low patronage of Pap smears in Accra (Adanu, 2002; Adanu *et al.*, 2006; Duda *et al.*, 2007).

Adanu *et al.* (2010) recommended that cervical screening should be perform as part of the general gynaecological examination even in the absence of symptoms suggestive of cervical cancer. Also the national health insurance scheme does not cover a Pap smear test. Adanu *et al.* (2010) reported that it cost GH 30 (US\$ 20) to undergo a Pap smear and GH 1.50 (US\$1) for the speculum which is used during sample collection. Presently, it cost GH 55 (US\$19) for a Pap smear at the Pathology Department, Medical School (personal communication, April 18, 2014). However 45% of the average Ghanaian live on less than US\$ 1 per day (Ibrahim Index of African Governance, 2008).

Thus it will be difficult to justify the need to spend more than they earn on preventive care when they are not having any symptom requiring medical attention. In order to reduce the incidence of cervical cancer, a national wide screening programme should be established by the Ministry of health and Ghana Health Service which is supported by the national health insurance scheme. However, until this happens there is the need for the gynaecology units to establish a protocol whereby every new gynaecology patient who is sexually active should have a Pap smear performed as part of the required investigations (Adanu *et al.*, 2010).



CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

The study was cross sectional.

3.2 STUDY SITE

The study site was the Fevers Unit of the Korle-Bu Teaching Hospital (KBTH). The Fevers unit, where patients with infectious diseases such as HIV/AIDS are managed by clinicians, is an important part of KBTH.

3.3 STUDY SUBJECTS

The study subjects were women of ages 18 years to 65 years old living with HIV that attend Antiretroviral Therapy (ART) clinic at KBTH Fevers Unit.

3.3.1 Inclusion criteria

- i. Women in the age range of 18 to 65 years of age living with HIV attending Antiretroviral Therapy (ART) clinic at KBTH's Fevers Unit.
- ii. Women who consented to the study.

3.3.2 Exclusion criteria

- i. Women who had undergone a hysterectomy.
- ii. Women who did not fall within the age range for this study
- iii. Women who were pregnant and have had a history of previous diagnosis or treatment for cervical neoplasia.
- iv. Women who did not consent to this study.

3.4 ETHICAL APPROVAL

Ethical approval for the study was obtained from the University of Ghana Medical School Ethical and Protocol Review Committee. In addition, informed written consent (Appendix I) was obtained from participating subjects before they were recruited into the study.

3.5 SAMPLE SIZE DETERMINATION

One hundred subjects were involved in this study. This number, based on 21.5% prevalence of HPV among women with normal cytology in West Africa (WHO/ICO Information Centre on HPV and Cervical Cancer, 2009), was generated using the formula:

$$N = [Z^2 (P) (1-P)] / (d)^2$$

Where:

N= desired sample size population

Z= the standard normal deviation (set at 1.96 corresponding to the 95% CI).

P= the proportion in the target population estimated to have the outcome.

d= Precision or degree of accuracy desired (set at 0.05).

3.6 SAMPLE COLLECTION

Upon arrival at the ART clinic at KBTH Fevers Unit, female patients were educated about cervical cancer and the benefits they stand to gain from participating in the project. They were informed that to screen for cervical cancer, they would have to undergo a pap smear which will be taken by a gynaecologist. This would be analysed in the laboratory after which those with any form of abnormality will be called for immediate medical attention. All this education was done in the English language or any suitable Ghanaian language for

each participant. Any further explanation was given to make sure that the patients really understood what the project was all about.

A structured questionnaire (Appendix II) was administered to consenting female patients in order to obtain relevant information that is useful for the project. These women were ushered into an enclosed room where their pap smears were taken by a gynaecologist using a pap kit which contained a glass slide, an Ayres spatula and a swab stick. The pap smears were sprayed with a cytology spray fixative to preserve the cervical cells prior to pap staining. The swab sticks were broken into the DNAgard (Biometrica Co, USA) which is photosensitive and thus was placed in a bag to prevent direct sunlight in order to avoid damaging the DNA of any HPV that maybe present in the cervical samples awaiting HPV DNA extraction. Cervical swab samples stored in DNAgard were kept at room temperature (25 -28°C).

All the cervical samples were assigned a pathological number for easy identification. The recent CD4+ T cell count of the women were obtained from their folders.

3.7 LABORATORY PROCEDURES

The pap smears were sent to the cytology laboratory of the Department of Pathology of the Medical School (KBTH) where they were stained with Papanicolau stain.

3.7.1 Papanicolau staining procedure

The slides were fixed into a paraffin based alcohol fixative for 15 min. Next they were introduced into absolute alcohol, 70% alcohol and 50% alcohol, each for 2 min. They were then immersed in distilled water for 3min. After 3min they were transferred into the nuclear stain, Harris' haematoxylin solution to stain for 3 min. The slides were then blued under a

weak stream of tap water 3-5min. After blueing the slides, they were reintroduced into increasing grades of alcohol namely 50 %, 70 %, 80 % and 96 %, for a minute each. The cytoplasmic staining was next done using orange G solution for 3 min. The slides were then washed with two changes of 96%alcohol. The slides were next stained in the polychromatic stain (Eosin Azure solution) for 3 min. The slides were then dehydrated in two changes of 96%alcohol.

Dehydration was continued in absolute alcohol for 5 min, then in equal parts of absolute alcohol and xylene. Clearing was done with xylene for 2 min after which the slides were mounted with DPX mountant. The stained Pap smears were then examined microscopically by a cytologist to assess the severity of cervical neoplasia among HIV infected women, using the Bethesda system of classification (Solomon *et al.*, 2002).

3.7.2 HPV DNA extraction

DNA extraction was done using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

To a cervical swab sample stored in DNAgard was added 20 μ L Qiagen protease stock solution (or proteinase K) and 300 μ L Buffer AL. The tube was vortexed for 15 seconds and then centrifuged at 5000rpm for 5 min after which the swab was removed from the tube and discarded. The filtrate obtained was pipetted into a 2ml Eppendorf tube and incubated at 56°C for 10 minutes, with occasional briefly centrifugation to remove drops from inside the lid. After the incubation, 400 μ L absolute ethanol was added to the tube, followed again by vortexing for 15 seconds.

The tube was then centrifuged briefly to remove drops from inside the lid and 700 μL of the content was carefully applied to a QIAamp mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and centrifuged at 8000rpm for 1 minute. The tube containing the filtrate was discarded and QIAamp mini spin column placed in a second 2ml collection tube. The QIAamp mini spin column was carefully opened and 500 μL of Buffer AW was added without wetting the rim. The cap was closed followed by centrifugation at 8000rpm for 1 minute. The QIAamp mini spin column was placed in a clean 2ml collection tube and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 500 μL Buffer AW2 was added without wetting the rim. The cap was then closed and centrifuged at full speed (14,000rpm) for 3minutes. The QIAamp mini spin column was placed in a new 2ml collection tube and the old collection tube was discarded with the filtrate. It was then centrifuged at full speed for 1 minute. This helped to eliminate the chances of possible Buffer AW2 carry over. The QIAamp mini spin column was placed in a clean 1.5ml microfuge tube and the old collection tube was discarded with the filtrate. The QIAamp mini spin column was carefully opened and 50 μL Buffer AE was added, incubated at room temperature (25 - 28°C) for 1 minute and centrifuged at 8000 rpm for 1 minute. The filtrate was equally divided into 2 separate 2ml tubes and stored at -20°C prior to HPV genotyping.

3.7.3 Genotyping of samples using nested PCR analysis

HPV-DNA detection and identification of the genotypes was carried out by nested multiplex PCR (Soltar *et al.*, 2004). Primers for the identification of high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 and low-risk genotypes 6/11, 42, 43, and 44 were used. The primers were used in four cocktails (Fig. 8), each containing four to five different primer pairs. .

