CONVENTIONAL PAP SMEAR AND HUMAN PAPILLOMA VIRUS DNA CO-TESTING IN HIV INFECTED WOMEN ATTENDING COMPREHENSIVE CARE CENTRE IN KENYATTA NATIONAL HOSPITAL

BY

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR THE AWARD OF DEGREE OF MASTERS OF SCIENCE IN CLINICAL CYTOLOGY

THE UNIVERSITY OF NAIROBI

2015

DECLARATION

I hereby declare that this dissertation is my original work under the guidance of the supervisors listed below and has not been previously submitted to the University of Nairobi or any other institution of higher learning.

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DEDICATION

To my beloved late husband Mr. P.K. Karuri, my late parents Mr. and Mrs. Muiruri and my beloved sons P. Kibogo, T. Muiruri and A. Mbuthia.

ACKNOWLEDGEMENT

I thank the almighty God for His strength grace and mercy that has enabled me reach this far.

I wish to thank the Ministry of Health for sponsoring me to pursue the Master of Science degree in Clinical Cytology, and KNH Research department for partially funding this dissertation.

I wish to extend my sincere gratitude to all those who contributed directly or indirectly to the success of this project.

Special gratitude to my supervisors Prof. L. Muchiri, Dr. W. Waweru , Dr. C.S. Gontier and Dr. M. Mungania for their tireless effort in guiding me through all the stages of this work.

Great appreciation to the CCC Staff, research assistants Simon Njoroge, Esther Nyambura, Mrs. Margaret Waweru of Obstetrics and Gynecology (Obs/Gyn) laboratory and all the study participants.

Sincere gratitude to all KNH cytology staff headed by Joyce Kiremu, and all staff of Kenya Aids Vaccine Institute (KAVI) - Molecular lab for their ever ready help.

Special gratitude to all staff, former and current students in the Department of Human Pathology.

My heartfelt appreciation to my sons and family members for their encouragement and moral support.

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LIST OF ABBREVIATIONS

AGC- Atypical Glandular Cells

AIDS- Acquired Immuno-Deficiency Syndrome

AIS- Adenocarcinoma in Situ

AOR- Adjusted Odds Ratio

ART- Antiretroviral Therapy

ARVs- Antiretroviral [agents]

ASC- H – Atypical Squamous Cells cannot exclude HSIL

ASCCP- American Society for Colposcopy and Cervical Pathology

ASCUS- Atypical Squamous Cell of Undetermined Significance

BP- Base Pairs

BTL- Bilateral Tubal Ligation

CCC- Comprehensive Care Centre

CI- Confidence Intervals

CIN- Cervical Intraepithelial Neoplasia

CP- Conventional Pap

DNA- Deoxyribonucleic Acid

DPX- Distyrene Plasticizer Xylene

EA- Eosin Azure stain

ERC- Ethics and Research Committee

ECC- Endocervical curettage

IUCD- Intra-uterine Contraceptive Device

HAART- Highly Active Antiretroviral Therapy

Hc2- Hybrid Capture 2

HIV- Human Immunodeficiency Virus

HPV- Human Papilloma Virus

HR-HPV- High Risk Human Papilloma Virus

HSIL- High Grade Squamous Intraepithelial Lesion

HSV- 2 Herpes Simplex Virus

IUCD- Intra-uterine Contraceptive Device

ID- Identification

IUCD- Intra-uterine Contraceptive Device

KAVI- Kenya Aids Vaccine Institute

KNH- Kenyatta National Hospital

LAB- Laboratory

LSIL- Low Grade Squamous Intraepithelial Lesion

NILM- Negative for Intraepithelial Lesion or Malignancy

Nm – Nanometer

NOS- Not Otherwise Specified

CO- Cut off

Obs/Gyn- Obstetrics and gynecology

OG- Orange Green

OR- Odds Ratio

OCP- Oral Contraceptive Pills

PAP- Papanicolaou Smear/ Conventional Papanicolaou

PCR- Polymerase Chain Reaction

PI- Principal Investigator

Rb- Retinoblastoma protein

RCS- Rapid capture system

RLUs- Relative light units

RNA- Ribonucleic Acid

SCC /SQC- Squamous Cell Carcinoma

SIL- Squamous Intraepithelial Lesion

SOP- Standard Operating Procedure

SPSS- Statistical Packages for Social Sciences

TBS- The Bethesda System

UK- United Kingdom

UON- University of Nairobi

VIA- Visual Inspection with Acetic Acid

VILI- Visual Inspection with Lugol's Iodine

WHO- World Health Organization

ABSTRACT

Background: Cervical cancer is the second most common cancer among women worldwide with 85% of cases occurring in developing countries. People living with Human Immunodeficiency Virus (HIV) have substantially higher risk for certain types of cancer, including cervical cancer compared to uninfected people of the same age. HIV infected women are at least five times more likely to be diagnosed with cervical cancer.

Pap smear has low sensitivity due to the fact that cytological criteria for the detection are not always present. Reflex Human Papilloma Virus Deoxyribonucleic Acid (HPV DNA) testing co-collected with conventional Pap smear is the preferred approach because it is more sensitive than a single repeat Pap test.

Objective: To determine the utility of co-testing by conventional Pap smear and HPVDNA testing in screening HIV infected women attending Comprehensive Care Centre (CCC) in Kenyatta National Hospital (KNH).

Study design: This was a cross- sectional descriptive study.

Setting: Comprehensive Care Centre (CCC), cytology laboratory, Kenyatta National Hospital (KNH) and Kenya Aids Vaccine Institute (KAVI) molecular laboratory, between August and December 2014.

Participants: A total of 119 HIV positive women aged 25 years and above were recruited from the CCC in KNH.

Material and methods: Demographic and clinical information was obtained by direct interview of the patients. Pap smears were collected by two nurses in a standard manner to ensure the quality of Pap smears and also collected HPV DNA samples using DNA Pap sampler and placed the brush into the preservative provided in the kit. Pap smears were stained using standard staining protocol, and then signed out by the principal investigator (PI) and the supervisors. The HPVDNA samples were preserved at -20^{o}C and processed in KAVI molecular laboratory and results were confirmed by the immunologist supervisor.

Results: A total of 119 patients were recruited. The mean age was 39.9 years (±6.96). About 63.9% were negative for intraepithelial lesion or malignancy (NILM) while 36.1% had atypical

squamous cells of undetermined significance (ASCUS) or worse on Pap smear. The commonest lesion reported was high grade squamous intraepithelial lesion (HSIL) (13.4%) followed by ASCUS (7.6%), squamous cell carcinoma (SCC) (6.7%) and low grade intraepithelial lesion (LSIL) (4.2%). Fourteen patients (11.7%) had infections, of these (9.2%) bacterial vaginosis and (2.5%) candida. High risk (HR) HPV DNA was prevalent in 36.27% cases. Out of a total of 66 women whose Pap smears were reported as NILM, (9%) of them were positive for HR-HPV DNA. Seven Pap smears were reported as ASCUS of which 4 were positive for HR-HPV DNA test. All the squamous cell carcinomas were positive for HR-HPV DNA. The correlation between Pap smear cytologic findings and HR-HPV DNA testing was found to be statistically significant with ($p=0.001$).

Conclusion

The majority of the high grade lesions and all squamous cell carcinomas detected in this study were positive for HR-HPV DNA. HPV DNA test also detected HR-HPV in normal Pap smears thus identifying HIV-positive women at risk of developing cervical intraepithelial neoplasia.

Recommendations

All HIV infected women are at high risk for cervical neoplasia and should have a mandatory cervical cancer screen as part of their management. HPV DNA testing should be preferably offered to identify HIV positive women with normal Pap smear who are at risk of CIN. Women with negative Pap smears but positive for HR-HPV should be closely followed up.

1.0 INTRODUCTION

Human papilloma virus (HPV) is a necessary cause of cervical carcinoma and intraepithelial neoplasm worldwide (1). Human papilloma virus belongs to the family of Papovaviridae which include double stranded DNA member papilloma virus and polyoma virus. HPV can be classified into low risk and high risk types. Low-risk HPV types are mainly found in genital warts and other benign lesions and include HPV types 6, 11, 40, 42, 44, 54, 61, 70, 72 and 81. The high risk HPV types are considered carcinogenic because they are found preferentially in precancerous lesions and cancer. The high risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 ,68, 73, and 82. Certain HPV types have strong association with and probably a causative role in the pathogenesis of cervical lesions which are predominantly high grade (1). Cervical cancer ranks as the first most frequent cancer among women in Kenya, and the second most frequent cancer among women between 15 and 44 years of age (2).

Tests for cervical cancer and premalignant lesions

Pap smear

Also known as Pap test, is a screening test used to detect pre-cancerous and cancerous uterine cervical lesions. The Pap smear test involves collection of sample from the uterine cervix using brushes or spatula and transferring them onto a glass microscope slide. This preparation is then fixed using liquid or spray fixative and sent to the laboratory where they are processed, examined and reported. The slides are sent to a cytology laboratory and evaluated by a trained cytologist who determines the cell classification. Pap smear is best collected if the woman is not menstruating, or has no other frank bleeding, unless there is high suspicion of vaginal neoplasia (cancer bleeding), because blood obscures proper screening of cells (Appendix viii).

Hybrid capture 2

Hybrid capture 2 has been the most widely used single assay in reporting HPV prevalence, incidence and viral load (1). The method is commercially available in form of a kit and uses Ribonucleic Acid (RNA) probes to detect viral DNA in the samples. The current commercial HPV detection Digenes' hc2 kit detects virtually all high risk oncogenic HPV types as well as low risk non oncogenic HPV genotypes. Digene's hc2 format is a proprietary nucleic acid hybridization signal amplification owned by Digene Corporation.

The assay is semi-quantitative as it defines multiple HPV types rather than specific types. The assay is highly sensitive, reproducible and specific. The test can easily be performed on a standard piece of equipment in a virology or cytology setting (3).

Other tests include visual inspection methods such as visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILI). These involve application of acetic acid or Lugol's iodine respectively to the cervix and observe color change of the cervical epithelium after specified time. The principle behind these techniques lies in the composition of cervical epithelium. However, one of the weaknesses of these visual inspection techniques is the high rate of false positive findings which may lead to unnecessary greater number of colposcopies. Postmenopausal women are not recommended for visual inspection test because of the shift in position of the transformation zone deep inside the endocervical canal making its visualization difficult (4).

Polymerase chain reaction

It is the most common and sensitive test for HPV detection and amplifies a specific piece of DNA using a small amount of template. It has the advantage of extreme sensitivity and versatility in that it can be used to detect more than one type of HPV when generated primers are used. Its major disadvantage is because of high sensitivity, and can amplify contaminating sequences, hence can have false positive results. Controls must strictly be used when using the test (5).

2.0 LITERATURE REVIEW

2.1 Human papilloma virus

Human papilloma virus belongs to a large group of viruses and the family is Papovaviridae. More than 120 different types of the human papillomavirus (HPV) have been isolated, more than 40 of these types infect the epithelial lining of the anogenital tract and other mucosal areas. Majority of the individuals, HPV infections are transient and asymptomatic with most new infections resolving within a period of 2 years. HPV infection has been firmly established as the primary cause of cervical cancer. It is not clearly understood why HPV infections resolve in certain individuals and result in cervical intraepithelial neoplasia in others, but several factors are thought to play a role; including individual susceptibility, immune status and nutrition, tobacco smoking, parity, co-infection with other sexually transmitted agents such as HIV, herpes simplex virus type 2 as well as viral characteristics such as HPV type, concomitant infection with other types, viral load, HPV variant and viral integration (6).

2.1.1 Classification of Human papilloma viruses

They are classified into oncogenic high risk and low risk types. Molecular techniques that detect HPV DNA and distinguish high-risk HPV types from low-risk HPV types have been introduced as an adjunct to cytology. Earlier detection of HR-HPV types may improve triage, treatment, and follow-up in infected patients (7).

2.1.2 Human papilloma virus and carcinogenesis

High-risk HPV E6 and E7 oncoproteins expressed in epithelial cells infected with HPV are associated with increased proliferation and in the abnormal differentiation of these cells. Once the E6/E7 proteins are the expression of infection of the cell with low-risk HPV, then these active proteins may induce benign neoplasms. However, when E6/E7 proteins are the expression of high-risk HPV infection, they promote the role of oncoproteins and they have the capacity to induce dysplastic and malignant epithelial lesions (8).

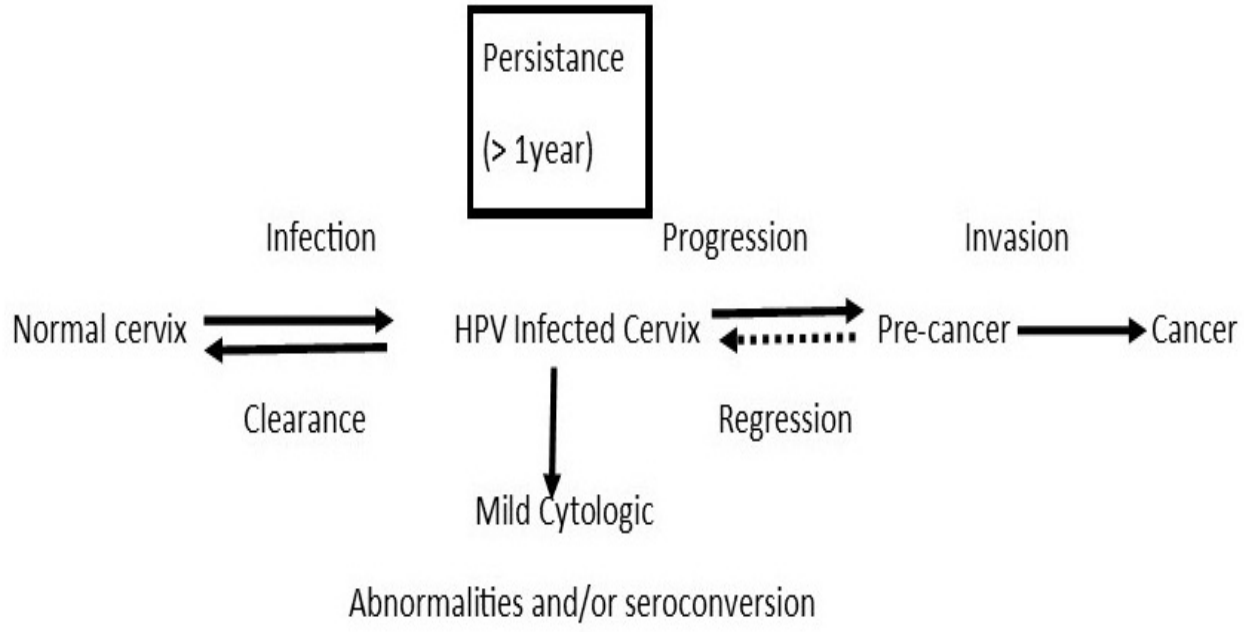


Figure 1. Natural history of HPV infection.

2.1.3 Persistence of infection

According to epidemiological evidence HPV may persist in squamous epithelium without producing any disease or recognizable lesions. It is widely accepted that persistence of HPV is essential for the development of precancerous lesion and cervical cancers. Persistence is uncommon compared with clearance especially in immunocompetent state as they are cleared before causing any disease. The importance of HPV clearance/persistence has been recognized recently, and the number of studies addressing these issues has increased substantially during the past few years. However, data are still incomplete and in part inconsistent as to the cofactors that regulate these events (9).

2.2 Laboratory diagnosis

Human papilloma virus cannot be grown in cell culture. In conventional Pap smears and liquid based preparations cytologists and cytotechnologist can see koilocytes features and hence HPV infection can be suspected. Polymerase chain reaction (PCR), nucleic acid probe and hybrid capture techniques are used for specific HPV detection. Studies have shown that PCR is more

sensitive and has been employed to establish that HPV is present in over 90% invasive cancers (5).

2.2.1 Prevention and control

A comprehensive approach to cervical cancer prevention and control involves health education, vaccinating girls before initiation of sexual activity, screening women for precancerous lesions and treatment before progression to invasive disease can significantly reduce the morbidity and mortality from the disease.

The HPV vaccines currently available are the bivalent vaccine (Cervarix™, GlaxoSmithKline Biologicals) and the quadrivalent vaccine (Gardasil™, Merck). The aim of these vaccines is to prevent infection from HPV types 16 and 18, since these two types are most carcinogenic and are responsible for majority of cervical cancers (10).

2.3 Methods for the detection of HPV infection

2.3.1 Non molecular techniques

The non-molecular methods include visual inspection, cytology and histology. These methods do not detect the actual presence of HPV but are indirect methods to detect the clinical sequel of the HPV infection. Cytology when used as a screening tool for cervical cancers is restricted to a correlation with presence of HPV. The best evaluated morphologic change of HPV infection is koilocytosis or koilocytic atypia which is a combination of nuclear atypia and the formation of a distinctive perinuclear halo. However in the presence of koilocytes in histological changes may be difficult to differentiate from artifacts due to fixation (11).

