Correlation of Human Papilloma Virus and Epstein Barr Virus co-infection with Cytological findings in Cervical Samples among Women Attending Kenyatta National Hospital, Nairobi

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Contents

DECLARATION

I, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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List of Abbreviations

5-FU 5-flourouracil

AIDS . . . . . Acquired Immune-Deficiency Syndrome

ASC-H . . . . . Atypical Squamous Cell cannot exclude High grade

ASC-US . . . . Atypical Squamous Cells of Undetermined Significance

BL . . . . . Burkitt's Lymphoma

CaCx . . . . . Cervical Cancer

CIN . . . . . Cervical Intraepithelial Neoplasm

CIS . . . . . Carcinoma In Situ

CMV . . . . . Cytomegalovirus

DES . . . . . Diethylstilboestrol

DNA . . . . . Deoxyribonucleic Acid

EBV . . . . . Epstein Barr Virus

HC-2 . . . . . Hybrid Capture 2

HGSIL . . . . . High-Grade Squamous Intraepithelial Lesion

HIV . . . . . Human Immunodeficiency Virus

HL . . . . . Hodgkin's Lymphoma

HPV . . . . . Human Papilloma Virus

HR . . . . . High Risk

IARC . . . . . International Agency for Research on Cancer

IC . . . . . Internal Control

ICC . . . . . Invasive Cervical Cancer

KNH . . . . . Kenyatta National Hospital

KSH . . . . . Kenyan Shilling

LBC . . . . . Liquid-Based Cytology

LEEP . . . . . Loop Electrosurgical Excision Procedure

LGSIL . . . . . Low-Grade Squamous Intraepithelial Lesion

LMP . . . . . Latent Membrane Protein

PAP . . . . . Papanicolaou

PCR . . . . . Polymerase Chain Reaction

RNA . . . . . Ribonucleic Acid

SSA . . . . . Sub Saharan Africa

STI . . . . . Sexually Transmitted Infection

TV . . . . . Trichomonas vaginalis

VIA . . . . . Visual Inspection with Acetic acid

VILI . . . . . Visual Inspection with Lugol's Iodine

WHO . . . . . World Health Organization

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Abstract

Background

Around the globe, cervical cancer is one of the commonest causes of death among women after breast cancer. Current research shows that the incidence of this disease is quite high in emerging nations as compared to developed ones. This statistic includes Kenya. Research has revealed that Human Papilloma Virus (HPV) is a major contributing factor to cervical carcinogenesis. But, beyond this, latest studies have also shown synergistic effects of carcinogenic factors, for instance, interaction between two or more viruses at different points of tumorogenesis. HPV and Epstein Barr Virus (EBV) are linked with 38% of these cancer cases.

Objectives

We used molecular testing to ascertain the presence of HPV and EBV co-infection in cervical samples of women from Kenyatta National Hospital (KNH) in Nairobi, Kenya. We then associated the existence of both viruses with cytology findings within the same sample population.

Materials and Methods

Two cervical brushes were used to collect cervical samples from each participant and placed in sterile containers, one containing a preservative for cytology and the other with a buffer for molecular tests. Liquid Based Cytology was performed and slides were stained with Papanicolaou stain for microscopy. Hybrid Capture 2 and Polymerase Chain Reaction (PCR) were performed for HPV and EBV respectively. Data analysis was performed using Stata and R software.

Findings

A total number of 114 samples were obtained and processed, and the mean age of the participants were 30-40 years and 40-50 years for HPV and EBV respectively. Of the 114 samples, 99 (86.8%) were normal, 11(9.6%) had microorganisms present, 32(28.1%) were positive for high risk HPV (HrHPV), 17(14.9%) were also positive for EBV.

The co-existence of HPV/EBV in the female genital tract was evident in 6.1% of the cases. On microscopy, the lowest precancerous lesion was ASC-US with 50% Hr HPV but without EBV, LSIL had 100% HrHPV DNA with 50% EBV while ASC-H had similar findings as LSIL.

Conclusion

HPV/EBV dual infection exists most commonly in women with cervical lesions. This is consistent with the fact that Hr-HPV is present in almost all cervical cancer lesions and is more frequent as the lesion grade progresses, and so does the EBV virus. However, a larger number of women evaluated in this study had viruses but did not have lesions.

Implementation

This being a baseline study, we suggest that a new research be done on women with lesions and generalize the prevalence of HPV/EBV co-infection. Most importantly, we recommend that women with positive HrHPV and negative Pap be critically enlightened and followed up to avoid them presenting these cases to the clinic only in late disease stage. This research also allows policy makers to consider changing the algorithms on cervical cancer testing of women above 30 years old whose Hr-HPV DNA is positive but with negative cytology and vice versa. Additionally, we challenge scientists and researchers to formulate an ideal vaccine for EBV, especially considering that it is implicated not only in many cases of cancers, but also with Malaria, which is currently the number one killer disease in Africa.