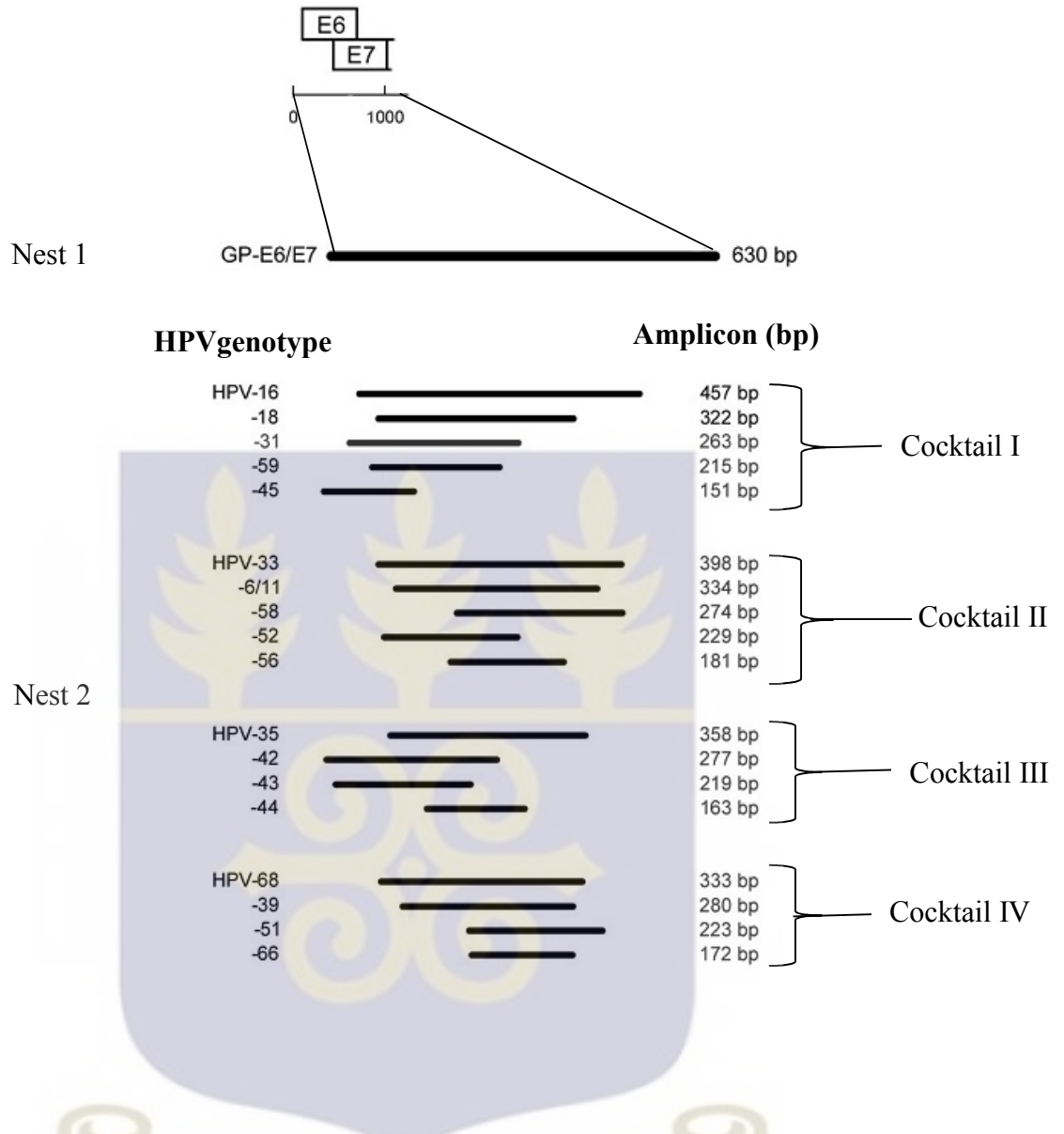


Fig.8: HPV detection and typing by GP-E6/E7 NMPCR. Diagram of PCR amplicons positions relative to the HPV-16 genome. Type specific nested PCR primers were arranged in four cocktails (Adapted from Soltar *et al.*, 2004).

The identification of each HPV type present was achieved by determining the size of the nested PCR amplification product by gel electrophoresis.

A single consensus forward primer (GP-E6-3F) and two consensus back primers (GP-E7-5B and GP-E7-6B) were used for first round PCR (nest 1). The PCR reaction mix of 50µl contained 1X PCR buffer (Promega, USA), 2.5 mM MgCl₂, 200 µM of each of the four oligonucleotide triphosphates (dNTPs) (Promega, USA), 15 pmols of each E6/E7 consensus primers and 1.25U of *Taq* polymerase (Promega, USA). Four microliters (4 µl) of DNA extracts was used as template for the amplification reactions. The cycling parameters for the first round PCR were: incubation at 94°C for 10 min, followed by 40 cycles of 1min denaturation at 94°C, 1min annealing at 55°C, and 1 min elongation at 72°C. The last cycle was followed by a final extension step of 7 min at 72°C.

In the second round PCR (nest 2), 1 µl of first round PCR product and 15 pmols of forward and reverse primers were used for genotyping. The other parameters that were used in the first round PCR mix were the same. The cycling parameters used were: 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 40°C, and 1.5 min elongation at 72°C and final extension of 7 min at 72°C.

3.7.4 Gel analysis of nested PCR products

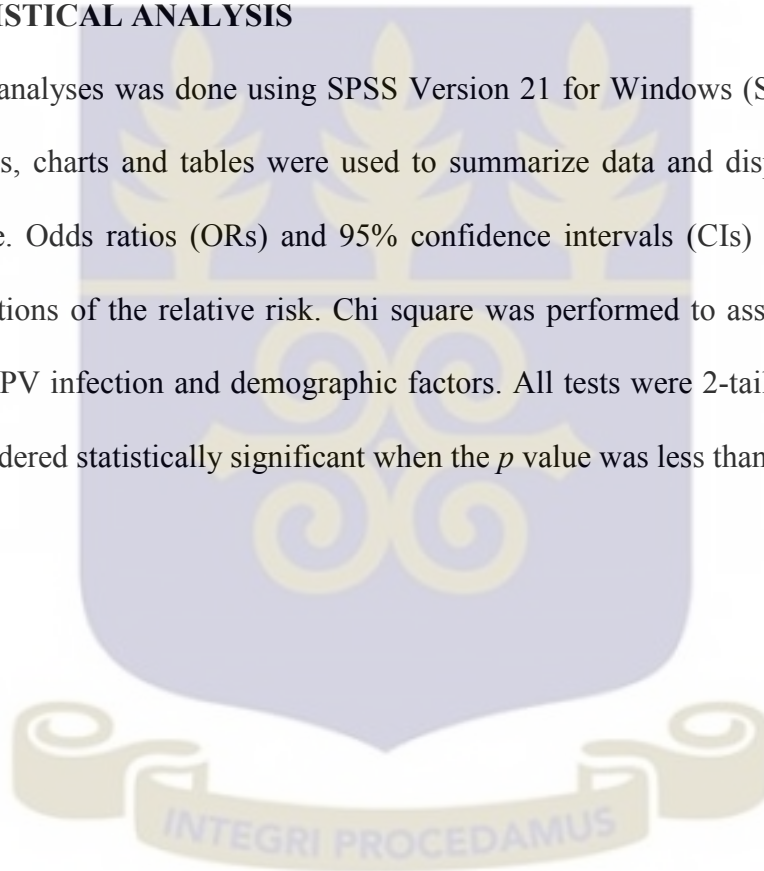
Following the PCR, 10 µl of each PCR product was added to 2µl of 6 X bromophenol blue or orange G loading dye and electrophoresed in 2% agarose gel stained with 0.5µg/ml ethidium bromide. The gels were prepared and run in 1x TAE buffer at 100V for 30-45 mins and were observed and photographed over a UV transilluminator at short wavelength using photographed using a Kodak EDAS 290 gel documentation system. The sizes of the

PCR products were estimated by comparing with the mobility of a standard 100bp DNA ladder.