2.3.2 HPV Testing methodologies

There are three types of nucleic acid hybridization formats used to detect HPV and they include direct nucleic acid probe methods, signal amplification and target amplification method. Each method has its own strengths and weaknesses i.e. signal amplification is a useful technology in the detection of viral loads and genotyping (8).

2.3.4 Hybrid capture

This assay distinguishes between high risk and low risk groups, but was not designed for genotyping single HPV. This is important, since with persistent infection the risk of a precancerous lesion is between 10 and 15% with HPV types -16/18, and below 3% for all other high risk types combined. Genotyping of HPV is very important to identify single oncogenic HPV types and to provide more information regarding risk-stratification as well as persistence of infection (12).

The method is not as sensitive as PCR but it does not have a problem of false positives inherent as in PCR since it does not depend on amplification of signals. It detects 16 HPV types while PCR can theoretically detect all types. The Digene hc2 test is approved in the United States to be used as an adjunct with Papanicolaou test for cervical cancer screening and it is being marketed in selected countries as primary cancer screen either in conjunction with or separate from Papanicolaou test. In the hc2 assay specific RNA probes are used which are directed towards individual DNA sequences comprising the HPV genotypes to be detected (1). The antibody is used both at the capture step and the detection step in which the antibody is labeled with a reporter molecule and is developed using a chemiluminescence detection system. In essence hc2 is an immunoassay. Based on the strength of their association with cervical cancer HPV genotypes detected by hc2 can be grouped into two groups namely: low risk HPV types 6, 11, 42 ,43 and 44 53 54 57 and 66, high risk HPV type 16, 18, 31, 33, 35, 39, 45, 52, 56, 58 59 and 68. The cocktail approach of the HPV hc2 test provides an excellent tool for the triage of patient with minor cytologic abnormalities on Pap test though it cannot determine the specific HPV type present (4).

2.4 Goal of HPV DNA testing

The test is not intended for use as a screening tool in the general population. It is designed to augment existing methods for the detection of cervical neoplasia and should be used in conjunction with clinical information. Some countries are now using HPV DNA testing as a primary screening test to triage those who will subsequently have a pap smear. In addition to changes in screening strategies, effective therapeutic and preventive vaccines have been developed that have the potential to contribute significantly to the control and prevention of cervical cancer (10).

Existing methods for the detection of cervical disease should be used in conjunction with clinical information. The test results should not be used as the sole basis for clinical assessment and treatment of patients. Its main utility is in reflex testing for women with ASCUS Pap smear results (13).

WHO recommend that where cytology programs are in place and meet quality indicator, Pap smear and HPV test followed by colposcopy should be done (44).

2.5 Cervical Cancer

2.5.1 Overview of Cervical Neoplasia

Cervical cancer develops in the lining of the cervix, the lower part of the uterus enters the vagina developing over time. Normal cervical cells may gradually undergo changes to become precancerous lesions and then cancerous. Cervical intraepithelial neoplasia is term used to refer to these abnormal changes. These lesions are classified according to the degree of cell abnormality. Low-grade squamous intraepithelial lesion (LSIL) is an indication of a minimal changes in the cells while high-grade squamous intraepithelial lesion (HSIL) indicates a higher degree of abnormality (14).

2.5.2 Incidence and Prevalence of Cervical Cancer

Worldwide there are over 500,000 new cases of cervical cancer occurring annually and in excess of 270,000 deaths, accounting for approximately 9% of female cancer deaths.
In Africa it is the commonest cancer in women with incidence frequently becoming equal with mortality in the absence of healthcare facilities to manage the conditions (15).

2.5.3 Squamous Intraepithelial Lesions

The following are some of the categories of lesions reported in the Bethesda reporting system on Pap smear.

2.5.3.1 Negative for intraepithelial lesion or malignancy

The report shows no evidence of neoplasia in the examined cells on a Pap smear. Approximately 91% are reported as Negative for intraepithelial lesions or malignancy (NILM). These are non-neoplastic lesion which includes non–infectious conditions, infections or cellular changes (16)

2.5.3.2 Atypical Squamous Cell of Undetermined Significance

Atypical squamous cells of undetermined significance (ASCUS) refer to changes that are more marked than those attributable to reactive changes. The cells qualitatively or quantitatively fall short of definitive diagnosis of squamous intraepithelial lesions. Example of conditions which show these cells includes atypical repair and atypia of atrophy. Atypical parakeratosis may also fall in this category. It is sometimes considered to be a waste basket for categories which presents with diagnostic problems and difficult to classify (17)

2.5.3.3 Low grade squamous intraepithelial lesion

Low grade squamous epithelial lesion is a lesion caused by low risk HPV which regress in most cases. Others are caused by high risk HPV which may persist and progress to HSIL or SCC.
It is a lesion of intermediate and superficial cells which show nucleur atypia, which include nucleur enlargement and hyperchromasia, irregular contours, slight chromatin coarseness and cytoplasmic cavities (koilocytes) (16).

2.5.3.4 High Grade Squamous Intraepithelial Lesion

High grade Squamous Intraepithelial Lesion are cytologic changes that affect cells that are smaller and less mature than cells in LSIL. The cells may occur singly, in sheets or form hyperchromatic crowded groups both retrospective and prospective studies suggest that across all HPV types, the rate of regression of cervical high-grade squamous intraepithelial lesions (HSIL) in 4–6 months is around 35%. Cervical HSIL associated with HPV-16 is less likely to regress than CIN2/3 associated with HPV types other than 16 (18).

2.5.3.5 Squamous Cell Carcinoma

Carcinoma of the cervix continues to be a leading cause of morbidity and mortality among gynecologic malignancies. Despite the institution of widespread cytologic screening, this remains the second most common malignancy diagnosed and the third leading cause of death worldwide among women. Among carcinomas of the cervix, squamous cell cancer remains the most common pathologic type. The factors that influence the prognosis in squamous cell cancer of the cervix, include age, diabetes, smoking, parity, anemia, and extent of disease. (19)

2.6. Glandular Abnormalities

2.6.1 Atypical Glandular Cells

Atypical Glandular Cells refers to cell changes that are between a benign reactive process and those of Adenocarcinoma in situ (AIS) or adenocarcinoma. Diagnosis of these glandular lesions in Pap smear is difficult because they are usually few in numbers. In a Pap smear cells are usually in sheets, strips and other shows crowding and overlapping. Hyperchromasia is mild and prominent nucleoli may be seen with cytoplasm which is fairly abundant (17).

2.6.2 Adenocarcinoma in situ

In order of frequency, cervical intraepithelial neoplasia (CIN), combined adenocarcinoma in situ (AIS)/CIN lesions, and solitary AIS are the most prevalent premalignant lesions of the uterine cervix

It is regarded as the precursor lesion to invasive adenocarcinoma. Diagnosis of this glandular abnormality is a challenge in Pap smears. The cells usually occur in sheets, clusters and strips with palisading arrangement of round to elongated nuclei which have luminal borders. The honeycomb pattern is completely lost with feathering effect and formation of rosettes. The cells show overcrowding, overlapping and they are hyperchromatic (16).

2.6.3 Endometrial adenocarcinoma

It is predominantly a tumour of postmenopausal women in the late 50s and early 60s with approximately 90% of the women presenting with postmenopausal bleeding while others may be asymptomatic. The most common are the endometrioid type and less common are serous, clear cell and mucinous type of adenocarcinoma. The cells occur singly or in small clusters with nuclear variation and loss of polarity. There is moderate hyperchromasia and chromatin is irregular. The presence of cytoplasmic neutrophils commonly referred to as bags of polyps and watery tumour diathesis may be seen (17).

2.6.4 Adenocarcinoma

Adenocarcinomas of the endocervix, endometrium, vagina and even the ovaries and fallopian tubes are sometimes detected with the Pap test. There is significant overlap in their morphologic features, so that a precise site of origin often cannot be established. Additional testing such as imaging studies and histological sampling is usually required for definitive classification and treatment (16).

2.7 Sensitivity and specificity

Sensitivity level of an assay is the detection limit and specificity is level of accuracy of an assay. Pap smear is less sensitive in detection of cervical intraepithelial lesion. Studies conducted in India, Zimbabwe and South Africa between 1999 and 2004 with a total of 32,839 participants, the sensitivity of conventional Pap smear ranged from 44%-78% while the specificity ranged from 91%-96% for HSIL threshold.

Goel et al conducted a prospective study on 400 women the sensitivity was 50% and 97% specificity (20, 21).

2.8 Epidemiological studies of HPV in HIV infected women

Human papilloma virus is known to be the major cause of cervical carcinoma and precancerous lesions worldwide. There are certain HPV types which are known to have strong association and probably play a major role in the pathogenesis of precancerous cervical lesions in HIV infected women. Some studies carried out in United States in the New Jersey Medical School showed that HIV infected women have increased incidence of squamous intraepithelial lesions (SILs) predominantly the high grade lesions. When using Pap smear test for cervical cancer screening about 6 to 30% of women diagnosed with ASCUS have shown to harbor squamous intraepithelial lesions (SILs) in a normal screening population (22).

In one of the studies 209 cervical swabs were collected from HIV positive women and of these 48% tested positive for HPV subtypes after performing DNA typing. About 63% of the Paps smears were reported as normal, benign cellular changes and ASCUS (favor reactive process). When HPV DNA testing was done the findings were as follows; positive for both high risk and low risk subtype was 19%, of this 32 were positive for high risk subtypes and 13 positive for low risk subtypes. The findings of the study drew a conclusion that HPV sub typing was able to identify a significant HIV positive group who are at risk of developing cervical intraepithelial neoplasia despite their Pap smears not showing significant abnormalities. In another study carried out in Brazil, hybrid capture 2 and PCR were used to determine the prevalence of HPV infection among HIV infected women. The study showed that 94% were infected with high risk genotype. Cervical cancer is the most common form of cancer in women in developing countries.

In Brazil, it has been ranked third most common type of cancer and the fourth cause of cancer deaths (23).

In a study carried out in Abuja Nigeria a total of 2,501 HIV positive women participated in a cervical cancer screen and treat program. The prevalence of precancerous lesion and cancer was 6% in HIV positive women. Majority of the cases of precancerous lesion and cancer were associated with high risk HPV infection. Commonly HPV types 16 and 18 and were found to account for the major cases (24,25).

The prevalence in sub- Sahara Africa was found to be high among sexually active women aged between25-35 years. Worldwide age adjusted HPV prevalence in women with normal cytology is estimated at 12% ranging from 5% in North America and 34 % in East Africa (26).

In another study carried out in Burkina Faso and South Africa the performance of two assays was compared. The detection rate for high-risk HPV DNA was done using cervical smears from 149 HIV infected women. Detection rate for care HPV was 37.6% while hc2 was 34.9%. The agreement between the two methods was excellent and were considered suitable for the detection of high risk HPVDNA in cervical samples (27).

A clinic based study conducted in Thika found the prevalence of HR HPV in HIV infected women at 49% and 17% in HIV negative women (26).

The immune system may clear HPV infection in Low grade squamous intraepithelial lesions (LSIL) spontaneously with tissues resolving to normal state in 47% of patients. About 20% may develop High grade Squamous Intraepithelial Lesion (HSIL). In developed countries, the highest rate of HPV infections has been observed among age 15-25yrs which might reflect transmission during the first sexual intercourse (27)

In Kenya, Tigoni rural women aged between age 25 to 60 years were screened for both cervical cancer and HIV. The study findings showed that 31.96% were positive for at least one type of HPV and the most frequent HPV types were 16, 56, 53, 35, and 39. The prevalence of high risk type among HIV infected women was 37% using polymerase chain reaction (PCR)(28).

2.9 HPV and Oncogenesis

The replication of DNA and RNA tumor viruses involve incorporation of the viral genome into the host cell chromosomes which induces several mutations that cause disruption of the normal balance between cell proliferation and cell death.

The role played by viral DNA E6 and E7 in stimulating cellular proliferation is crucial. In this process, E6 acts by inhibiting p53 while E7 acts by binding retinoblastoma protein. These result in inhibition of apoptosis leading to unregulated cell proliferation. The major steps of carcinogenesis include specific high risk HPV type infection in the cervix, viral persistence and progression to invasive cancer (29).

2.10 Biomarkers

Two biomarkers p16 and Ki-67 or MIB1 are useful for identification of SIL, but they are not reliable for grading (HSIL versus LSIL).The Ki-67 antibody shows positive nuclear staining in over 30% of nuclei in the upper epithelial layers of a SIL, and p16 is strongly expressed by nuclei and cytoplasm of dysplastic cells in both LSIL and HSIL associated with high-risk HPV types. Normal, atrophic, reactive and metaplastic squamous epithelial cells in contrast, are negative for p16 and Ki-67 is expressed only by parabasal cells (30).

2.11 Problem statement

Screening for precancerous lesions in a clinical setting by conventional Pap smear is not effective in all cases since Pap smear has low sensitivity due to the fact that cytological criteria for the detection are not always present. In addition the low sensitivity may be due to a variety of other reasons i.e. sampling, quality of smears including abundant inflammatory cells, subjectivity and experience in cytologic interpretation and absence of cytologic phenotypic expression of HPV. Sensitivity of Pap smear could potentially be lower in HIV infected women. Reflex HPV DNA testing co-collected with conventional Pap smear is the preferred approach because it is more sensitive than a single repeat Pap test; it also represents a good quality measure and with appropriate re-review reduces the rate of ASCUS/ASC-H diagnosis (31).

2.12 Justification

Although Pap smear has proved to be the method of choice for mass screening and prevention of cervical cancers, it has its own limitations with regard to sensitivity, specificity and reproducibility. A combination of methods has been proposed in attempt to improve the sensitivity of the Pap smear. Molecular method technology presents high sensitivity for HSIL and age dependent sensitivity. Other studies have shown improvement in detection of CIN 3 cases. Apart from improvement in sensitivity there will be a larger spacing of time between screenings and virtual reduction in number of consultations in a screening program. HPV DNA testing would reduce human error which may occur as a result of human fatigue especially when detecting lesser numbers of abnormal cells in cervical smears. Currently the clearest role for HPV DNA testing is to improve diagnostic accuracy and limit unnecessary colposcopy in patients with borderline or mildly abnormal cytologic test results. Visual examination methods lack specificity and quality control is a challenge. They are also known not to work well in the screening of post-menopausal women due to the fact transformation zone receeds deep into cervical canal (entropion) and cannot be visualized well by VIA (32,33).

The study findings will create awareness of the distribution of HR HPV among HIV infected women who are at higher risk of cervical neoplasm and these may provide new or improved strategies for prevention of cervical cancers in immuno-compromised women. The study will identify gaps for more research mostly in molecular testing for HR HPV subtypes.

2.13 Research question

Can co-testing by conventional Pap smear and HPV DNA test improve screening of cervical neoplasia in HIV infected women?

2.13.1 Broad objective

To determine the utility of co-testing by conventional Pap smear and HPVDNA test in screening HIV infected women attending CCC in KNH.

2.13.2 Specific objectives

Primary objectives

1. To determine the prevalence of cervical lesions among HIV infected women attending CCC in KNH by conventional Pap smear.
2. To determine the prevalence of HR HPV among HIV infected women attending CCC in KNH.
3. To determine the prevalence of HR-HPV among HIV infected women with an abnormal Pap smear attending CCC in KNH.
4. To determine if co-testing by conventional Pap smear and HPV-DNA test improves screening for cervical lesions among HIV infected women attending CCC in KNH.

Secondary objective

1. To determine the cost of Pap smear and HPV DNA tests.

3.0 METHODOLOGY

3.1 Study design

This was a cross- sectional descriptive study conducted between September 2014 and January 2015.

3.2 Study Area

Study was conducted at the Comprehensive Care Centre in Kenyatta National Hospital which is the largest referral and teaching hospital in Kenya. Comprehensive Care Centre is an outpatient HIV/AIDS care clinic. Patients come from Nairobi and its environs and the clinic attends to average 2400 patients monthly of which about 50% are women. Clients who test HIV positive from the various departments of KNH are referred to the clinic. It also receives clients tested elsewhere and willing to be followed up at the clinic. All women of child bearing age are routinely screened for cervical cancer using VIA /VILI tests. Those who test positive for VIA /VILI are considered eligible for Pap smear.

3.3 Study population

All HIV infected women aged 25 years and above.

Inclusion Criteria

- HIV infected women aged 25 years and above.
- Those sexually active.
- Those who gave consent to participate in the study.
- Those eligible for Pap test.