The results obtained from the cervical cytology were compared with the HPV genotypes detected to determine the genotypes that were responsible for causing cervical cancer in HIV infected women.

3.8 STATISTICAL ANALYSIS

Statistical analyses was done using SPSS Version 21 for Windows (SPSS Inc, Chicago, IL). Graphs, charts and tables were used to summarize data and display figures where appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated as approximations of the relative risk. Chi square was performed to assess the association between HPV infection and demographic factors. All tests were 2-tailed and differences were considered statistically significant when the *p* value was less than 0.05



CHAPTER FOUR

4.0 RESULTS

4.1 SOCIO-DEMOGRAPHIC DATA

A total of 100 participants (mean age 40.54 ± 3.32 years) were involved in the study (Table 1). The 30 - 39 years age groups had the highest frequency (45%) of women. Majority of the women were Akans (41%), ended their education at the primary level (53%), were married (38%), were traders (67%) and earned GH¢100 (43%) in a month.

4.2 SOCIO-DEMOGRAPHIC FACTORS AND HPV INFECTION

Women who were less than 40 years were 0.348 times more likely of having HPV infection than those who were more than 40 years (p value = 0.02). Marital status however, did not have a significant association with HPV (p value = 0.312).

Women who earned \leq GH¢500.00 had 0.039 times chances of HPV infection than those who earned $>$ GH¢ 500 but this was statistically insignificant (p value = 0.843). Women whose education was below tertiary level had 2.05 times chances of being infected with HPV but this was also statistically insignificant (p value = 0.15).

4.3 RISK FACTORS AND HPV INFECTION

4.3.1 Sexual behaviour characteristics

Table 2 shows the sexual behaviour characteristics of the women. At the time of the study, 34% of the women had partners who they were living together with. Majority of the women (54%) have not had a sexual partner for the past six months. Having had three life time partners was the highest (35%).

Table 1: Socio-demographic characteristics of the subjects

Variable	Category	Percentage (%)
Age	20 – 29 years	7.0
	30 - 39 years	45.0
	40 - 49years	35.0
	50 – 59 years	10.0
	60 – 69 years	3.0
Ethnicity	Ga-Dangme	16.0
	Akan	41.0
	Ewe	23.0
	Northern	19.0
	Other	1.0
	Level of education	Primary
	Secondary	18.0
	Tertiary	7.0
Marital status	None	22.0
	Single	20.0
	Married	38.0
	Widowed	21.0
	Divorced	21.0
Average income (GH¢)	100	43.0
	100 - 250	27.0
	250 - 500	10.0
	>500	4.0
	NA	16.0
Occupation	Trader	67.0
	Unemployed	15.0
	Hair dresser	3.0
	Seamstress	5.0
	Others	10.0

Table 2: Sexual behaviour characteristics

Variable	Category	Percentage%
Sexual partners	Yes	34.0
	No	4.0
	N/A	62.0
Sexual partners last 6 months	No	54.0
	Yes	46.0
Sexual partners life time	1	4.0
	2	19.0
	3	35.0
	4	16.0
	5	15.0
	>5	11.0
	N/A	1.0
Sexual frequency (times per month)	1-14	45.0
	N/A	55.0
Age at first coitus (years)	10-14	10.0
	15-19	65.0
	20-24	17.0
	25-29	5.0
	30-34	2.0
	N/A	1.0
	N/A	1.0
Contraceptive use	Always	35.0
	Sometimes	23.0
	Never	42.0

Women who had less than or equal to three sexual partners had 1.48 chances more of HPV infection than those who has more than three sexual partners. However this was not statistically significant (p value = 0.36). Those who have had <5 lifetime partners had 0.75 chance less of HPV infection than those who has >5, though this was also not statistically significant (p value = 0.574).

Majority of the women (65%) had their first sexual intercourse within the age group of 15-19 years of age with a mean age of 17.98 (\pm 5.22). Teenagers had 1 times chance of being infected with HPV than adults but this was not statically significant (p = 1.00). Women who had no sexual intercourse had 0.711 times of having HPV infection than those who had between 0-14 times per month of HPV infection. However this was not statistically significant with (p value = 1).

Women who always used contraceptives had 1.369 times more chances of HPV infection than those who never or sometimes used contraceptive but this was not statistically significant (p value = 0.49).

4.3.2 History of pregnancy

The history of pregnancy of the women is shown in Table 3. Out of the 100 women, 94% have had a history of pregnancy. The highest age group for first time pregnancy was 15-19 years (41%) with a mean age of 20.8 \pm 3.5 years. Most of the women (30%) have been pregnant for than five times.

Teenagers had 0.816 times of having HPV infection than adults but this was not statistically significant (p value = 0.646).

Table 3: History of pregnancy

Variable	Category	Percentage%
First pregnancy age	10-14	3.0
	15-19	41.0
	20-24	31.0
	25-29	12.0
	30-34	6.0
	N/A	6.0
	32.00	1.0
Number of times pregnant	1	14.0
	2	21.0
	3	14.0
	4	12.0
	5	9.0
	>5	30.0
Miscarriage	None	78.0
	1	15.0
	2	5.0
	6.	1.0
	7.	1.0
Abortion	1	27.0
	2	10.0
	3	9.0
	4	1.0
	5 and above	6.0
	None	47.0
History of pregnancy 1st sex	10-14	10.0
	15-19	65.0
	20-24	17.0
	25-29	5.0
	30-34	2.0
	N/A	1.0
Contraceptive use	Always	35.0
	Sometimes	23.0
	Never	42.0

4.4 ASSOCIATION BETWEEN CD⁺ 4 COUNT AND HPV INFECTION

More HPV genotypes were detected in women with CD⁺ count < 350 cells/ μ l compared to those with CD⁺ count > 350 cells/ μ l (Fig. 9). In addition, women whose CD⁺ count was < 350 cells/ μ l had 0.66 times chances of being infected by HPV but this was not statistically significant (p value = 0.36).

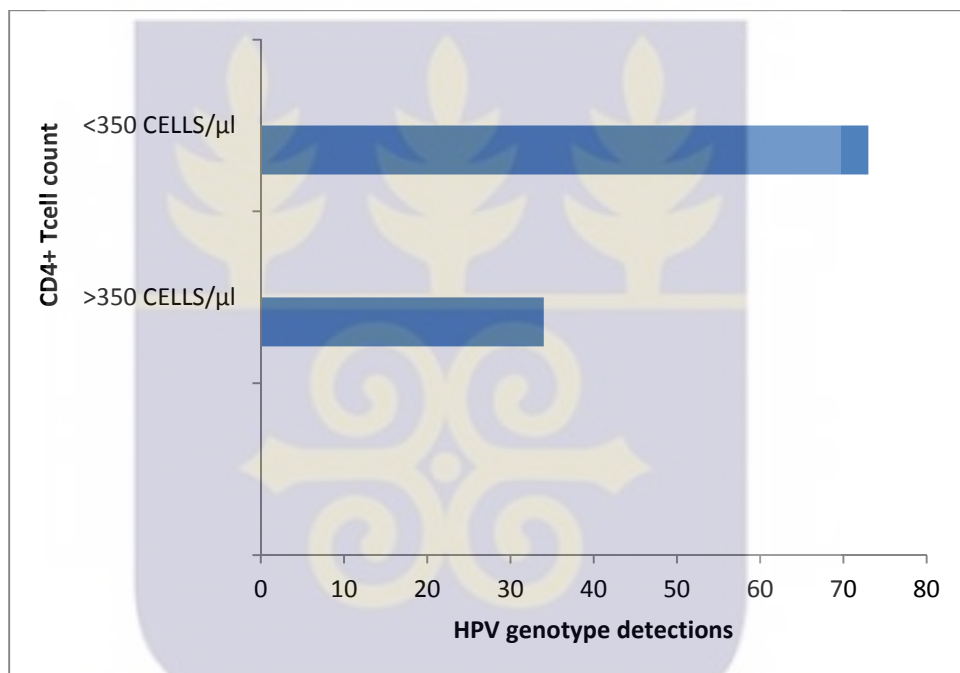


Figure 9: Association between of the CD⁺ 4 count of participants and HPV infection.