Exclusion criteria

- HIV infected but had undergone hysterectomy/ablation.
- Those who were pregnant.
- Those with known cervical lesions and were on therapy.

Recruitment

Recruitment of the study participants and specimen collection was done at the CCC in KNH. Potential participants were identified either by the principal investigator or the research assistants. The participants were screened for inclusion and exclusion criteria through direct interview. Those who fulfilled the inclusion criteria were informed about the study and written informed consent was obtained from those who agreed to participate in the study (Appendix 1a). Convenience and Consecutive sampling was used until the required sample size was achieved.

Data collection procedure

Clinical procedure

After obtaining informed consent the participants social-demographic characteristics and necessary clinical information was obtained by direct interviewing of the participants by the principal investigator or the research assistants and the information captured in the pre-designed questionnaire.

Specimen handling

The cervical specimen for Pap and HPV/DNA were co-collected by two nurses or OBs/Gyn resident under speculum examination. The Pap specimen was applied on a frosted end glass slide, fixed immediately with 95% alcohol both of which are contained in the Pap smear collection kit and labeled properly.

The HPV/DNA sample was placed in a transport tube containing a liquid preservative, tightly capped and placed in a zip lock bag. The principal investigator packed the samples in a cooler box and transported them to store at -20^0C refrigerator in KAVI molecular laboratory.

Laboratory procedure

The Pap smears were stained by the principal investigator using Papanicolaou stain protocol (Appendix IV).

The principal investigator performed the primary microscopic evaluation of the Pap smears followed by signing them out with the supervisors. The Bethesda System 2001 for reporting cervical cytology was used during examining and also while reporting the smears (Appendix V).

The HPV/DNA samples were processed in KAVI molecular laboratory by principal investigator, a scientist and a technologist. On the assay day all the reagents were prepared using instructions on the user manual by the principal investigator under instructions of a scientist and a laboratory technologist. The equipment's were set in preparation for the runs. Test samples were thawed and all working reagents brought to ideal working temperatures as per the steps in the user manual. The results were interpreted by the principal investigator with the immunologist supervisor.

3.4 Quality Assurance

Pap smear

All Pap smears were collected by two qualified nurses or OBs/Gyn resident who are already skilled in Pap smear collection in a standard procedure to ensure quality of Pap smears and also removed HPVDNA samples using DNAPAP sampler brush and placed it into a transport tube which contained liquid preservative.

Pap smears were stained in KNH Cytology laboratory using standard operating procedures (SOPs) prepared and approved by the laboratory-in-charge. Old deteriorated stains were discarded and replaced with fresh batches where necessary. For microscopic examination the principal investigator first screened all the smears and signed out with two supervisors (Prof. L Muchiri and Dr. W. Waweru).

The third supervisor reviewed those results which showed discrepancies and played the role of a tie breaker.

HPVDNA samples

The principal investigator transported the samples for HPVDNA testing using a cool box and preserved them at appropriate temperature -20^0C in a refrigerator with automated temperature monitoring system at KAVI molecular Laboratory for two months.

Internal Quality Control samples were incorporated in the test kit. The study samples, three negative and positive calibrators (one low risk and one high risk control) were set to run in the same microplate but in different wells and processed simultaneously as per steps in the user manual. Timing and incubation temperatures as per the user manual were strictly adhered to.

All steps were critically followed up to the end of the procedure. Interpretation and quality control of result. (Appendix XII)

3.5 Sample size determination

The number of samples for the study population was calculated using prevalence of 26.7% as was observed in a study to assess the prevalence and identified associated risk factors for precancerous cervical cancer lesions among HIV-infected women in resource-limited settings in Kenya. HIV- infected women attending the ART clinic at the Nazareth Hospital between June

2009 and September 2010. The prevalence of precancerous lesions (CINI, CINII, CIN III, ICC) was (26.7%), using Fishers formulae (34).

$$n = \frac{z^2 P(1-p)}{d^2}$$

n= required sample size

Z= Statistic for level of confidence on normal distribution

Critical value set at 1.96 which corresponds to 95% confidence interval

P= Expected prevalence of proportions expected at to a particular characteristics=26.7%

D= degree of precision set at plus or minus 10%

$$n = \frac{1.96^2 \times 0.267(1-0.267)}{0.1^2} = 75$$

Sample size of 75 with non-respondent rate of 20 % = 75*20/100 =15, the overall sample size is 75+15=90 approx. 100

Although the calculated sample size was 100 an additional 19 samples were added to cover for unsatisfactory smears.

3.6 Ethical consideration

1) Approval to conduct the study in the Comprehensive Care Centre was obtained from KNH/UON ethical review committee - **P304/05/2014**.

2) Permission to conduct the study was also obtained from the manager in charge of Comprehensive Care Centre.

3) All eligible clinic attendees were offered the opportunity to participate without discrimination.

4) Informed consent was obtained from all the study participants.

5) Permission to use KAVI laboratory was sought and obtained from Laboratory Director.

6) Patients privacy and confidentiality was strictly observed.

7) All procedures were performed in a standard manner to minimize harm, and maximize benefit to study participants.

8) Results were sent to the patients´ files in a timely manner to inform clinical decisions.

3.7 Data management and statistical analysis

The data was collected and stored in hard cover register, Microsoft excel as well as SPSS software. Information stored in soft copies was protected from access from unauthorized persons by a password. All records were identified by study identification numbers. All the data was analyzed using SPSS version 20. All statistical tests were performed at 5% level of significance (95% confidence levels). Chi square test was used to calculate the measure of association. Descriptive statistics was presented as proportions and percentages in form of tabulation charts and graphs.

4.0 RESULTS

4.1 Characteristics of study participants

A total of 119 participants were recruited into the study with age range between 25-55yrs mean age of 39.88 (1SD±6.96years). Figure 1 show a peak at age 40-44 years 32 (26.7%).

Figure 2: Age (years) distribution

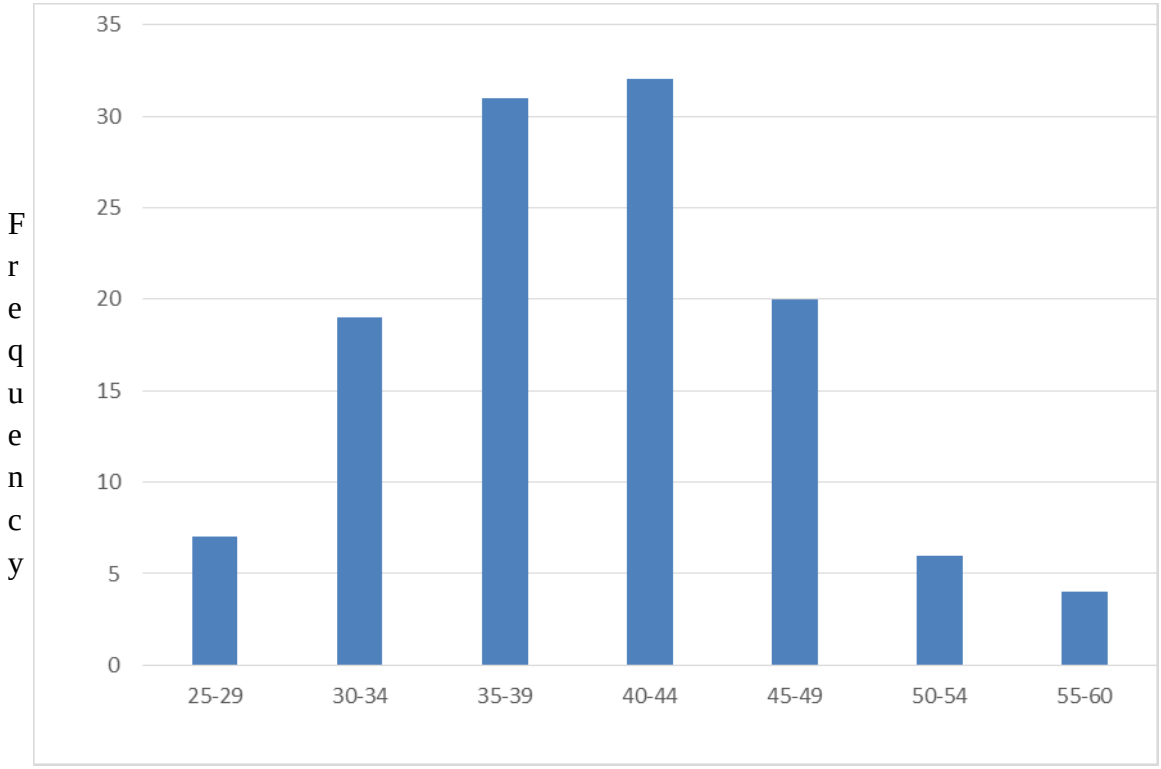


Table 1. Descriptive Statistics

	Minimum	Maximum	Mean	Std. Deviation
Age	25	56	39.88	6.96
Age of the first Intercourse	13	28	19.4	2.84
Number of pregnancies	1	10	2.88	1.79

The minimum age at first intercourse was 13 and the number of pregnancies was 2.88 per woman (Table 1).

Parity and sexual history

Parity ranged from 1-10 with a median of 2.9. Median age at first sexual intercourse was 19 years (Table 1).

Table 2: Characteristics of study participants

Variable	Frequency	%
Marital Status		
Married	**65**	**56.3**
Single	**34**	**28.6**
Divorced	**9**	**7.6**
Widowed	**6**	**5**
Separated	**3**	**2.5**
Reproductive History		
Contraceptive use		
Condom	**81**	**71**
Injection	**18**	**15.9**
IUCD	**6**	**5.2**
Implant	**5**	**4.2**
None	**5**	**4.2**
OCP	**3**	**2.6**
BTL	**1**	**0.8**
Condoms		
Always	**69**	**57.9**
Sometimes	**42**	**35.3**
Never	**8**	**6.7**
Pap smear screening		
Yes	**38**	**31.9**
No	**81**	**68**
Cervical appearance		
Normal	**65**	**54.6**
Eroded	**37**	**31.2**
Inflamed	**5**	**4.2**
Suspicious	**2**	**1.7**

Marital status

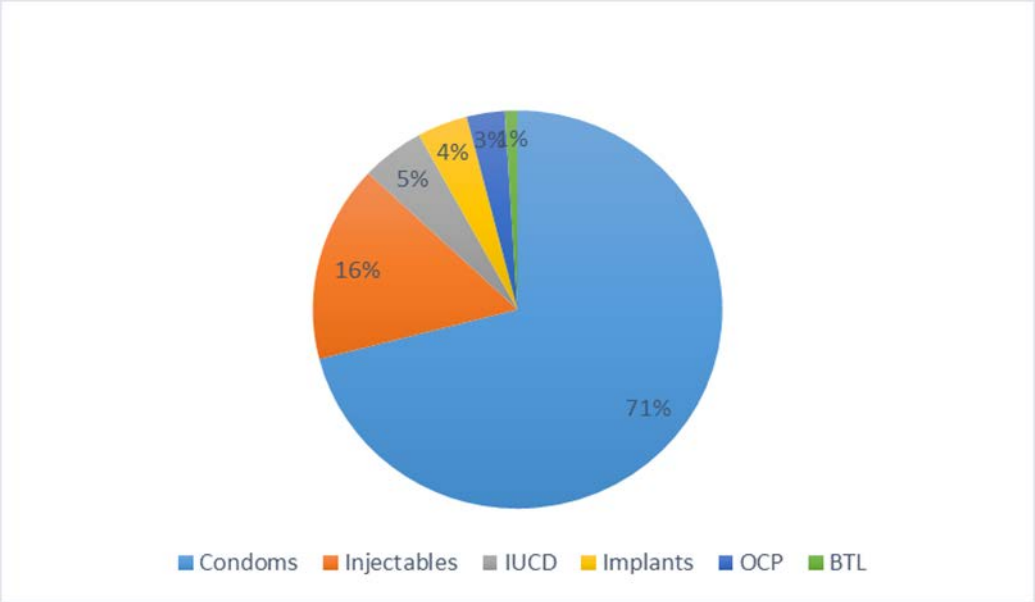
Majority of women (56.3%) reported they were married, while 28.6% were single.

Contraceptive use

Contraceptive use was high with 114 (95.7%) reporting being on some contraceptive method and a small number 5 (4.2%) were not using any contraceptive method.

The most commonly used contraceptive method was condom, 81 (71.0%; n= 114) followed by injectables 18 (15.9%); IUCD 6 (5.2%); implants 5 (4.4%); OCP 3 (2.6%) and BTL 1 (0.8%). However, 42(35.3%) of the women were reported using condoms inconsistently.

Figure 3: Contraceptive method used



Pap smear and cervical appearance

During their life time 38 (31.9%) had ever had a pap smear whereas 81 (68%) had never had one (Table 2). The appearance of the cervix was normal in 65 (54.6%) and 37 (31.1%) were reported to have erosions (Table 2).

4.2 Prevalence of cervical lesions on Pap smear

Out of a total number of 119 participants, 43 (36.1 %) had an abnormal Pap smear (ASCUS and above). High Grade Squamous Intraepithelial Lesions (HSIL) was the most common abnormality accounting for 13.4% followed by squamous cell carcinoma at 6.7% (Table 3). Of the 43 participants with abnormal lesions 9(20.9%) were reported as ASCUS, 5(11.6%) were LSIL, 2(4.6%) were ASC-H, 16(37.2%) were HSIL, 8(18.6%) were SCC, 3(6.9%) were HSIL/AGC, AGC and Adenosquamous carcinoma (Table 4).

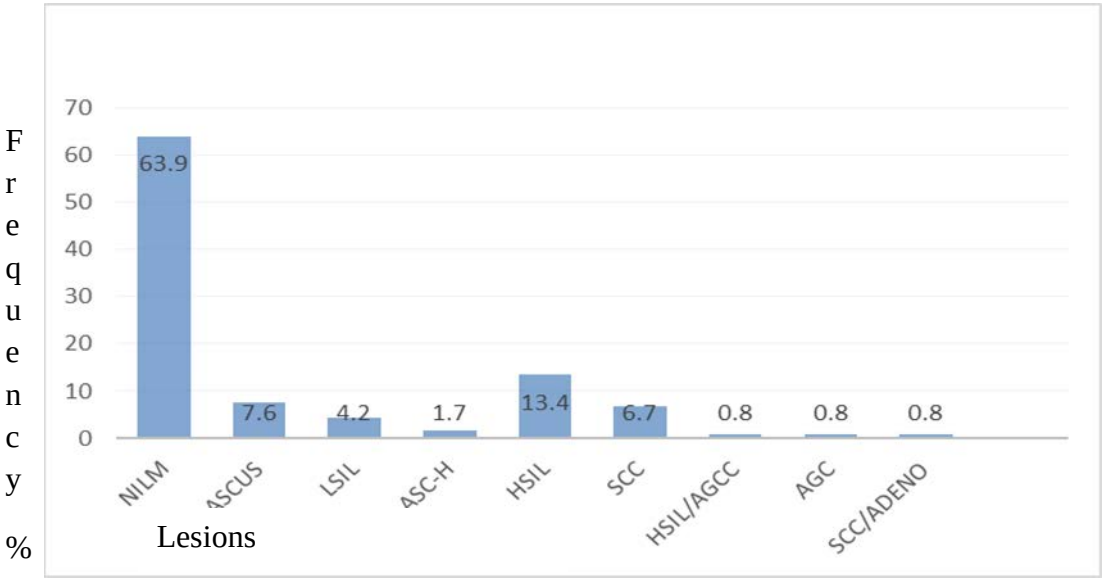
Table 3: Pap smear results (n=119)

	Frequency	Percent
NILM	76	63.9
ASCUS	9	7.6
LSIL	5	4.2
ASC-H	2	1.7
HSIL	16	13.4
SCC	8	6.7
HSIL/AGC	1	0.8
AGC	1	0.8
Adenosquamous	1	0.8
Total	119	100

Table 4: Abnormal Pap smear results (n=43)

Pap smear lesion	Frequency	Percent
ASCUS	**9**	**20.9%**
LSIL	**5**	**11.6%**
ASC-H	**2**	**4.6%**
HSIL	**16**	**37.2%**
SCC	**8**	**18.6%**
HSIL/AGC	**1**	**2.3%**
AGC	**1**	**2.3%**
Adenosquamous Ca	**1**	**2.3%**

Figure 4: Pap smear results



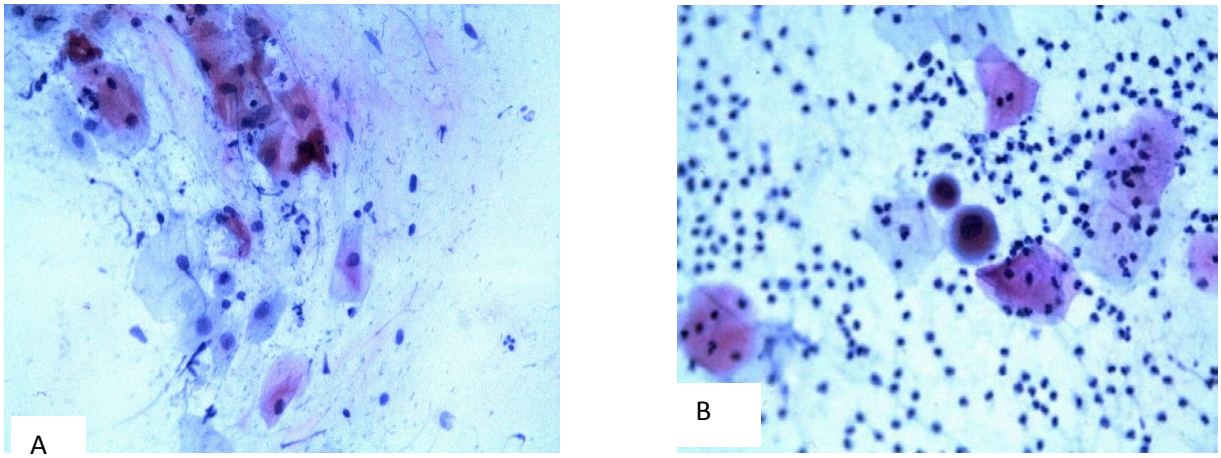
Prevalence of infections among the HIV infected women

Fourteen patients (11.7%) had infections; of these, 11(9.2%) had bacterial vaginosis and 3(2.5%) had Candida infection.