4.5 CYTOLOGICAL FINDINGS

Fig. 10 shows the cytological findings detected. Only seven (7%) of the 100 women whose Pap smears were cytological by examined showed cytological abnormalities. These were classified as either high grade squamous intraepithelial lesion (HGSIL), low grade intraepithelial lesion (LGSIL), atypical squamous cell of unknown significance (ASCUS) or atypical squamous cell - cannot ignore high grade (ASC-H). LGSIL recorded the highest percentage (3%).

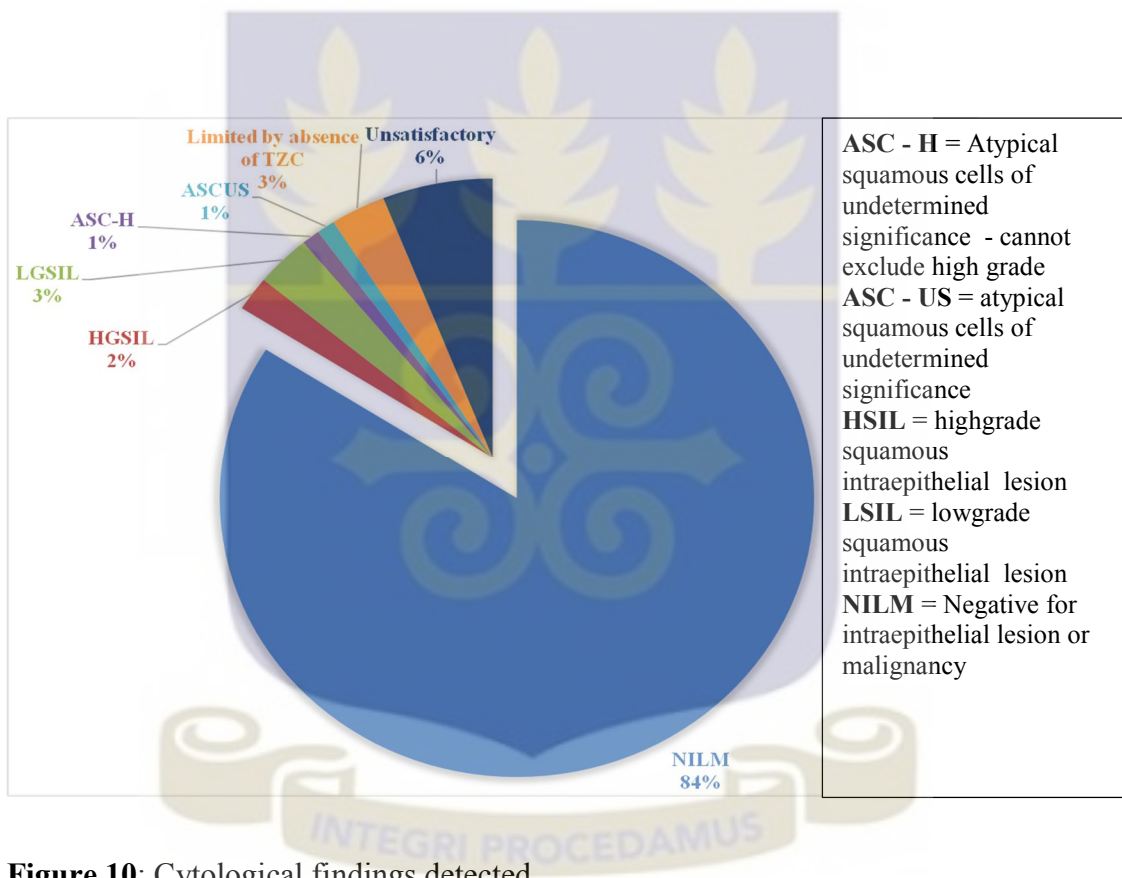


Figure 10: Cytological findings detected

4.6 GENITAL INFECTIONS

Genital infections which were detected were bacteria vaginosis, vaginitis, atrophic cervicitis and candidiasis (Fig. 11). Vaginitis recorded the highest frequency of 12% with atrophic vaginitis and candidiasis recording the lowest infection detected of 4%.

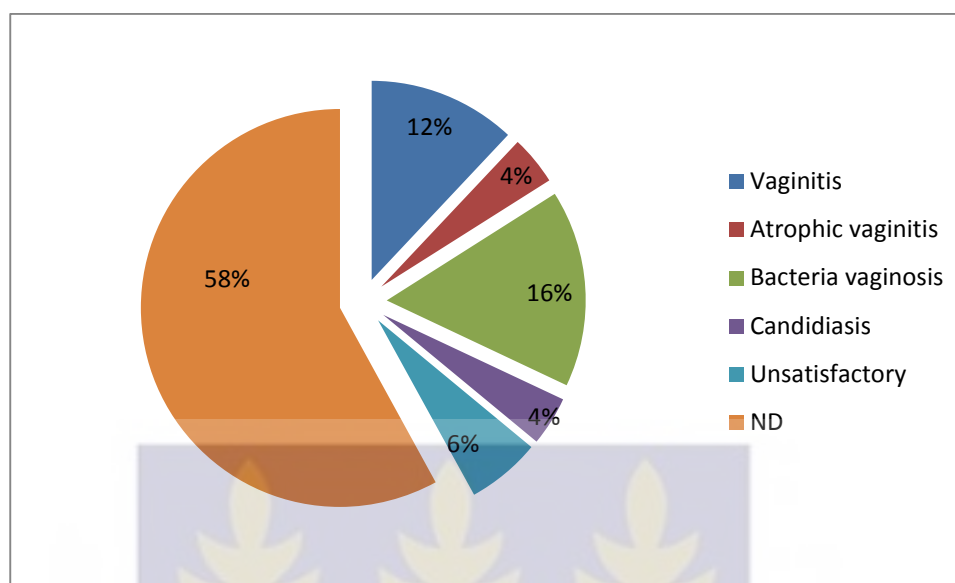


Figure 11: Genital infections detected.

4.7 HPV GENOTYPE DETECTION

Seventeen HPV genotypes (12 high risk and 5 low risk) were detected by PCR. Both single and multiple infections were also detected. The genotypes detected had the expected DNA fragment sizes (Figs 12a and b).

A total HPV prevalence of 61.4% was observed at the end of this study. The prevalence of hrHPV infection was 54%. Genotypes 35 (12%) which is a high risk and 42 (12%) which is low risk were the most prevalent (Fig. 13). Genotypes 16 (2%), 39 (2%), 59 (2%) and 56 (1%) were the least prevalent. There were 41% single HPV infections (Fig. 14) and a total of 27% multiple HPV infections. Of the multiple infection, double infections (13%) were the highest (Fig. 14). The 40 -49 age group (mean age 40.53 ± 5.58) had the highest HPV detection frequency (Fig. 15).

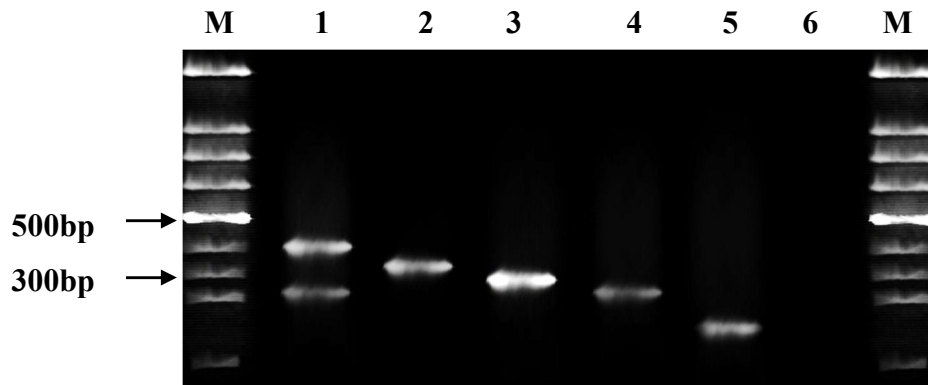


Figure 12a: A representative agarose gel electrophoregram of second round PCR products from Cocktail II.