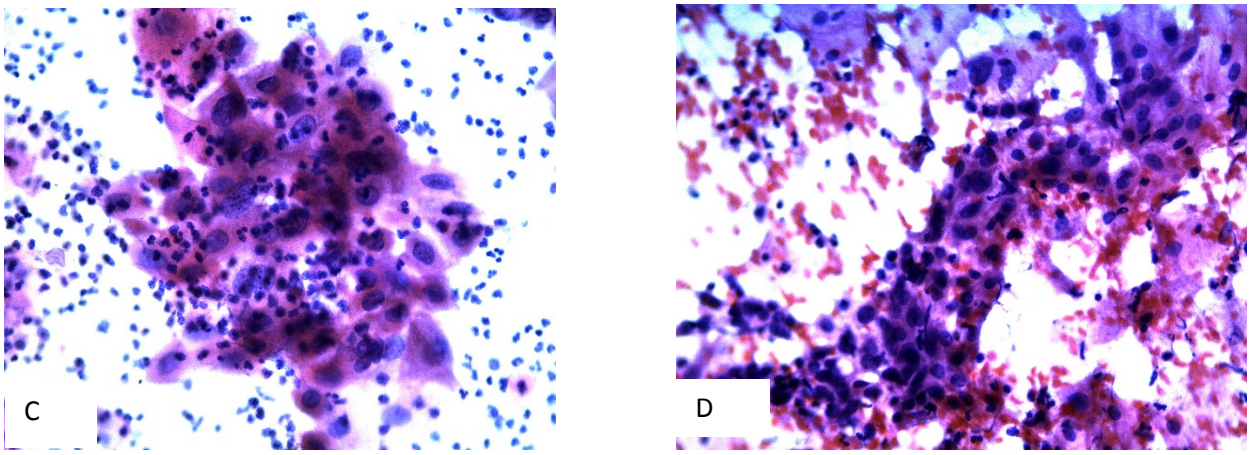
Table 5: Prevalence of infections among the HIV infected participants

Infection	Prevalence
Bacterial vaginosis	11 (9.2%)
Candida	3 (2.5%)
Total	14 (11.7%)

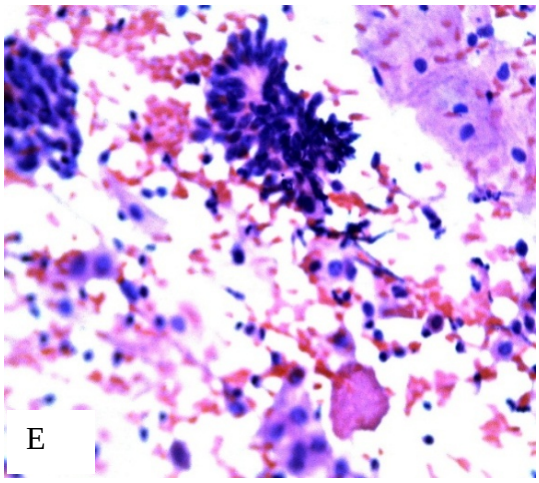
Photomicrographs of intraepithelial lesions and infections (Leica) (x40, Pap stain)

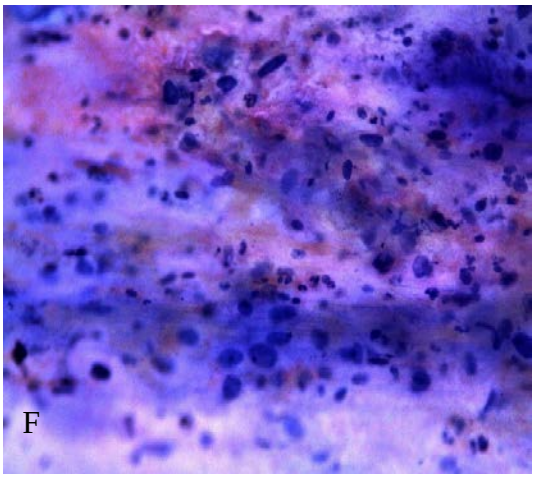


A. Atypical squamous cells of undetermined significance (ASCUS) (x40, Pap stain). B. Atypical squamous cells cannot exclude HSIL (ASC-H) (x40, Pap stain).

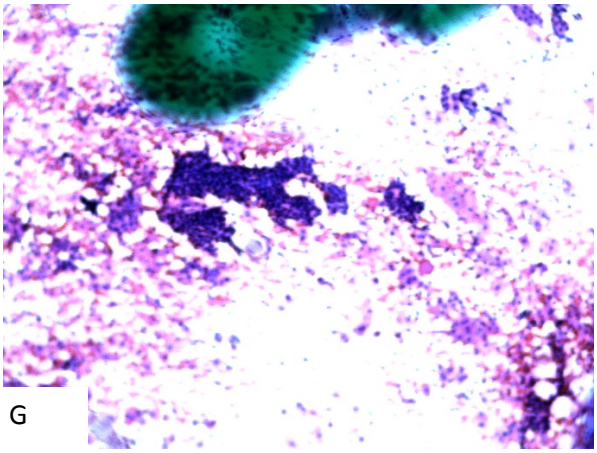


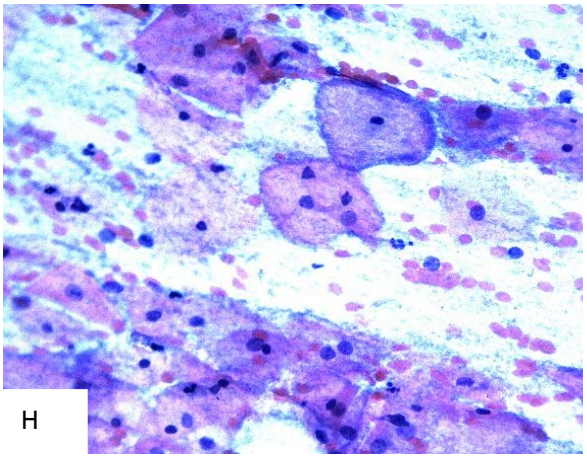
C.High grade squamous intraepithelial lesion (HSIL) (x40, Pap stain). D.Squamous cell carcinoma (SCC) (x40, Pap stain).





E. Rosettes´ in a case of adenosquamous carcinoma (x40, Pap stain). F. Invasive squamous cell carcinoma (SCC) with tumour diathesis (x40, Pap stain).





G. Adenosquamous carcinoma(x10, Pap stain). H. Bacteria vaginosis (BV) (x40, Pap stain).

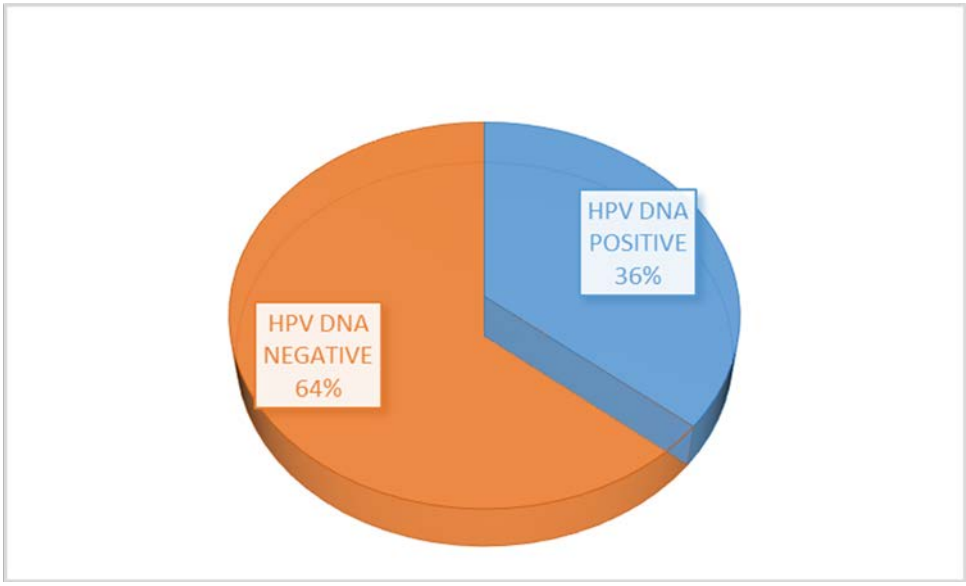
4.3 High risk HPV DNA test results

The High risk HPV DNA test by hc2 was carried out for one hundred and two cases. It was positive in 37(36.27%) cases (Table 6).

Table 6: HPVDNA Test Results

Result	Frequency	Percent
Positive	**37**	**36.27**
Negative	**65**	**63.73**
Total	**102**	**100**

Figure 5: Results High risk HPV infection



4.4 HPV DNA among HIV infected women with abnormal Pap smear results.

A total of 102 participants had a HPV DNA test done. Out of these 36(35.2%) had abnormal Pap smear (ASCUS and above) of this, 28(77.7%) were positive for HR HPV DNA and 8(22.3%) were negative for HR HPV**.**

Table 7: The Comparison for both Pap smear and HPV DNA tests

	High risk HPV DNA results		
Pap smear	**Positive (%)**	**Negative (%)**	**Total**
NILM	**9(13.6%)**	**57(86.4%)**	**66**
ASCUS	**4(57%)**	**3(43%)**	**7**
LSIL	**4(80%)**	**1(20%)**	**5**
ASC-H	**0**	**1(100%)**	**1**
HSIL	**13(87%)**	**2(13%)**	**15**
SCC	**6(100%)**	**0**	**6**
HSIL/AGC	**0**	**0**	**0**
AGC	**0**	**1(100%)**	**1**
Adenosquamous Ca	**1(100)**	**0**	**1**
Total	**37**	**65**	**102**

Table 8: The results of both Pap smear and HPV DNA tests

HR HPV DNA			
Lesions	Positive	Negative	Total
NILM	9(24.3%)	57(86.3%)	66
ASCUS	4(10.8%)	3(8.1%)	7
LSIL and ASC-H	4(10.8%)	2(5.4%)	6
HSIL and above	20(54%)	3(8.1%)	23
Total	37	65	102

4.5 Relationship of Pap smear results and HPV DNA test

The correlation between Pap smear and HPV DNA test was statistically significant with **(p=0.001).**All Pap smears with carcinoma were positive for HR-HPV test**.**

Table 9: Correlation of Pap smear results and HPV DNA

HR HPV DNA				
Pap smear result	Positive	Negative	Total	P value
Normal	9(24.3%)	57(87.6%)	66	**0.001**
ASCUS/LSIL	8(21.6%)	4(6.1%)	12	
ASC-H above	20(50.0%)	4(6.1%)	24	
Total	37	65	102	

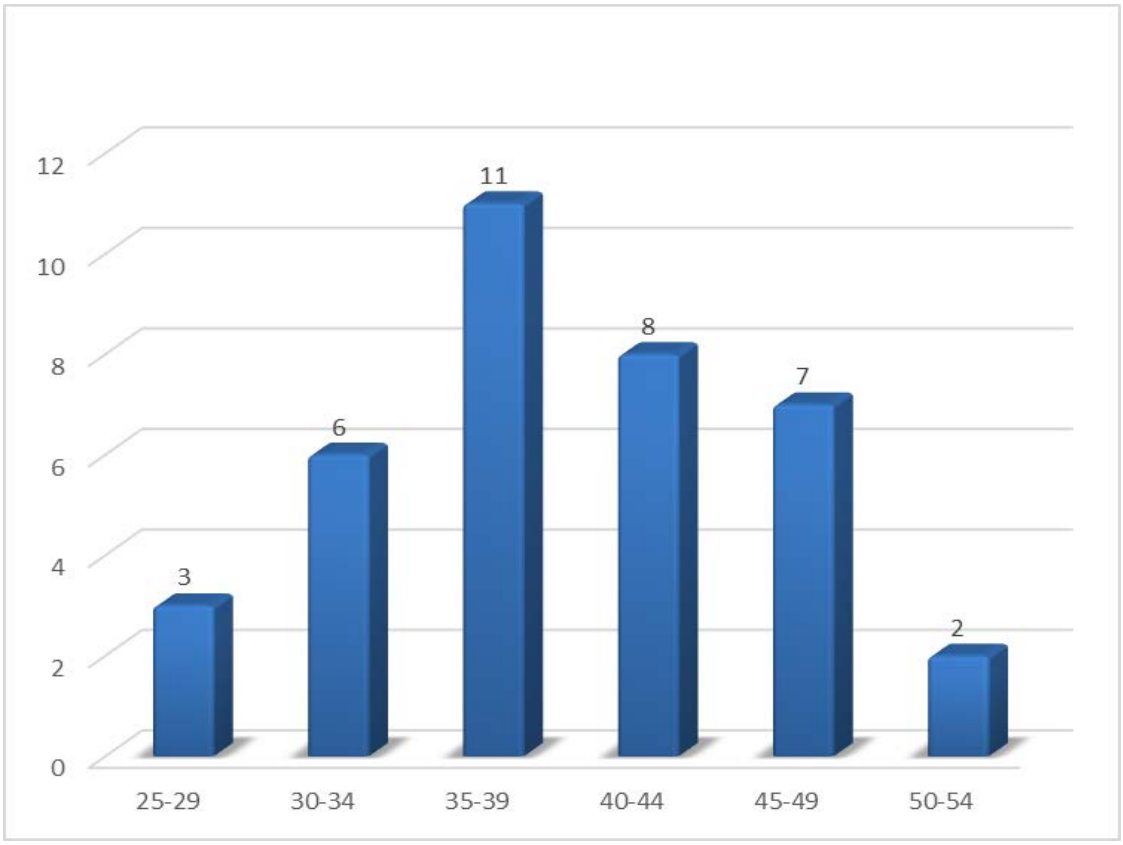
The results of both High grade Lesions in Pap smear and HPV DNA test.

A total of 15 participants had HSIL, out of which 13(87%) tested positive for HR HPV DNA and the rest, (2) were negative. All the cases of SCC and adenosquamous carcinoma lesions were positive for high risk HPV DNA.

4.6 Age group distribution and HR HPV test results

The prevalence of HR HPV showed a normal distribution, and increased with age in this cohort of women, peaking in the 35 - 39 year age group and declining thereafter.

Figure 6: HR HPV DNA positivity by Age



The cost of Pap smear and HPV DNA test.

Cost of a Pap smear at KNH CCC was charged at Ksh 500, HPV DNA Hybrid capture2 test at KAVI, UoN, Molecular laboratory was Ksh.2400 totaling to Ksh 2900. In a private hospital laboratory in the same neighborhood, the cost of HPV hybrid capture 2 would be Ksh 4000 and Pap smear Ksh 1500. The total cost of both tests to the client if they had paid outside the study would therefore have been Ksh.5500. A rapid HPV DNA test just coming into the market will cost about Ksh. 470. (The current´s exchange rate of 1USD = Ksh 96).

HPVDNA testing identifies risk for developing CIN earlier than Pap smear and this factor when considered downstream the patient will save money.

5.0 DISCUSSION

A total of 119 HIV positive patients attending the Comprehensive care centre at Kenyatta National Hospital were studied.