Lane 1 = genotypes HPV33/52; Lane 2 =HPV6/11; Lane 3 =HPV58; Lane 4 = HPV52, Lane 5 = HPV56; Lane 6 = negative control; Lanes M = 100bp DNA ladder.

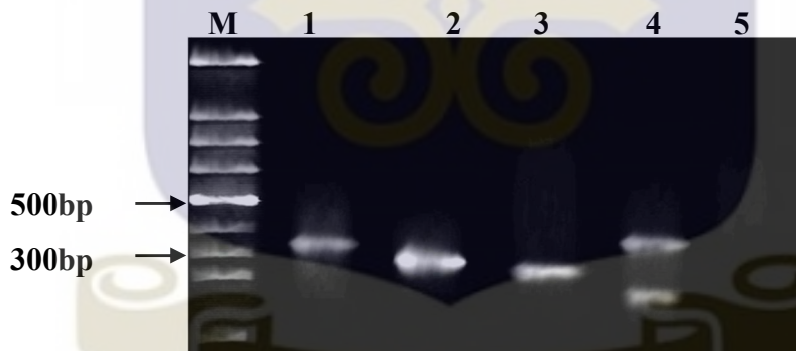


Figure 12b: A representative agarose gel electrophoregram of second round PCR products from Cocktail IV

Lane 1 = genotypes HPV68; Lane 2 =HPV39; Lane 3 = HPV51; Lane 4 = HPV66/68, Lane 5 = HPV56; Lane 5 = negative control; Lane M = 100bp DNA ladder.

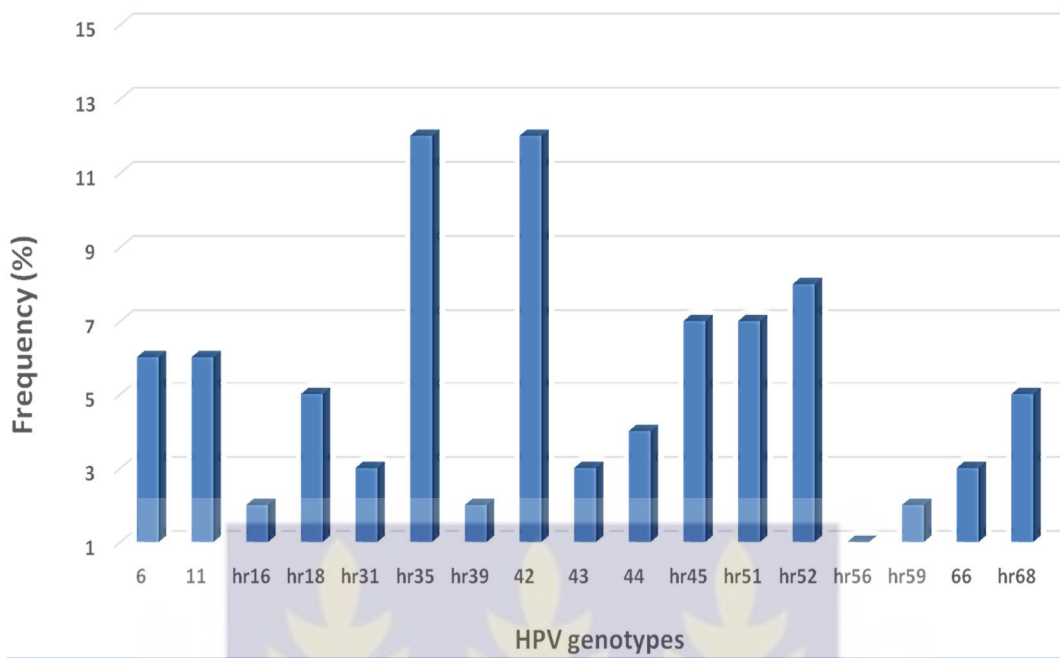


Figure 13: Distribution of the HPV genotypes. (hr = high risk)

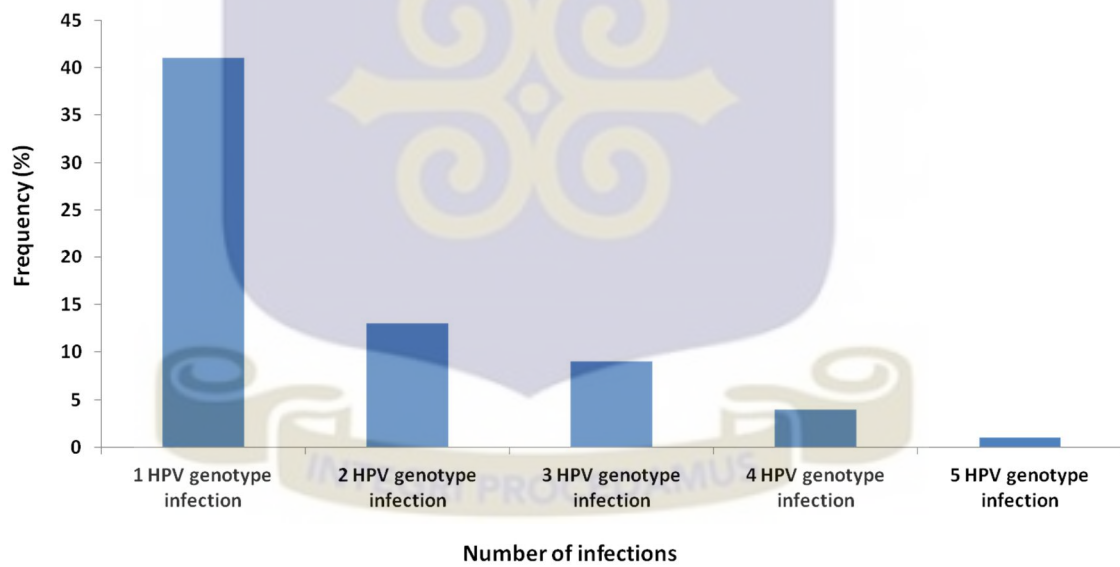


Figure 14: Distribution of both single and multiple HPV infections.

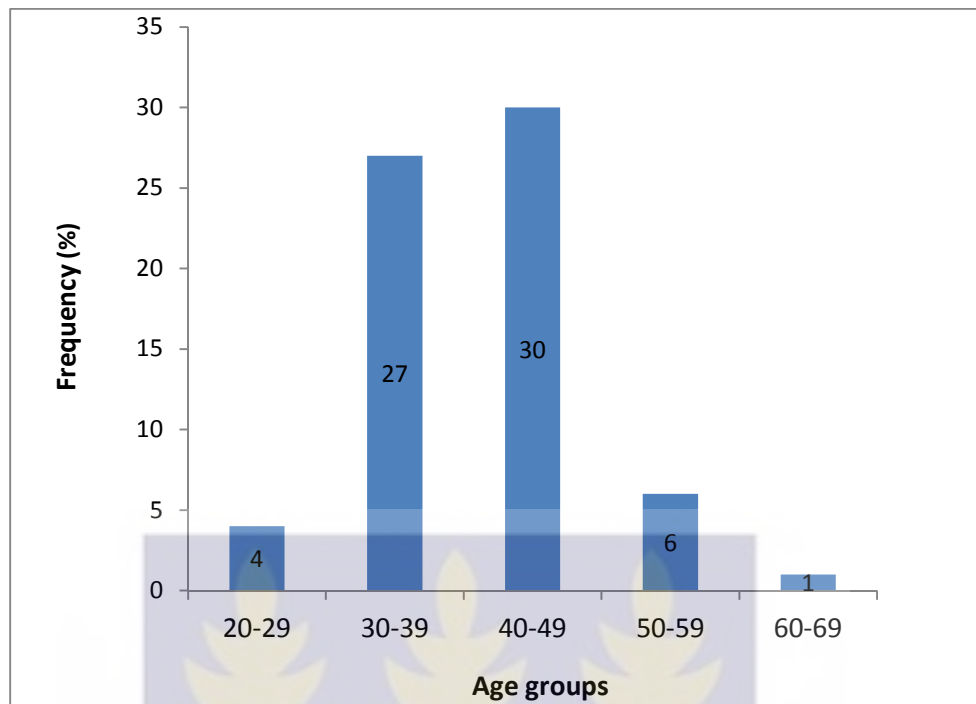


Figure 15: Percentage distribution of the various HPV genotypes detected against the various age groups.