The mean age of the study participants was 39.9 years (1SD ± 6.96), range 25-56 years. Of the 119 participants who met the inclusion criteria, 36.1 % had an abnormal Pap smear (ASCUS and above). Of the 43 participants with abnormal lesions, 7.6% were reported as ASCUS, 4.2% were LSIL, 1.7% were ASC-H, 13.4% were HSIL, 6.7% were SCC and 6.9% were HSIL/AGC, AGC and Adenosquamous carcinoma.

In a study by Memiah et al. in Kenya 2012) the prevalence of precancerous lesions (LSIL, HSIL, and ICC) lesions was 26.7% which is comparable with this study at 24.3% (34). Participants from both studies were from the same resource-limited setting and were on ARVs. In a similar study done by Parham et al. (2006) the prevalence of LSIL, HSIL and SCC was 23.3%, 32.6% and 20% respectively which was higher than this study (35).

The reasons for this high-prevalence could be partially attributed to the fact that the women in this study were severely immunosuppressed (median CD4+ count 165/μL). The participants recruited were HIV-infected women attending the tertiary care hospital to receive antiretroviral therapy, and to be eligible for such treatment they had to meet the criteria of having a CD4+ count of <200/μL or had recently experienced some other AIDS defining illness, while this study included participant who were sexually active for the last six months and on ARVs. Another possible explanation for the high rate of severe cervical abnormalities detected in Parham et al study was the use of monolayer liquid cytology said to have a higher sensitivity than conventional smear. In a study by Firnhaber et al. (2013) from South Africa a higher prevalence of HSIL at 33% was observed (36).

The high prevalence of HSIL was attributed to women living longer due to ART use. South Africa also has one of the highest HIV prevalence in the world (37). The difference can be explained by the fact that in the South Africa study the cohort was of over 1000 participants compared to this study which had 119. The high prevalence of squamous intraepithelial lesions among HIV infected women is occasioned by persistent HR-HPV infection which is a risk factor for cervical neoplasia (36).

It is well established that HPV infection is a contributor to cervical neoplasia and invasive cancer, and that it progresses to squamous intraepithelial neoplasia more frequently and rapidly in HIV-infected women than in the general population (36). HIV-positive women have a 2-fold to 4-fold greater rate of HPV infection than HIV-negative women.

The prevalence of HPV among HIV-positive women is associated strongly with low CD4 counts and high HIV viral load (36).

The prevalence of high risk HPV infection in this study was 36%, this can be attributed to the low level of HPV infection awareness among the study participants. The age-adjusted global figure of HPV DNA in women with normal cytology in large meta-analysis studies was 10.5% and 31.6% for Eastern Africa in which PCR-based assays were used for HPV DNA detection of cervical cells (28). The highest prevalence of HPV occurs among adolescents and young adults between the ages of 15 and 25. More than 75% of new HPV infections occur in individuals of this age range. This increased risk for infection among younger women has been postulated to be related to the lack of adaptive immune responses and/or the relatively large area of cervical epithelium undergoing squamous metaplasia in this age group, which may enhance the opportunity for HPV DNA to infect the basal cell layer where it can then proliferate **(**38).The findings of this study compares well with those by Isaakidis et al. in India (2008) (39), and Muchiri L et al. in Kenya (2012) (28) who reported a prevalence of 32% and 37% respectively. These similarities could be explained by the fact that the study participants were of the same age group, (25-55+ years), the age group associated with persistent HPV infection. A similar study done by De Vuyst et al. (Kenya) (2012) (40) showed a prevalence of 52.6% which was higher than the current study. The difference could be attributed to the fact that De Vuyst et al. selected women between ages 18 – 55 years thus increasing detection of transient HPV while the current study included women above 25 years. ARVs are now more readily available than earlier in the HIV/AIDS epidemic. This may contribute to the lower prevalence of HPV in this study. Luchters et al. (41) reported a prevalence of HR HPV at 84% among HIV- infected pregnant women in Kenya. The higher prevalence can be explained by increased risk factors - the study participants being married, pregnant and a more advanced WHO clinical staging for HIV disease which are considered significant predictors of HR-HPV infections. High risk-HPV infection among HIV-infected pregnant women is common and in the majority of women persists until three months

post-delivery. In the current study no data was sought on clinical staging and participants were not pregnant.

The HPV prevalence in samples with abnormal cytology (ASCUS and above) in this study was 78%. Bosch et al (1995) (42) reported the prevalence of HPV in HIV infected women with cervical lesion to be between 50-85% which is similar to this study. In a study by Muchiri L et al. (28) a prevalence of 52% was reported which was lower than this study. This difference could be explained by the fact that study participants were from peri-rural community with majority having one sexual partner during their life time thus reducing the risk of acquiring HPV infection. The current study was based in an urban community with mixed ethnic groups who were attending CCC. In this study 78% participants had abnormal pap smears (lesions of ASCUS and above) and were positive for HR-HPV, of this 25% were carcinoma and all were 100% positive for HR-HPV DNA which is similar to a study by Bosch et al. (1995) (42) in which the results of their study showed 99.7% high risk HPVDNA using PCR based assay. A study by Firnhaber et al. (2013) (36) compared screening methods in HIV positive women using Pap smear and hybrid capture 2. HPV DNA using hybrid capture 2 testing was found to be more sensitive method, but less specific than conventional Pap smear. It was also interesting to note , but not unexpected, that more than half of the lesions which were HSIL and above (54%) were positive for high risk HPV DNA, further proving evidence of the importance of co-testing using Pap smear and HPV DNA test. The study findings indicate that HPV infection was frequent in the study participants and associated with squamous intraepithelial lesions, suggesting that this population follows an epidemiological pattern common with other HPV-infected women, who remain at high risk for cervical neoplasia; as such, aggressive screening and management are justified for HIV/HPV co-infected women (39).

In this study HPV DNA testing picked a significant percentage (13.6%) who had negative Pap smears and required follow up. A similar study by Hameed et al. (2001) reported a prevalence 63% of normal Pap smear and positive for HPV DNA which was higher than this study (22). This could be accounted by the fact that in Hameed et al. study HPV typing was done for both low risk and high risk HPV DNA, while this study tested for HR-HPV only.

HPV typing identifies a significant group of HIV-positive women who are at risk of developing cervical intraepithelial neoplasia, although they may not show significant abnormalities on their

Pap smears. HPV DNA testing identifies risk for developing CIN earlier than Pap smear and this factor when considered downstream the patient will save money. HPV DNA testing would also reduce errors caused by human misinterpretation especially when abnormal cells are few in a sample (7).

5.1 Conclusion

More than 20% of the HIV infected women in this study had high grade lesions requiring further active intervention.

Majority of the high grade lesions detected in this study were positive for high risk HPV DNA, including all cases of invasive carcinoma. HPV DNA test also detected HR-HPV in women with normal Pap smears thus identifying HIV-positive women at risk of developing cervical intraepithelial neoplasia.

5.2 Recommendations

1. All HIV infected women in the CCC are at high risk for cervical neoplasia and should have a mandatory cervical cancer screen as part of their management.

2. HPV DNA testing identifies HIV-positive women who have normal Pap smear but are at risk of developing cervical intraepithelial neoplasia. This test is therefore more useful in identifying cervical neoplasia risk than a repeat Pap smear.

3. Women with a positive high risk HPV DNA test and an abnormal Pap smear result should be offered appropriate therapy while those with negative Pap smear results and positive high risk HPV DNA should be followed up closely.

5.3 Limitations

1. There were no significant limitations in this study.

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APPENDICES

APPENDIX 1a: INFORMED CONSENT EXPLANATION

Introduction and objectives of the study

My name is Joyce Wanjiru Karuri, a Master's of Science (clinical cytology) post graduate student in the Department of Human Pathology at the University of Nairobi. I am conducting a study on conventional Pap smear and HPV DNA co-testing in HIV infected women at the Comprehensive Care Centre in KNH using facilities at cytology laboratory and KAVI Molecular laboratory.

Purpose of Study

The study aims to determine the utility of co-testing by conventional Pap smear and HPVDNA test in screening HIV infected women attending Comprehensive Care Centre in KNH.

Benefits and Risks

There may be no direct benefit to the participant but the information provided by the results may be used for better management of your condition by the attending doctor, as well as improved cervical cancer screening programs for women with HIV.

The early diagnosis of cervical premalignant and malignant lesions and other abnormalities such as infections will guide the clinician to offer a comprehensive management.

Some of the information required from the study participants may be personal.

There is minimal pain during sample collection and the procedure is minimally invasive, and would be no different from the usual Pap smear collection

I humbly request you to join the study and allow use of your specimen to determine the presence of pre-cancerous and cancerous lesions. Other conditions e.g. infections may be revealed and will be indicated in the report and this will improve your management and follow up.

Participation in this study will be voluntary and it is part of your routine evaluation and you are free to withdraw any time without losing the benefits to which you are entitled in this institution.

Pap collection explanation for study participants

You will be prepared to lie on an examination couch.

A nurse or a doctor will insert a speculum in your vaginal canal and visualize the cervix.

The cervix will be sampled using Pap smear sterile collection devices in the kit.

A second sample for HPV DNA will be collected using sterile DNA sampling devices and the sampler placed in a preservative.

The principle investigator will take both samples to the laboratory for examination.

Confidentiality

Names will not be included in the study materials and you will be identified by study numbers. Questionnaire will be kept under key and lock and only the principal investigator will access it. Questionnaires will be kept for one year then destroyed. Any information given to us will remain confidential and will be for your own benefit.

Your name will only appear in the laboratory result form that will be sent to your patient file.

You will get your results in the usual manner during your next visit.

Withdrawal from study

In case you feel unable to continue to participate in the study you are free to withdraw without loss of any benefit or quality of management to which you are entitled in this clinic and Kenyatta National Hospital.

Contact Information

If you have any question regarding the study please contact me, Joyce Wanjiru Karuri University of Nairobi P.O BOX 19676-00202 Nairobi on Mobile number 0721319898 or my supervisor, Prof. L.W. Muchiri, P.O BOX 19676-00202 Nairobi Tel: 726300 Ext. 43774 or Prof. M. L. CHINDIA, The Secretary, KNH/UON Ethical Research Committee, Tel: 726300-9 Ext 44102.

CONSENT FORM

I……..after reading and being explained the purpose of the study and what it entails, do hereby give consent to participate in the study fully aware of the benefits and risks.

I am aware that I can withdraw from this study without loss of any benefit or quality of management to which I am entitled.

Participants Signature/Thumb print ……Date……..

Doctor/Nurse……Date……..

Principal investigator……Date…….

APPENDIX 1B: FOMU YA IDHINI

KICHWA CHA UTAFITI:

KULINGANISHA KIPIMO CHA PAP SMEAR NA HPV DNA KWA WANAWAKE WALIOGUNDULIWA KUWA NA VIRUSI VIA UKIMWI KATIKA CCC KWENYE HOSPITALI KUU YA KENYATTA.

Jina langu ni Joyce Wanjiru Karuri mwanafunzi wa chuo kikuu cha Nairobi idala ya Human Pathology. Ningependa kufanya utafiti ambao nitawaelezea. Tafadhali soma ujumbe ufuatao kwa makini. Ujumbe huu utaelezwa kwa njia ya kingereza na Kiswahili. Una uhuru wa kuchagua lugha ambayo utaelewa vyema

MAELEZO KWA UFUPI NA NJIA YA UTAFITI HUU

Njia ya kizazi yaweza kuwa na dalili za ugojwa wa saratani ambao huletwa na virusi vya HPV kwa wanawake ambao wako na virusi vya ukimwi. Utatolewa vipimo mala mbili na njia ya uchunguzi ni rahisi.

Hali yako ya kua na virusi vya HPV itajullikana na uchunguzi kwa njia ya Pap smear yatalinganishwa.

FAIDHA YA UCHUNGUZI NA MADHARA YA UTAFITI HUU KWAKO

Majibu ya HPVDNA italinganishwa na ile ya Pap smear. Vipimo vyote viwili vina uwezo wa kugundua dalili ya saratani ambayo kipimo cha Pap smear peke yake kingekosa. Dalili ya magojwa mengine yaweza kupatikana. Hivyo basi utafaidhika kutokana na uamuzi bora wa uchunguzi na uamuzi bora wa matibabu utakayopewa..Hakuna faidha yeyote ya kifedha utakayopata kutokana na utafiti huu.

Utaratibu wa kupata vipimo vyote viwili kwa njia ya Pap smear hauna madhara yeyote, muhudumiwa aweza kuhisi usumbufu kiwango kidogo ile anaweza vumilia

Matokeo yako itashugulikiwa kwa njia ya siri na hakuna yeyote anayeruhusiwa atakayesisoma. Hakuna malipo ya ziada utakayo hitajika kwa kuhusika katika uchunguzi huu.

Majina halisi hayatatumiwa ila nambari ndizo zitakazotumiwa.

Matokeo yatawekwa kwa njia ya siri na ni mchunguzi pekee yake ambaye atayapata.

Watakao shiriki katika uchunguzi huu itakua kwa njia ha hiari bila kushuritishwa. Kutoshiriki hutapoteza kwa njia yeyote haki yako kuhundumiwa unavyostahili.

Majibu ya uchunguzi huu utapata kwa njia ya kawaida wakati wa kufuata kiliniki yako ya Kawaida.

Maelezo ya vipimo kwa washiriki

Muuguzi ama daktari atakutayarisha kupimwa.

Utalala kwa kitanda cha kupimiwa.

Ataingigiza speculum katita njia yako ya uzazi ili aweze kuona sehemu ambazo zinaweza kuwa na shida.

Kipimo cha Pap kitachukuliwa na vifaa safi ndani ya Pap smear kit.

Kisha utatolewa kipimo cha pili HPV DNA na vifaa safi ndani ya DNA sampling kit.

Mchuguzi atapeleka vipimo vyotee viwili katika chumba ha utafiti .

Ukiwa na swali yeyote unaweza wasiliana nasi wakati wowote kupitia nambari zifuatazo iwapo una swali lolote;

MUCHUNGUZI, JOYCE WANJIRU KARURI, Chuo Kikuu Cha Nairobi S.L.P. 19676-00202 Nairobi Numbari ya simu 0721319898 Msimamizi Prof. L.W. Muchiri S.L.P 19676-00202, Nairobi Nambari ya Simu 726300 Ext. 4377, Prof. M.L CHINDIA Maadali ya utafiti ya KNH/UONERC S.L.P. 20732-0200 Nairobi Kenya. Nambari ya simu 726300-9 Ext. 44102.

IDHINI YA MSHIRIKI

Kama utashiriki tafadhali tia sahihi yako kwenye pengo lilioachwa hapa chini

Mimi----------nimesoma na nimeelewa nia

ya uchunguzi huu, utaratibu utaotumika kuchukua kipimo, faida na madhara yanayohusika

na uchunguzi huu. Nimekubali kushiriki kwa hiari bila kushurutishwa.

Sahihi ya mushirika----------Tarehe ----------

Sahihi ya shahindi----------Tarehe……...

APPENDIX II: QUESTIONNAIRE

PROJECT TITLE: CONVENTIONAL PAP SMEAR AND HUMAN PAPILLOMA VIRUS DNA CO-TESTING CONENTIONAL PAP SMEAR AND HPV DNA TESTING IN HIV INFECTED WOMEN ATTENDING CCC IN KENYATTA NATIONAL HOSPITAL

Variable

Study number----------**[code: dm0]**

1. Age …… years **[code: dm1]**
2. Are you currently using modern family planning method **[dm2]** (1) Yes… (2)No…If yes which one? [**code: dm21**]

1) Natural…...
2) OCP……
3) IUD…….
4) Injectable…….
5) Condoms……

Others ….Specify……..

3. Experience contact or post coital bleeding 1) Yes……2) No……..**[code:dm3]**
4. Ever had previous Pap smear 1) Yes……2) No……**[code: dm4]**
5. Condom use 1.always/ 2.sometimes/ 3.never **[code:dm5]**
6. Sex partners in the last 6 months 1) Yes……. 2) No.…… **[code:dm6]**
7. Age at first intercourse……**[code:dm7]**
8. Marital status……**[code:dm8]**

1) Singled /
2) widowed/
3) divorced/separated
4) married

9. Number of pregnancies……**[code:dm9]**
10. L.M.P……**[code:dm10]**

APPEARANCE OF THE CERVIX [code: dm CX]

1) NORMAL……..
2) ERODED…….
3) INFLAMMED…….
4) SUSPICIOUS……..
5) OTHERS……..SPECIFY…….

DATE OF SPECIMEN COLLECTION: ……..

Previous Pap report…….Date…….

LAB REPORT

BETHSEDA CLASSIFICATION [code: BC]

Adequacy 1.Yes……2. No……3.Specify…….
4. Negative…….5.ASCUS……6.LSIL…….

7. Inflammatory/ 8.Reactive……..9.ASC H…….10.HSIL……11.SCC……
12. AGC……13.AIS……14.ENDOCERVICAL ADENOCARCINOMA……

HPV DNA RESULTS [code: HD]

1. HR-HPV Positive…… 2.HR-HPV Negative……

COMMENTS

PRINCIPAL INVESTIGATOR'S NAME……SIGN……..

PATHOLOGIST'S NAME……. SIGN…….

DATE…….

APPENDIX 111: PROCEDURE OF PAP STAINING

Principle of the stain (43)

Haematoxylin stains the nuclei blue by dye-like formation. The eosin azure solution being acidic stains the cytoplasm which is basic so that the eosin has affinity for the mature cells while light green has affinity for the young endocervical cells. Orange G also being an acidic dye has an affinity for the cytoplasm of the oldest superficial cells and also stains keratin(36).

1. Slides smears were smears were fixed in 95% ethanol.
2. They were hydrated through ethanol grades of 80%, 70% and then 50%.
3. Smears were rinsed with distilled water.10dips.
4. They were stained in Harris Haematoxylin for 4minutes.
5. They were rinsed with tap water.
6. Smears were differentiated in 0.05%acid water 10dips
7. They were rinsed in tap water and blued in running tap water 10 dips.
8. They were rinsed in 95% ethanol.
9. Smears were stained with O.G for 2 minutes.
10. They were rinsed in 95% ethanol 10 dips.
11. They were stained in EA50 for 4 minutes
12. Smears were rinsed in 95% ethanol10dips
13. They were dehydrated in 3 changes of absolute alcohol.
14. They were cleared in 2changes of xylene 10 dips.
15. They were mounted in DPX.

RESULTS

Pap smear results will be reported using the Bethesda System 2001;

Detailed below,

Sample report

Pap smear

Specimen adequacy

Satisfactory smear for evaluation

Endocervical cells present

Interpretation

Low grade squamous intraepithelial lesion (LSIL)

Comment

Repeat Pap smear after 12 months

APPENDIX 1V: THE BETHESDA SYSTEM FOR REPORTING CERVICAL CYTOLOGY (2001) (16)

- The Bethesda System-2001 consists of several components, as outlined below, and is recommended for reporting cervical cytology.

1. SPECIMEN TYPE

- Indicate conventional (Pap smear) vs. liquid-based preparation

2. SPECIMEN ADEQUACY

- Satisfactory for evaluation (describe presence or absence of endocervical or transformation zone component and other quality indicators), e.g., partially obscuring blood, inflammation, etc.)
- Unsatisfactory for evaluation (Specify reason).
- Specimen rejected/not processed (specify reason).
- Specimen processed and examined, but unsatisfactory for evaluation of epithelial abnormality because of (specify reason).

3. GENERAL CATEGORIZATION (Optional)

- Negative for Intraepithelial Lesion or Malignancy.
- Epithelial Cell Abnormality:
- Others: see /Result (e.g. endometrial cells in a woman (40 yr. of age or older).

4. INTERPRETATION/RESULT

A. Negative for Intraepithelial Lesion or Malignancy

- When there is no cellular evidence of neoplasia, state this in the General
- Categorization above and/or in the Interpretation/Result section of the report whether or not there are organisms or other non-neoplastic findings.

1. Organisms:

- *Trichomonas vaginalis*
- Fungal organisms morphologically consistent with *Candida spp.*
- Shift in flora suggestive of bacterial vaginosis.
- Bacteria morphologically consistent with *Antinomyces spp.*
- Cellular changes consistent with herpes simplex virus
- Reactive cellular changes associated with
- Inflammation (includes typical repair)

- Radiation
- Intrauterine device (IUD).
- Glandular cells status post hysterectomy
- Atrophy.

3. Others

- Endometrial cells (in a woman 40 years of age or older)
- Specify if (negative for squamous intraepithelial lesion)

B. Epithelial Cell Abnormalities

Squamous cell:

- Atypical squamous cells
- Of undetermined significance (ASC-US).
- Cannot exclude HSIL (ASC-H).
- Low-grade squamous intraepithelial lesion (LSIL)
- Encompassing: HPV/mild dysplasia/CIN1).
- High-grade squamous intraepithelial lesion (HSIL)
- Encompassing: moderate and severe dysplasia, CIS, CIN 2 and CIN 3).
- With features suspicious for invasion (if invasion is suspected).
- Squamous cell carcinoma

2. Glandular Cell:

- Atypical

  Endocervical cells (NOS or specify in comments)
- Endometrial cells (NOS or specify in comments)
- Glandular cells (NOS or specify in comments).
- Atypical
- Endocervical cells, favor neoplastic.
- Glandular cells, favor neoplastic
- Endocervical adenocarcinoma in situ.
- Adenocarcinoma
- Endocervical
- Endometrial

- Extra uterine
- Not otherwise specified (NOS)

C. Other Malignant Neoplasm: (specify)

- Carcinoma
- Sarcomas
- Other tumours

ANACILLARY TESTING

- Provide a brief description of the test method(s) and report the result so that it is easily understood by the clinician.

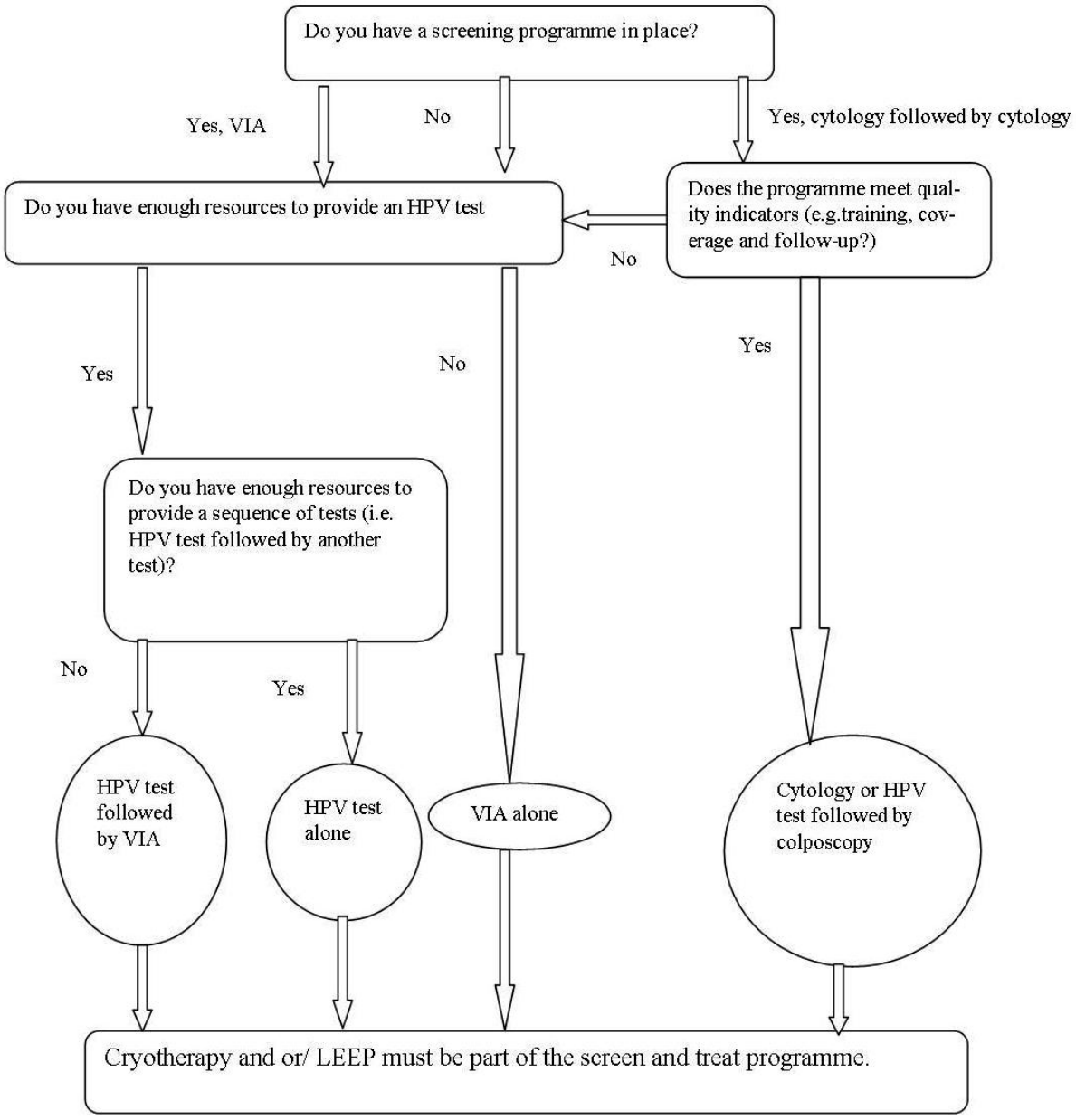
AUTOMATE REVIEW

- If specimen was examined by automated device, specify the device and the result.

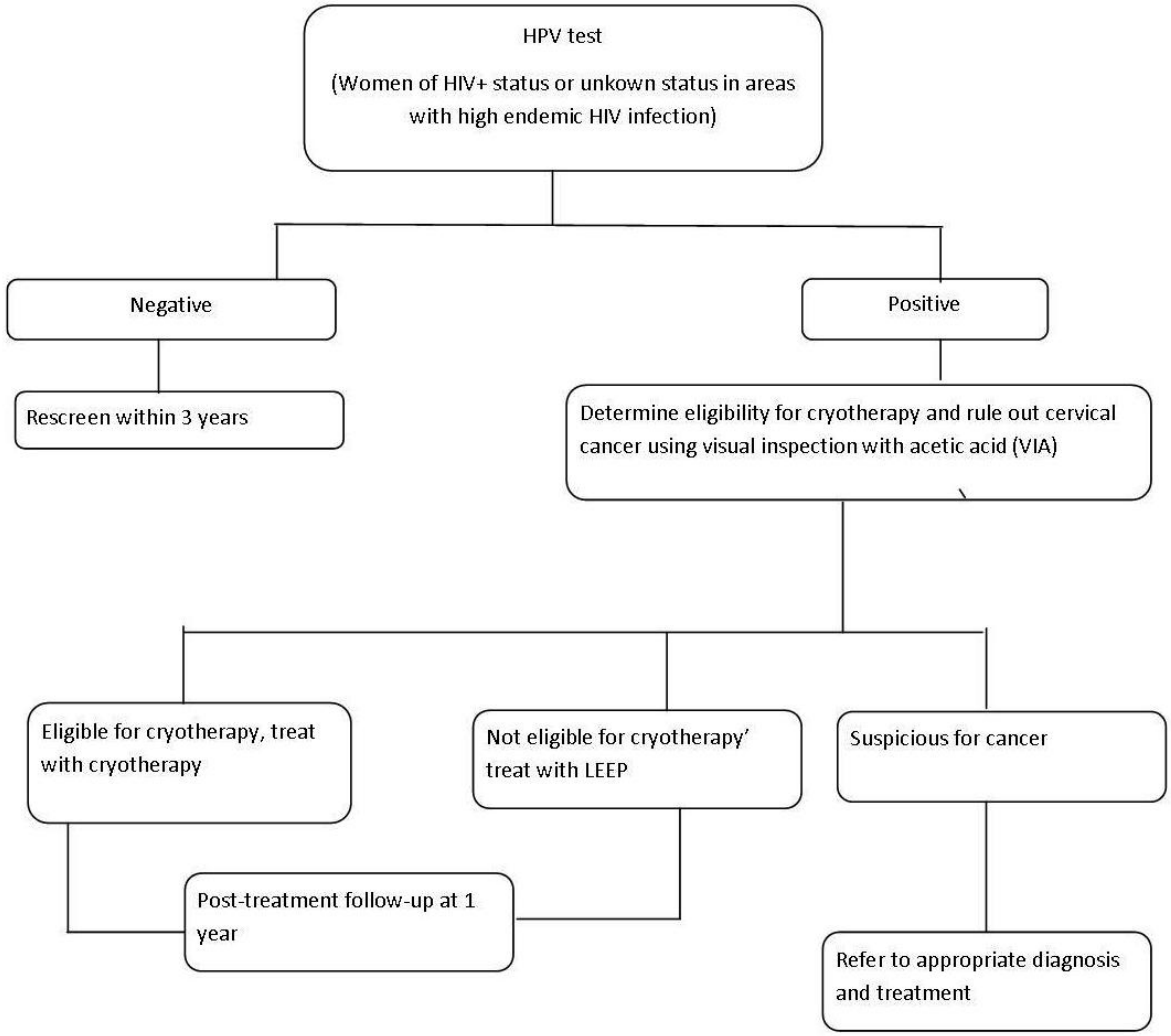
EDUCATIONAL NOTES AND SUGGESTIONS (optional)

- Suggestions should be concise and consistent with clinical follow-up guidelines by professional organizations (references to relevant publications may be included).

APPENDIX V: FLOW CHART FOR WHO RECOMMENDATION IN GENERAL POPULATION (44)

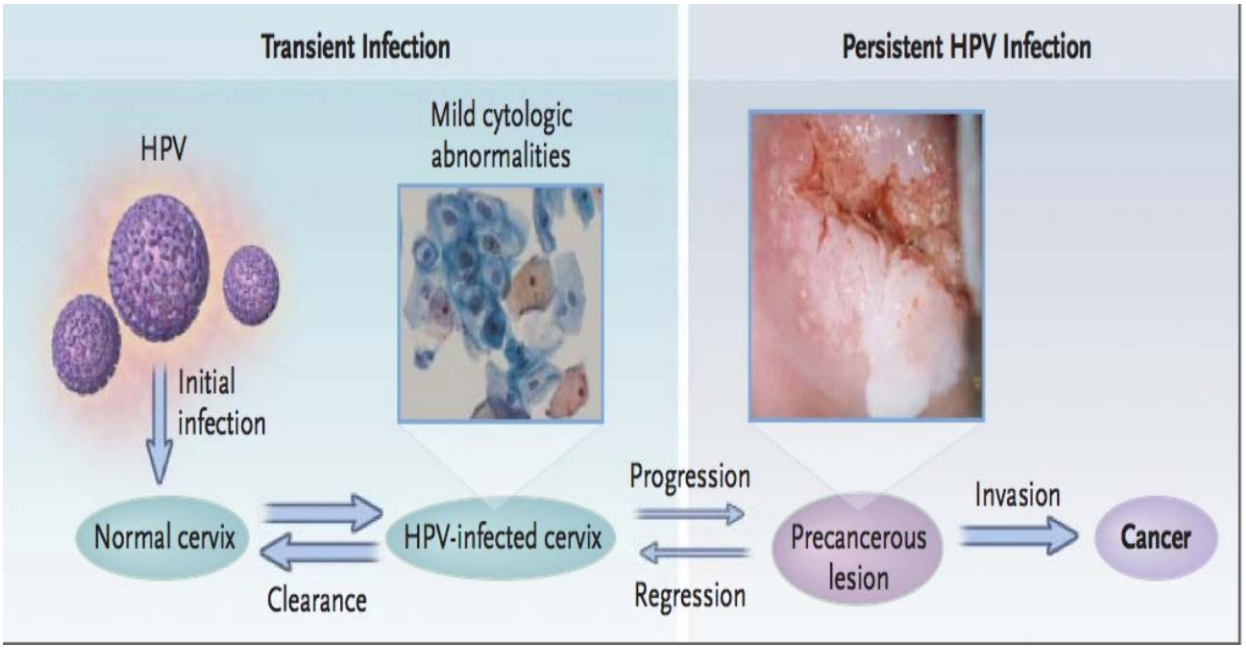


APPENDIX VI: FLOW CHART SHOWING WHO RECOMMENDATION OF HIV INFECTED WOMEN OR UNKNOWN STATUS IN AREAS OF HIGH ENDEMIC HIV INFECTION (44).



.

APPENDIX VII: HPV NATURAL HISTORY (45)



APPENDIX VIII: SAMPLE COLLECTION PROCEDURE FOR PAP SMEAR AND HPV DNA (46)

Pap smear and HPV DNA collection procedure

1) Upon signing of the consent and patients assurance the patient was placed in a lithotomy position.

2) The speculum was lubricated and inserted into the vagina until the cervix came into full view.

3) The cervix broom was inserted and rotated at 360 degrees at the columnar junction or any suspicious area.

4) It was then removed and the smear was on a slide and the broom discarded.

a. DNA Pap cervical sampler brush was inserted 1-1.5cm into the os of the cervix until the largest outer bristles of the brush touched the ectocervix. It was rotated three full turns in a counter clockwise direction.

b. The brush was inserted into the bottom of the transport tube and the shaft snapped off at the score line and the tube capped securely.

c. The speculum was slowly withdrawn and removed.

d. The patient was advised to come for the results during the next visit.

e. The specimens were stored at -20 degrees for 2months prior to testing.