4.8 ASSOCIATION BETWEEN HPV GENOTYPE AND CYTOLOGICAL FINDINGS

With the exception of ASC-H which was associated with only one HPV genotype, the rest were associated with multiple HPV genotypes, of both high and low risk types (Table 4). The most common hrHPV were 35 and 45, whilst the most common low risk HPVs were 42 and 44. With the genital infections, only bacteria vaginosis was associated with multiple HPV genotypes (Table 5).

Table 4: HPV genotypes in relation to the cytological findings..

Cytological finding (n)	HPV genotypes	
	High risk	Low risk
HSIL (2)	35,45,52,56	42
LSIL (3)	35	44
ASC-H (1)	45	
ASC-US (1)	51	42,44,66

Table 5: HPV genotypes in relation to the genital infections

Infections (n)	HPV genotypes	
	High risk	Low risk
Bacteria vaginosis (16)	16, 18, 35, 66, 68	6, 11, 42, 43, 44
Vaginitis (12)	52	
Atrophic cervicitis (4)	39	
Candidiasis (4)	45	



Women who have NILM have 3 times more chances of HPV infection than those who have cervical neoplasia. However this was not statistically significant (p value= 0.275). Also women who have NILM have 1.4 times more chances of multiple HPV infection than those who have cervical neoplasia. However this was not statistically significant with (p value= 0.712). Women who have genital infections have 0.088 times more chances of HPV infection than those who have no genital infection. This was statistically significant (p value = 0.004).



CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

This study determined the prevalent HPV genotypes in women living with HIV at the Fever's Unit of the Korle-Bu Teaching Hospital. In the present study, a very high prevalence (61.4%) of HPV was recorded. This is close to the prevalence of 63% reported by Palefsky *et al.* (1999) in HIV infected women in the United States but was high than the HPV prevalence (52.4%) recorded by McDonald *et al.*, (2014) in HIV positive women. This indicates that policy makers need to implement measures to reduce the prevalence rate thereby reducing morbidity and mortality rate that are associated with HPV induced cervical cancer.

A total of seventeen HPV genotypes were detected in the women. The high risk genotypes were 16,18,31,35,39,45,51,52,56,59,66 and 68 whilst the low risk genotypes were 6,11,42,43 and 44. A total high risk HPV prevalence of 54% was recorded at the end of the study. This is higher than the 45% high risk HPV prevalence recorded by Musa *et al* (2013). High prevalence of hr HPV genotypes in these women indicates that their chances of developing cervical cancer is high.

Among the high risk HPV genotypes, 35 (12%) had the highest frequency which was similar to what Akarolo-Anthony *et al.* (2013) reported in their study of HPV in HIV Nigeria women. In case of the low risk HPV genotypes, 42 (12%) recorded the highest frequencies. Genotypes 16 (2%), 39 (2%), 59 (2%) and 56 (1%) were the least prevalent high risk HPV genotypes. A prevalence of 27% multiple HPV infections which was lower than the 36% reported by Palefsky *et al.* (1999) was observed in this study. For single HPV

infections a prevalence of 41% was recorded. The high prevalence of non 16 and 18 hrHPV genotypes that were detected in this study was similar to the findings by Akarolo-Anthony *et al.* (2013), McKenzie *et al.* (2010), Bollen *et al.* (2006) and Luque *et al.* (2006).

Lifestyle factors such as sociocultural characteristics, nutritional, environmental, sexual behaviour etc. were accessed and this explained the varying prevalence of hrHPV and cervical cancer incidence across populations. Amongst the socio-demographic factors, age was found to have a significant effect on HPV in this study and this agrees with what Burk *et al.* (1996) reported. However marital status, level of education, number of sexual partners and history of pregnancy had no association with HPV. Previous studies have found that education is a relevant factor for HPV prevalence (del Amo *et al.*, 2009). Some studies concluded that education is a protective factor against HPV infection (Khan *et al.*, 2005) whereas others determined that educational level had no relationship with HPV infection and, in one case, a higher educational level was found to be associated with higher HPV prevalence (Sherpa *et al.*, 2010).

In assessing other risk factors that increase the chances of HPV infection, smoking was not found to be a risk factor as all the women had no smoking experience. Most of the women also reported that they never or sometimes used contraceptive during sex and this may explain the high HPV prevalence in this study. Multiple sexual partners is known to increase the risk of HPV infection and this study recorded a higher incidence of multiple sexual partners which is similar to what Peyton *et al.* (2001) reported that multiple sexual partners makes one susceptible to various high risk genotypes.

CD4 T cell count which gives an indication of the level of immune suppression was also accessed as one of the risk factors. A CD4 T cell count value of > 350 cells per/ μ l was

reported by Sabin *et al.* (2009) as the level to initiate highly active antiretroviral therapy (HAART). However there was no significant association between HPV and CD4 T cell count and this was similar to the findings by Isaakidis *et al.* (2013).

Pap smears examination for the presence of any cytological abnormalities showed that 84% of the women reported negative for squamous intraepithelial lesion or malignancy (NILM). However hrHPV genotypes being detected (16, 18, 31, 35, 45, 51, 52, 59, 66 and 68) does not eliminate the chances of future cervical abnormalities since it takes years for the disease to manifest based on certain underlining conditions such as immune status.

Cervical abnormalities detected were HSIL and LSIL which are the pre invasive lesions. The other abnormalities detected were ASCUS and ASC-H which were based on the quality and quantity of epithelial cells. Women who reported HSIL and ASC-H must undergo colposcopy and biopsy to access the level of malignancy. However women who reported for ASCUS and LSIL must undergo a repeated Pap smear after 6 months to 1 year to find out if the lesion will change to a HSIL or will be resolved by then. This study did not proceed to determine any cytological malignance from the various squamous lesions detected thus cannot be compared with the finding of Attoh *et al.* (2010) who looked at the prevalence of HPV in confirmed cervical cancer cases. LSIL had the highest prevalence of 3%, HSIL (2%) and ASCUS and ASC-H recording 1% respectively. These findings were lower than what Groesbeck *et al* (2006) reported in their study: HSIL (32.7%), LSIL (23.3%), ASCUS (17.3%) and SCC (20%). Their findings have been reported as being the highest prevalence of abnormal cytology ever reported in any population. Isaakidis *et al.* (2013) also reported LSIL and HSIL of 14% and 5%, respectively, in HIV infected women in Mumbai, India.

HPV genotypes were detected in the women who had other infections. For those who had bacteria vaginosis 6, 11, 42, 43, 44 (low risk genotypes) and 16, 18, 35, 66, 68 (high risk genotypes) were detected. For vaginitis, HPV genotypes 52 (high risk), atrophic vaginitis HPV genotype 39 (high risk) and candidiasis genotypes 45 (high risk) were detected. Women who had infections had 0.088 chances of HPV infections than those who had no infection (p value = 0.0004). This confirms what Samoff *et al.* (2005) reported about *Chlamydia trachomatis* increasing the chances of hrHPV infection.