Source: QIAGEN (46)

DNAPAP Cervical Sampler Transport Medium

Manual Insert Ref 5122-122 Lot 148862 Exp 2016-08-30

APPENDIX 1X: HPV DNA TESTING
TYPE OF EQUIPMENT: HPVDNA TEST TYPE (46)

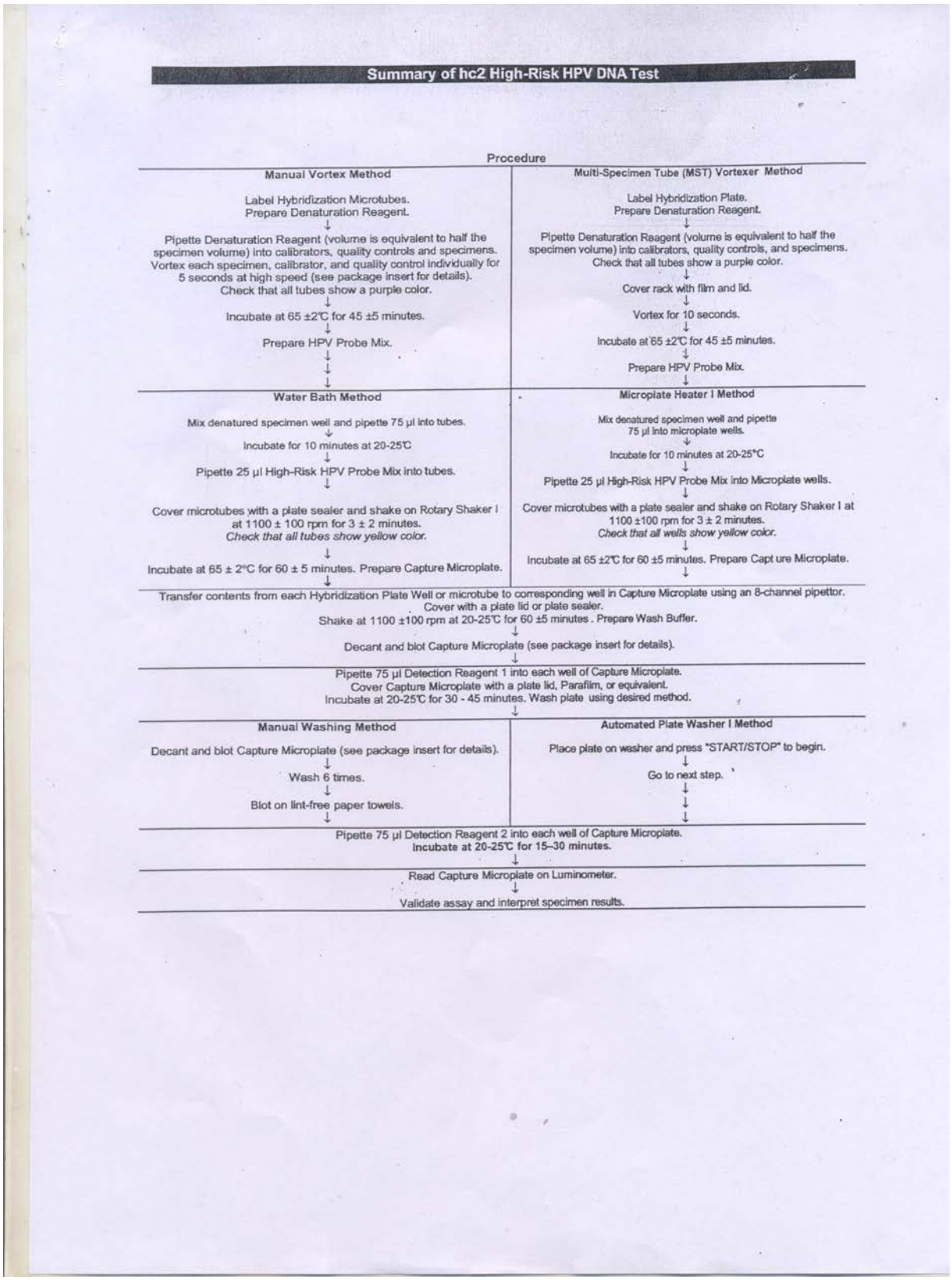
Hybrid capture V.II, (hc2) is a machine to test HPV DNA utilizing the hybrid capture system technology which is a registered trade mark of QIAGEN, Inc. Germany. REF 5197-1330.A

PRINCIPLE OF THE PROCEDURE

The HPVDNA test using Hybrid capture (hc2) technology is a signal amplified hybridization antibody capture assays that that utilizes microplate chemiluminescent detection. Specimens containing the target DNA hybridize with a specific HPVRNA probe. The resultant RNA; DNA hybrids are captured on onto the surface of the microplate well coated specific for RNA: DNA hybrids. The immobilized hybrids are reacted with alkaline phosphatase conjugated antibodies specific for RNA: DNA hybrid and are detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase light is emitted which is measured as relative light units on a luminator. The intensity of the light emitted denotes the presence or absence of the target DNA in the specimen. An RLU measurement equal or greater than the cut off value indicates the presence of HPV DNA sequence in the specimen. An RLU measurement less than the cut off indicate the absence of specific HPV DNA sequence tested or HPVDNA sequence below the detection limit of the assay. High volume samples throughout testing with the Hc2 HPVDNA test can be performed utilizing the rapid capture system (RCS).The instrument processes up to 352 specimens in 8 hours.

To enable high volume sample throughout testing all the procedural steps of the assay are performed by the rapid capture system (RCS) with the exception of specimen denaturation, chemiluminescent signal detection, and result reporting.

Appendix X: Protocol for processing HPV DNA samples (46)



Summary of hc2 High-Risk HPV DNA Test

Procedure

Manual Vortex Method

Label Hybridization Microtubes.
Prepare Denaturation Reagent.
↓
Pipette Denaturation Reagent (volume is equivalent to half the specimen volume) into calibrators, quality controls and specimens.
Vortex each specimen, calibrator, and quality control individually for 5 seconds at high speed (see package insert for details).
Check that all tubes show a purple color.
↓
Incubate at 65 ±2°C for 45 ±5 minutes.
↓
Prepare HPV Probe Mix.
↓

Multi-Specimen Tube (MST) Vortexer Method

Label Hybridization Plate.
Prepare Denaturation Reagent.
↓
Pipette Denaturation Reagent (volume is equivalent to half the specimen volume) into calibrators, quality controls, and specimens.
Check that all tubes show a purple color.
↓
Cover rack with film and lid.
↓
Vortex for 10 seconds.
↓
Incubate at 65 ±2°C for 45 ±5 minutes.
↓
Prepare HPV Probe Mix.
↓

Water Bath Method

Mix denatured specimen well and pipette 75 µl into tubes.
↓
Incubate for 10 minutes at 20-25°C
↓
Pipette 25 µl High-Risk HPV Probe Mix into tubes.
↓
Cover microtubes with a plate sealer and shake on Rotary Shaker I at 1100 ± 100 rpm for 3 ± 2 minutes.
Check that all tubes show yellow color.
↓
Incubate at 65 ± 2°C for 60 ± 5 minutes. Prepare Capture Microplate.
↓

Microplate Heater I Method

Mix denatured specimen well and pipette 75 µl into microplate wells.
↓
Incubate for 10 minutes at 20-25°C
↓
Pipette 25 µl High-Risk HPV Probe Mix into Microplate wells.
↓
Cover microtubes with a plate sealer and shake on Rotary Shaker I at 1100 ±100 rpm for 3 ± 2 minutes.
Check that all wells show yellow color.
↓
Incubate at 65 ±2°C for 60 ±5 minutes. Prepare Capt ure Microplate.
↓

Transfer contents from each Hybridization Plate Well or microtube to corresponding well in Capture Microplate using an 8-channel pipettor.
Cover with a plate lid or plate sealer.
Shake at 1100 ±100 rpm at 20-25°C for 60 ±5 minutes . Prepare Wash Buffer.
↓
Decant and blot Capture Microplate (see package insert for details).
↓

Pipette 75 µl Detection Reagent 1 into each well of Capture Microplate.
Cover Capture Microplate with a plate lid, Parafilm, or equivalent.
Incubate at 20-25°C for 30 - 45 minutes. Wash plate using desired method.
↓

Manual Washing Method

Decant and blot Capture Microplate (see package insert for details).
↓
Wash 6 times.
↓
Blot on lint-free paper towels.
↓

Automated Plate Washer I Method

Place plate on washer and press "START/STOP" to begin.
↓
Go to next step.
↓
↓
↓

Pipette 75 µl Detection Reagent 2 into each well of Capture Microplate.
Incubate at 20-25°C for 15–30 minutes.
↓

Read Capture Microplate on Luminometer.
↓
Validate assay and interpret specimen results.

Appendix XI: Interpretation of Quality Control and Results of Test (46)

ASSAY CALIBRATION VERIFICATION CRITERIA

Assay Calibration Verification is performed to ensure that the reagents and furnished Calibrator and Quality Control materials are functioning properly, permitting accurate determination of the assay cutoff value. The hc2 High-Risk HPV DNA Test requires calibration with each assay, therefore, it is necessary to verify each assay using the following criteria. This verification procedure is not intended as a substitute for internal quality control testing. The DHCS v.2 and Digene Qualitative Software with Version 4.01 or later DML 2000 Assay Protocols for HPV automatically verify the criteria below.

1. Negative Calibrator
   The Negative Calibrator must be tested in triplicate with each assay. The Negative Calibrator mean must be ≥ 10 and ≤250 RLU's in order to proceed. The Negative Calibrator results should show a coefficient of variation (%CV) of ≤ 25%. If the %CV is >25%, discard the value with a RLU value furthest from the mean as an outlier and recalculate the mean using the remaining two values. If the difference between the mean and each of the two values is ≤ 25%, proceed to step 2. Otherwise, the assay calibration verification is invalid and the assay must be repeated for all patient specimens. Accordingly, do not report patient specimen results.

2. Calibrator
   The High-Risk HPV Calibrator (HRC) must be tested in triplicate with each assay. The Calibrator results should show a coefficient of variation (%CV) of ≤ 15%. If the %CV is >15%, discard the calibrator value with a RLU value furthest from the mean as an outlier and recalculate the mean using the remaining two calibrator values. If the difference between the mean and each of the two values is ≤ 15%, proceed to step 3. Otherwise, the assay calibration verification is invalid and the assay must be repeated for all patient specimens. Accordingly, do not report patient specimen results.

   The assay calibration verification described above for the Negative Calibrator and Calibrator is performed automatically by the DHCS v.2 and the Digene Qualitative Software and printed on the data analysis report. **The DHCS v.2 Software and Digene Qualitative Software with Version 4.01 or later DML 2000 Assay Protocols for HPV automatically verifies the High-Risk HPV Calibrator %CV is ≤ 15%.** However, the Digene Qualitative Software (v1.0.2 and v1.0.3) will NOT invalidate the assay unless the %CV is >25% for the High-Risk HPV Calibrator. Therefore the user must manually verify that the %CV calculated by the DML 2000 Instrument software is ≤ 15% and proceed as indicated for Situation 1 in the table below. If the %CV of the Calibrator replicates falls between 15 and 25% refer to the Instructions in Situation 2 or 3 in the table below and proceed with the indicated "User Action."

Situation	Report %CV for the HRC Replicates	Action Taken by Digene Qualitative Software	User Action
1	≤ 15%	Assay reported as "Valid"	Results may be reported; no further action required.
2	Between 15% and 25%	No outliers removed and assay reported as "Valid"	Remove the Calibrator RLU value farthest from the mean. Recalculate the %CV of the Calibrator with the two remaining values. If the %CV of the two remaining RLU values is > 15%, the assay is invalid. The results must not be reported. If the %CV of the remaining RLU values is ≤ 15%, recalculate the assay cutoff, then recalculate the RLU/cutoff ratio of each specimen using this cutoff. These recalculated values may be reported.
3	Between 15% and 25%	One outlier removed and assay reported as "Valid"	Assay is invalid. Results must not be reported. Assay must be repeated.
4	> 25%	One outlier removed and assay reported as "Invalid"	Assay is invalid. Results must not be reported. Assay must be repeated.

In order to manually calculate the %CV as required in Situation 2 above, the user should divide the standard deviation (n-1) of the remaining replicate RLU values by the mean of the remaining replicate RLU values (HRC) and multiply that result by 100.

To calculate the %CV using Microsoft® Excel (supplied with the Digene Qualitative Software), the user can calculate the standard deviation of the Calibrator replicates using the formula *STDEV* and determine the mean RLU of the Calibrator using the formula *AVERAGE*. Once these two values are obtained, divide the STDEV by the AVERAGE and multiply the result by 100 to obtain the %CV.

(STDEV/AVERAGE) * 100 = %CV

If there are any questions related to calculating %CVs, recalculating the assay cutoff, or recalculating the RLU/cutoff of the specimens, please call your local QIAGEN Representative.

To determine Calibrator reproducibility and estimate the frequency in which manual recalculations may be necessary, the results from three clinical evaluations involving 152 assay runs performed with the hc2 High-Risk HPV DNA Test were compiled. The results showed that the average %CV for these 152 runs was 8.1. Considering all three replicates of the calibrator per test run, calibrator reproducibility of >15%CV was observed for only 17 out of 152 runs (11.2%), with 10 out of these 17 test runs resulting in %CV between 15-25 (Situation 2). For the 17 test runs that yielded a %CV >15, a single outlier was removed and the %CV recalculated. Following the User Action for Situation 2, only one of the test run's %CV remained >15, invalidating the test run. The %CVs of the remaining 151 test runs were calculated for an average %CV of 6.0.

3. **The Calibrator mean (HRC%) and Negative Calibrator mean (NC%) results are used to calculate the HRC%/NC% ratio. These ratios must meet the following criteria to verify the assay calibration** before the specimen results can be interpreted:

Assay Calibration Verification Acceptable Ranges

$2.0 \leq HRC\% / NC\% \leq 15$

4. Calculate the HRC%/NC% ratio. If the ratio is ≥ 2.0 and ≤15, proceed to the next step. If the ratio is <2.0 or >15, **the assay is invalid and must be repeated**. All patient specimens should be repeated within the run.

Note: Acceptable ranges for the Negative Calibrator and Calibrator have been established only for the DML 2000 Instrument.

CUTOFF CALCULATION

Once an assay has been validated according to the criteria stated above, the Cutoff Value for determining positive specimens is HRC X.

Example Cutoff Calculation:		
	NC RLU Values	HRC RLU Values
	97	312
	101	335
	91	307
Mean RLU Value	96	318
%CV	4.9	4.7
HRCX/NCX	N/A	3.31

Therefore, Positive Cutoff Value is (HRCX) = 318.

All specimen RLU values should be converted into a ratio to the appropriate Cutoff Value. For example, all assays should be expressed as Specimen RLU/Cutoff Value.

Notes: RLU/CO values and Positive/Negative results for all specimens are reported in the *DML 2000 Data Analysis Report.*

For the Rapid Capture System instrument application, the RCS HPV software protocol has been programmed to apply a Calibration Adjustment Factor (CAF) of 0.8 to the mean of RLU value of the valid Positive Calibrator replicates. This CAF is necessary so that the performance characteristics of the assay remain equivalent to the manual test procedure. This change only applies to assays performed using the Rapid Capture System instrument application. Therefore, it is critical to select the correct software protocol for use with each specific test method in order to generate accurate test results. All specimen RLU values should be converted into a ratio to the appropriated Cutoff (CO) Value. For example, all assays should be expressed as Specimen RLU/CO Value.

QUALITY CONTROL

Quality control samples are supplied with the hc2 High-Risk HPV DNA Test. Consult the Digene Hybrid Capture System Version 2 User manual or the DHCS v.2 Software Interactive Operator's Guide or Digene Qualitative Software user manuals for instructions on how to input the Lot Numbers and Expiration Dates of the quality controls. These quality controls must be included in each assay, and the RLU/CO of each control must fall within the following acceptable ranges for the run to be considered valid. **If the quality controls do not fall within these ranges, the assay is invalid and must be repeated.** Accordingly, no patient results should be reported for any invalid run.

Quality Control	HPV Type	Expected Result (RLU/Cutoff Value) High-Risk HPV Probe			
		Minimum	Maximum	Average	%CV
QC1-LR	Low-Risk (HPV 6)	0.001	0.999	0.5	25
QC2-HR	High-Risk (HPV 16)	2	8	5.0	25

1. The Quality Controls provided in the kit are cloned HPV DNA targets and are not derived from wild-type HPV. This is the same type of material used for the calibrators supplied with the *hc2 High-Risk HPV DNA Test.*

2. This Quality Control material will not act as an appropriate quality control for the processing of Specimen Transport Medium, PreservCyt Solution or SurePath Preservative Fluid.