Given the fact that the women living with HIV who participated in this study had a high HPV prevalence and had a high prevalence of non 16 and non 18 hrHPV genotypes, the current HPV vaccines may not be so effective to be used as a therapeutic measure in this section of the population. Also an increase in sample size as compared to the size of 100 used in this study will enable detection of other genotypes as well as to be able to give a more conclusive report of the HPV prevalence.

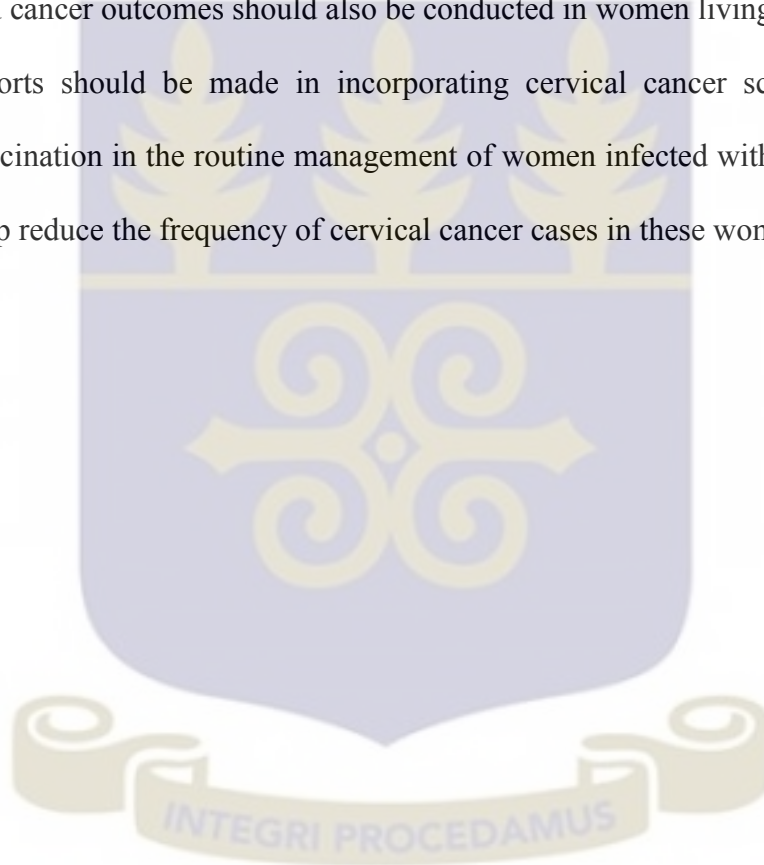
5.2 CONCLUSION

Seventeen HPV genotypes (12 high risk and 5 low risk) were detected in this study. hrHPV35 (12%) and low risk HPV42 (12%) were the most prevalent. Genotypes 16 (2%), 39 (2%), 59 (2%) and 56 (1%) were the least prevalent. Both single (41%) and multiple (27%) HPV infections were recorded in the study. A total HPV prevalence of 61.4% was observed at the end of this study. The prevalence of high risk HPV infection was 54%. The age group that recorded the highest HPV prevalence was 40-49 years. Of the 100 women whose Pap smears were cytologically examined only 7% had cytological abnormalities and 84% of the women reported NILM. Squamous cell lesions namely LSIL (3%), HSIL (2%) ASCUS (1%) and ASC-H (1%) were reported. Genital infections which were

associated with HPV genotypes were bacteria vaginosis, vaginitis, 53 atrophic vaginitis and candidiasis. Bacteria vaginosis was the most prevalent infection (16%).

It is recommended that

1. More work should be done in the area of HPV in HIV infected women to be able to get a clearer picture of cervical cancer in these women.
2. Longitudinal investigations of HPV genotype-specific risks for cervical precancer and cancer outcomes should also be conducted in women living with HIV.
3. Efforts should be made in incorporating cervical cancer screening and HPV vaccination in the routine management of women infected with HIV as these will help reduce the frequency of cervical cancer cases in these women



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APPENDICES

APPENDIX I

Informed Consent Form

Subject number:

Date _____

CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

TITLE OF PROJECT: HUMAN PAPILLOMAVIRUS GENOTYPES IN WOMEN LIVING WITH HIV ATTENDING ANTIRETROVIRAL THERAPY CLINIC AT THE KORLE-BU TEACHING HOSPITAL, ACCRA.

The contents will be explained to the participants in the language that the she is most comfortable with. This form describes the nature and purpose of the study, explanation of procedures, discomforts and risks, benefits, alternative therapies, refusal/withdrawal, rights and complaints, confidentiality, research authorization for use, and disclosure of your health care information.

Nature and Purpose of the Study

The study is been conducted by the team of investigators from University of Ghana (University of Ghana School of Allied Health Sciences, University of Ghana Medical School,

You are being invited to participate in a research project because you are HIV positive, over 18 years old, are here for routine genital examination, have a uterus, have not been treated for abnormal cervix, are not pregnant, and do not have genital warts (fleshy mass in the genital area). There are several types of HPV (Human Papillomavirus) and some can cause cervical cancer. The purpose of the study is to find what type/s of HPV can be found in the cervix of HIV infected women and if they have HPV, to see if these are HPV type/s that can cause cervical cancer.

Benefits

There are no direct benefits by participating in this project.

However, you can also request for your HPV test results. In case you want your HPV test results, please indicate this to the nurse explaining the study to you. We will also request you let us have a telephone number which we will use to contact you to come for your HPV test results which would be in a sealed envelope for your collection from the Doctor in Charge of the Clinic.

If you are found to be HPV positive, it will be documented in your folder and a plan for yearly screening for 1. genital warts and 2. Paps smear for CIN will also be documented and explained to you. You will be referred to the Gynaecologist for Paps smear and will be followed up at the Adult HIV clinic. The regularity of the Paps smear will be tied to one of the 6 monthly CD4 tests. If you develop genital warts, you will be treated with a drug.

Explanation of Procedures:

The procedure will be well explained to you in the language you are most comfortable with before commencement. You will be asked to answer a questionnaire about your age, education level, occupation, ethnicity, marital status, income, pregnancies, and risk factors for HPV infection such as sexual partners, smoking, birth control and sexually transmitted infections. During your study samples from your cervix (opening of your womb) will be collected for an HPV test. These specimens are sent to the laboratory for testing. A female assistant will always be present for this examination if the examiner is a male.

Discomforts and Risks

You may feel some discomfort when the speculum (instrument to open the vagina) is inserted. Slight lower abdominal cramping may occur when specimens are collected from the cervix and opening of the cervix. Some slight bleeding (spotting) from the cervix may occur during the Pap smear and HPV collection.