3. The Quality Controls provided with this test kit must be used for internal quality control. Additional controls may be tested according to guidelines or requirements of local, and/or country regulations or accrediting organizations.

INTERPRETATION OF SPECIMEN RESULTS

Note: The hc2 High-Risk HPV DNA Test cutoff of 1pg/ml is equivalent to 100,000 HPV copies/ml or 5,000 HPV copies per assay.

1. STM Specimens with RLU/Cutoff Value ratios ≥1.0 are considered "Positive."
2. Specimens with RLU/Cutoff Value ratios <1.0 are considered "Negative" or "None detected" for the 13 HPV types tested. High-risk HPV DNA sequences are either absent or the HPV DNA levels are below the detection limit of the assay.
3. When testing PreservCyt specimens, if the RLU/CO ratio of a specimen is ≥1.0 and <2.5, QIAGEN recommends that the specimen be retested. When testing PreservCyt specimens, if the RLU/CO ratio of a specimen is ≥1.0 and <2.5, QIAGEN recommends that the specimen be retested. If the initial retest result is positive (≥1.0 RLU/CO), the specimen can be reported as positive and no further retesting needs to be completed. However, if the first retest result is negative (<1.0), then a second retest (third result) needs to be completed to generate a final result. The result of the second retest is considered the final result and is to be reported.
4. If the RLU/Cutoff ratio of a specimen is close to but less than 1.0 and high-risk HPV infection is suspected, consider alternate testing methods and/or a repeat specimen.
5. Because this assay only detects high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, be aware that other low-risk HPV types may be present in the specimen. If testing specifically for the presence of sexually transmitted low-risk HPV, it is necessary to use the hc2 HPV DNA Test, which detects low- and high-risk HPV DNA types.

PERFORMANCE CHARACTERISTICS

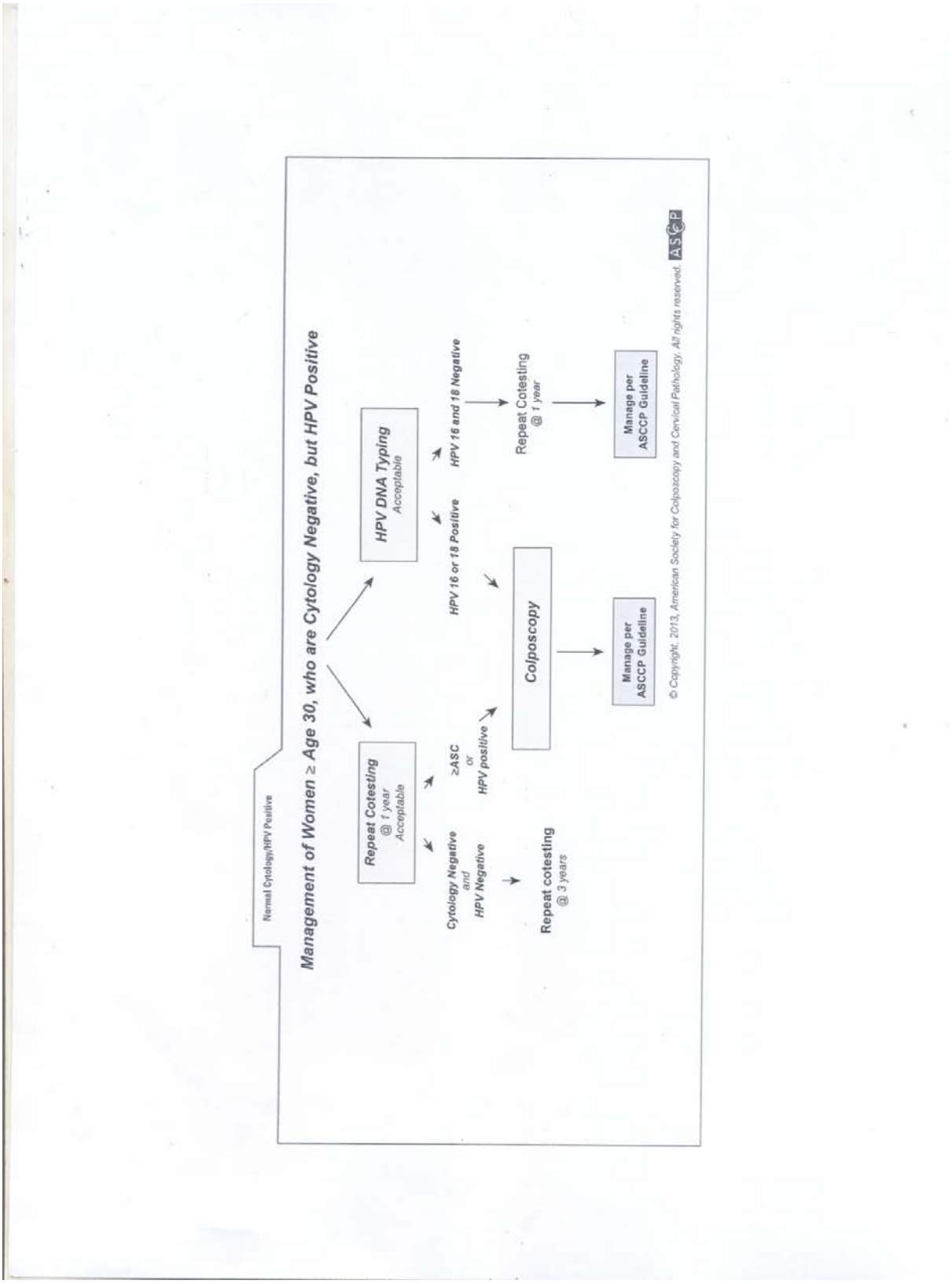
CLINICAL PERFORMANCE WHEN SCREENING PATIENTS WITH NORMAL PAP SMEAR RESULTS AS AN AID IN THE ASSESSMENT OF RISK FOR PATIENT MANAGEMENT

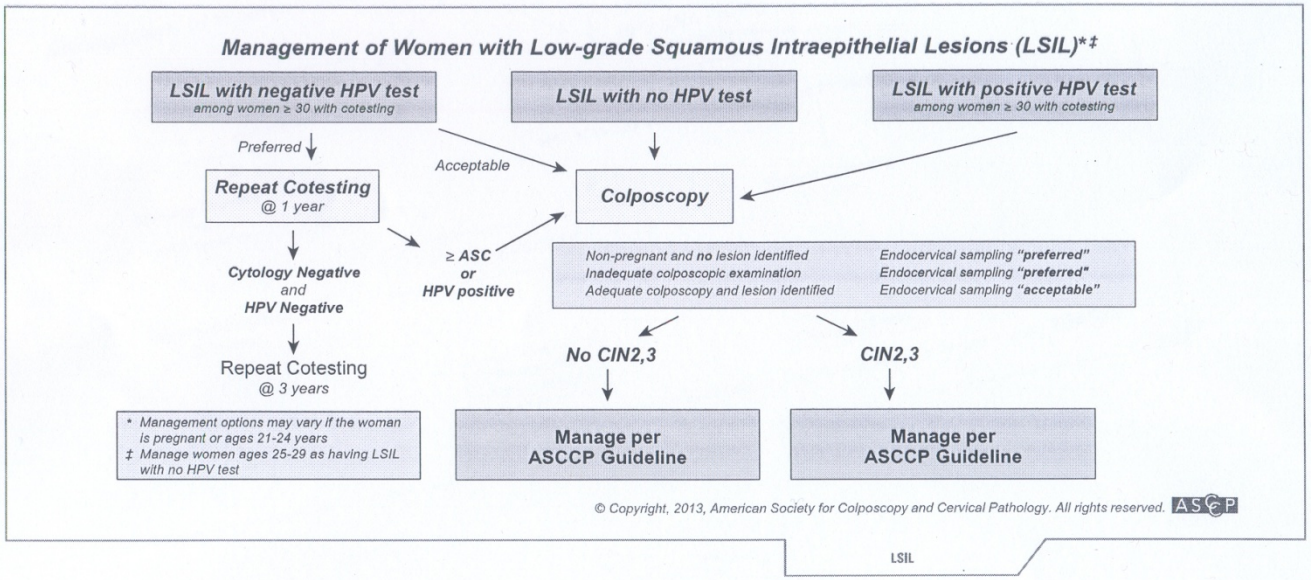
The results of eight independent clinical studies conducted by prominent medical, academic, and government institutions at centers in the United States and abroad are described below. The studies utilized the established Pap methods in use in the countries in which the study was conducted. In all but two cases, the Bethesda Grading System was utilized to interpret the Pap results. For cervical cancer screening equivalent terminology in the European Community refer to the European Guidelines for Quality Assurance in Cervical Cancer Screening[42]. In addition, high-grade cervical disease was diagnosed through the use of colposcopy-directed biopsy for each study. These studies assessed the clinical usefulness of the hc2 High-Risk HPV DNA Test in comparison to the Pap smear for older women (generally over 30-35 years old). All but one study also performed prospective HPV testing using the hc2 High-Risk HPV DNA Test.

The studies were cross-sectional general population screening studies utilizing the hc2 High-Risk HPV DNA Test, unless otherwise noted below. As indicated, 2 of the 8 screening studies were conducted in the United States; 2 in Europe, 2 in Latin America, 1 in Africa, and 1 in Asia.

The performance of the hc2 High-Risk HPV DNA Test observed from six cross-sectional studies are summarized, in tables 3 and 4, for women aged 30 years and over and diagnosed with histologically confirmed high-grade cervical neoplasia (defined as CIN3 or more severe).

Appendix XII: American Society of Colposcopy and Cervical Pathology Algorithms (47)




Management of Women with Low-grade Squamous Intraepithelial Lesions (LSIL)*‡
LSIL with negative HPV test
among women ≥ 30 with cotesting
LSIL with no HPV test
LSIL with positive HPV test
among women ≥ 30 with cotesting
Preferred
Acceptable
Repeat Cotesting
@ 1 year
Colposcopy
Cytology Negative
and
HPV Negative
≥ ASC
or
HPV positive
Non-pregnant and no lesion identified
Inadequate colposcopic examination
Adequate colposcopy and lesion identified
Endocervical sampling "preferred"
Endocervical sampling "preferred"
Endocervical sampling "acceptable"
No CIN2,3
CIN2,3
Repeat Cotesting
@ 3 years
* Management options may vary if the woman is pregnant or ages 21-24 years
‡ Manage women ages 25-29 as having LSIL with no HPV test
Manage per ASCCP Guideline
Manage per ASCCP Guideline
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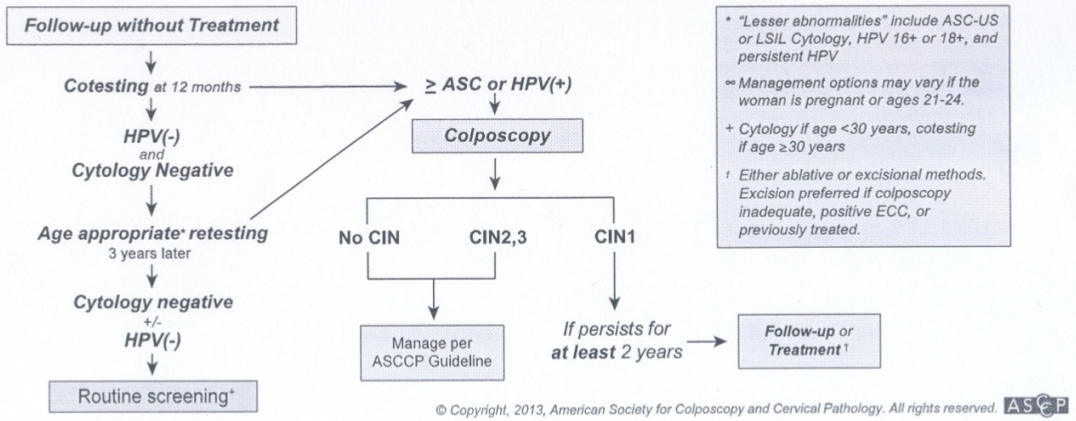
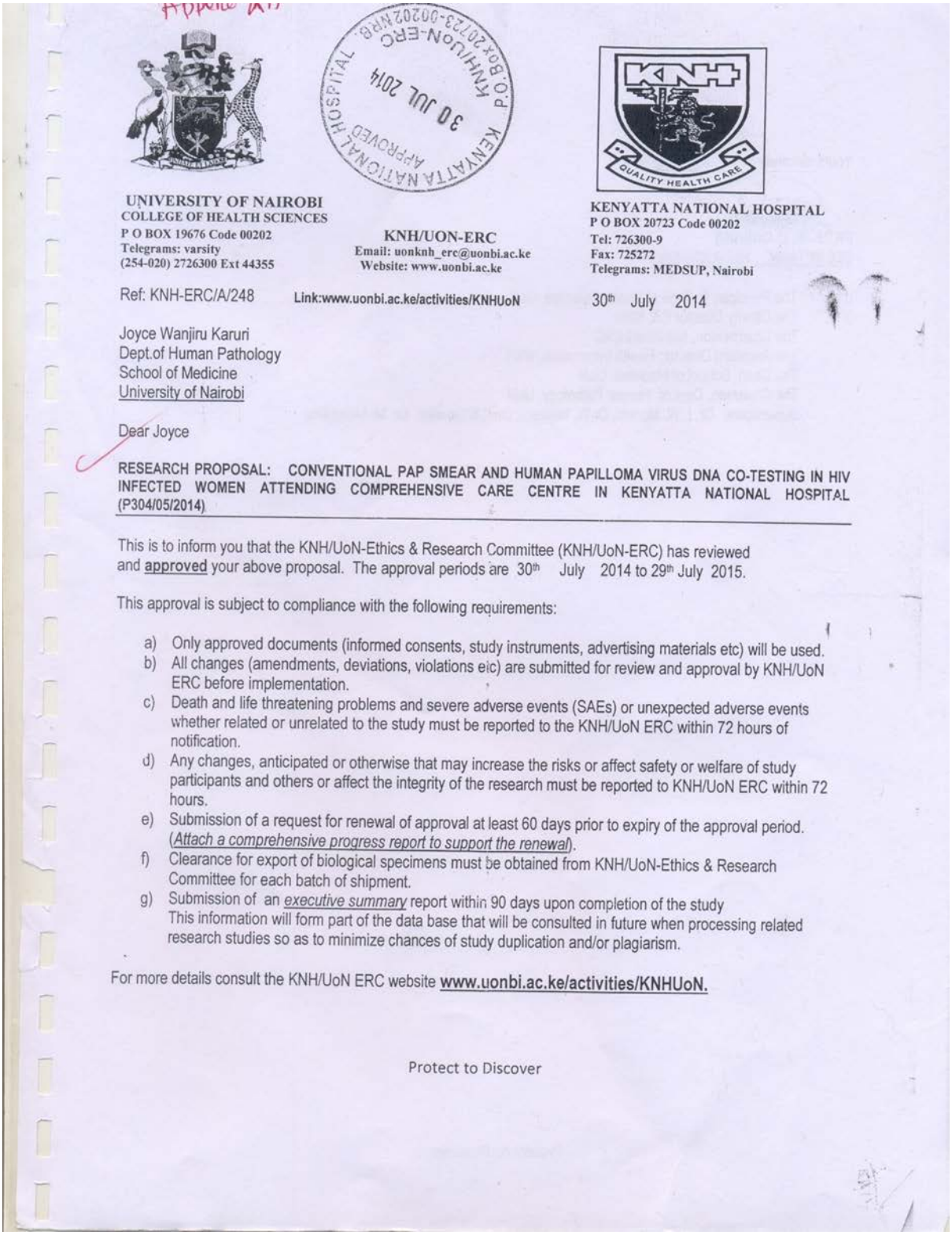
ASCCP
LSIL

CIN1 Preceded by Lesser Abnormalities

Management of Women with No Lesion or Biopsy-confirmed Cervical Intraepithelial Neoplasia — Grade 1 (CIN1) Preceded by "Lesser Abnormalities"*∞



Appendix X111: Ethical Approval



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Ref: KNH-ERC/A/248 Link:www.uonbi.ac.ke/activities/KNHUoN 30th July 2014

Joyce Wanjiru Karuri
Dept.of Human Pathology
School of Medicine
University of Nairobi

Dear Joyce

RESEARCH PROPOSAL: CONVENTIONAL PAP SMEAR AND HUMAN PAPILLOMA VIRUS DNA CO-TESTING IN HIV INFECTED WOMEN ATTENDING COMPREHENSIVE CARE CENTRE IN KENYATTA NATIONAL HOSPITAL (P304/05/2014)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 30th July 2014 to 29th July 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Protect to Discover