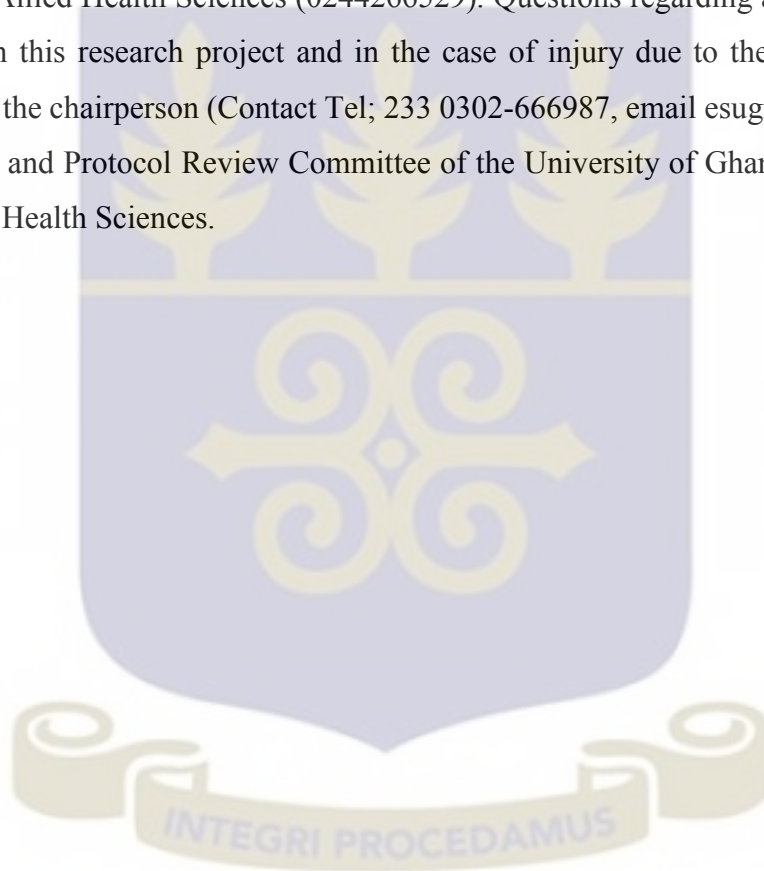
Confidentiality and Research Authorization for Use and Disclosure of Your Health Care Information. All information gathered from the study will remain confidential to the fullest extent of the law. Your identity as a participant will not be disclosed to any unauthorized persons; only the researchers will have access to the research materials, which will be kept under lock. The study participants will be coded with the code known only to the principal investigator and research staff. The principal investigator will only break the code if you require further treatment.

Refusal/Withdrawal

Participation in this study is voluntary; refusal to participate will involve no penalty or loss of medical care. You are free to withdraw consent and discontinue participation in this project at any time. Withdrawal or refusal to participate will not affect the care you receive from the Fevers Unit

Rights and Complaints

Any questions concerning the research project, participants can call Richard Harry Asmah, School of Allied Health Sciences (0244266529). Questions regarding any rights issues as a person in this research project and in the case of injury due to the project should be directed to the chairperson (Contact Tel; 233 0302-666987, email esugms@yahoo.com) of the Ethical and Protocol Review Committee of the University of Ghana Medical School, College of Health Sciences.



Consent to participate in Research

I,

Confirm that I have read the written information (or have had the information read to me) for the study HUMAN PAPILLOMAVIRUS GENOTYPES IN WOMEN LIVING WITH HIV ATTENDING ANTIRETROVIRAL THERAPY CLINIC AT THE KORLE-BU TEACHING HOSPITAL, ACCRA.

and that the study procedures have been explained to me by study staff during the consent process for this study.

Confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided.

Understand that I grant access to data to authorised persons described in the information sheet.

Have been given time and opportunity to consider taking part in this study.

Tick as appropriate (this decision will not affect your ability to enter the study):

I consent to participate in the above research study.

Signature of Subject:

Date.....

Signature of Interviewer:

Date.....

Name of

Impartial Witness:

Signature of

Impartial Witness:

Date.....

Appendix II

QUESTIONNAIRE

Project Title: HUMAN PAPILLOMAVIRUS GENOTYPES IN WOMEN LIVING WITH HIV ATTENDING ANTIRETROVIRAL THERAPY CLINIC AT THE KORLE-BU TEACHING HOSPITAL, ACCRA

Subject No.....

Date.....

Personal Profile

1. Age.....

2. Educational Level: None Primary Secondary Tertiary

3. Occupation.....

4. Region.....

5. Marital status: Single Married Separated Divorced Widowed

6. Level of income: <GH¢ 100 GH¢ 100-250 GH¢ 250-500 >GH¢ 500

Risk Factors for HPV infection

Sexual Partners

7. Marital status-married, single, cohabiting, not married but in a sexual relationship

8. If married, are you the only wife of your husband? Yes No

9. If no how many wives does your husband have?

10. How many sexual partners have you had in the last 6 months and in your life?.....

How often do you have sex .in a month? 0-14, 15-29, >30

History on Pregnancy

12. Have you ever been pregnant? Yes No

If yes, what was your age at first pregnancy?

How many times have you been pregnant?

How many of your pregnancies resulted in miscarriage or abortion?

How many of your pregnancies resulted in still birth?

Smoking status

13. Do you smoke tobacco? Yes No

If yes, how long have you been smoking?

How many cigarettes on average do you smoke per day?

14. Have you smoked before? Yes No

15. If yes how long did you smoke?

16. Do you use any other tobacco-related product? (snuff) Yes No

.If yes, how long have you been using it?

How many tobacco-related products on average do you smoke per week?

Contraceptive Use

17. Do you use condoms? Always Sometimes Never

Other Sexually Transmitted Infection

When were you diagnosed of HIV?

18. Are you using anti-retroviral therapy? Yes No

19. Type of anti-retroviral therapy

20. If yes, how long have you been using it.....

21. Have you ever had the following 1. Vaginal discharge Sore on genital area.

Yes No

When was it diagnosed?

How was it treated?

Capsules/tablets 2. Vaginal tablets or pessaries 3. Vaginal cream 4. injections

LMP

Ethnicity-.....

CD4+ COUNT.....

Age of 1ST Sexual encounter.....



Appendix III**REAGENT PREPARATION****1) Working stock concentration- HPV general PCR and cocktails for genotyping primers.**

Stock concentration of the primer (C1) = 1000 μ M

Volume of the stock concentration used in making the working concentration (V1) = x

Working stock concentration (C2)= 10 μ M

Volume of the working stock solution to be used (V2) = 200 μ l

$$C1 \times V1 = C2 \times V2$$

$$\text{Therefore } V1 = \frac{C2 \times V2}{C1}$$

$$V1 = \frac{10\mu\text{M} \times 200\mu\text{l}}{1000\mu\text{M}}$$

$$V1 = 2\mu\text{l}$$

2 μ l of the primer stock was pipetted from the whole primer stock after thawing it from the -20°C. It was added to 198 μ l of the nuclease free water to make up the 10 μ M primer working concentration. The primer working solution was then vortexed and kept at -20°C to be used for the general PCR and HPV genotyping

2) Working stock concentration for dNTPs (dATPs, d GTP, d CTP, d TTP)

Stock concentration of the dNTPs (C1) = 100mM

Volume of the stock concentration used in making the working concentration (V1)
= x

Working stock concentration (C2) = 10mM

Volume of the working stock solution to be used (V2) = 100 μ l

$$C1 \times V1 = C2 \times V2$$

$$\text{Therefore } V_1 = \frac{10\text{mM} \times 100\mu\text{l}}{100\text{Mm}}$$

$$V_1 = 10\mu\text{l}$$

10 μl of the dNTPs stock was pipetted from the whole primer stock after thawing it from the -20°C. It was added to 90 μl of the nuclease free water to make up the 10mM primer working concentration. The primer working solution was then vortexed and kept at -20°C to be used for the general PCR and HPV genotyping

3) 1X Tris acetate (TAE) Buffer.

Stock concentration of TAE buffer (C1) = 50X

Volume of the stock concentration used in making the working concentration (V1) = x

Working stock concentration (C2) = 1X

Volume of the working solution to be used (V2) = 500ml

$$C_1 \times V_1 = C_2 \times V_2$$

$$\text{Therefore } V_1 = \frac{1 \times 500\text{ml}}{50}$$

$$V_1 = 10\text{ml}$$

10ml of the stock 50x was transferred into a 500ml volumetric flask and topped up to the 500ml mark of the flask with distilled water. The buffer was gently agitated to mix and was kept at room temperature.

4) 2% agarose gel preparation.

2 grams of the agarose powder was weighed with a weighing balance and was poured into a heat resistant bottle. 100ml of 1X TAE buffer was added to the

agarose powder and mixed. The solution was then micro waved in order to dissolve the agarose powder properly.

After microwaving the solution was allowed to cool under running water but not to solidify after which 2 μ l of ethidium bromide was added and mixed. The solution was then poured into a gel casting tray with the comb inserted in the tray in order to create wells for loading PCR products.