Chapter 1

Introduction

1.1 Background

Cancer of the cervix is the most prevalent malignant neoplasm among women in Central America, Sub Saharan Africa (SSA) and East Africa.[3,8,26,29] For every hundred (100) women who are diagnosed with cancer, twenty two (22) have cervical cancer. In SSA,cervical cancer accounts for more deaths among women than any other cancer.[3,33] The mortality is as high as 85%.[26,37] The women who live in rural areas of SSA are more vulnerable. They account for 60-75% of cervical cancer cases.[3,34] This is because the affected women are unscreened, untreated, or present to the clinic late with advanced disease, largely due to limited access to knowledge and health care.[3,36,41]

The gravity of the danger that cervical cancer poses has been under-appreciated especially in the face of other competing health priorities such as Malaria, Human Immunodeficiency Virus (HIV) and Tuberculosis. This is because of insufficient epidemiological data, inadequate awareness, deficient human and economic resources, poor implementation of cancer control policies and a dearth of political will to address this complex emergency.[1,14,25,33,44]

Cervical cancer has an estimated mortality rate of 35 per 100,000 in East Africa.[3,36,43] and the incidence in sub-Saharan Africa continues to be very high, comprising 22.2% of all cancers. The incidence of this disease in poor nations is twice above that of industrialized ones.[12,19,33,41] Additionally, incidence rates are expected to rise to 22.2 million diseases per annum with 14 million cancer deaths worldwide unless preventive measures are adopted.[9,19,40,41] In advanced countries where they have successfully implemented winning screening programs, mortality rates have often fallen below 5 per

100,000 women.[2,3,44]

Cervical Cancer is caused by HPV in 99.7% of the cancer patients.[22,30,44] However, recent studies have shown that synergistic elements of carcinogenic components, such as two or more viruses inter-relating at distinct phases of tumorigenesis may play a role.[11] HPV and EBV are DNA viruses which are correlated with 38% of all virus linked cancers from an approximation of 12% of cancers contributed by viral infections worldwide.[37] The two viruses mentioned above have been reported to co-exist in the cervix as well as other parts of the body such as the larynx, and probably causing cancer. EBV has been said to be an inducer or co-factor in carcinogenesis such as nasopharyngeal carcinoma, gastric carcinoma and lymphoepithelioma like carcinomas..[5,6,11,13]

Chapter 2

Literature Review

2.0.1 Biology of Cervical Cancer

Cervical cancer is a disease that involves the abnormal proliferation of the cells of the cervix. It is a preventable and treatable disease if identified at a sufficiently early stage.[3,7,26,43] The canal of the cervix is continuous from the uterine body through the internal os (orifice or opening) into the vagina through the external os.[26,38]

Cervical cancer evolves from definite pre-cancerous lesions generally known as cervical intraepithelial neoplasia or squamous intraepithelial lesions.[24,44] The ectocervix is lined by squamous cells while the mucin-producing glands in the wall of the cervical canal are lined by columnar cells[3]. A percentage of about 80% to 90% of tumours of the cervix squamous cell carcinoma. The cancer cells envelop the outward surface of the ectocervix. Cervical cancer frequently commences where the ectocervix joins the endocervix known as the transformation zone or squamo-columnar junction.[38,44] Cervical cancer can begin in the squamous cells (squamous cell carcinoma), columnar cells (adenocarcinoma) or a combination of both.[26,38,44] These epithelial cells lie on a surface known as the basal lamina or basement membrane.[26]

When these cells in the epithelium bypass this membrane and infiltrate the stroma which is the primary connective tissue of the cervix, Invasive Cervical Cancer (ICC) ensues.[26] The primary cancer may invade the regions neighbouring the uterus and cervix or adjacent organs, like bladder or rectum in the later stages. Conceivably, this cancer can metastasize to distant sites of the body through the bloodstream or the lymphatic system.[26,38]

2.0.2 Aetiology of Cervical Cancer

The current research has reached a consensus that specific types of Human Papilloma Viruses (HPV) are the primary aetiological actant for cervical cancer and its precursor lesions.[15,22,30,44] HPV is detected in virtually all (99.7%) invasive cervical cancers.[2,15,22,26,28,30,44] It is sexually transmitted and its genes activate certain oncogenes (cancer-causing genes)[28,28,44] which interact with virally extracted and endogenous cellular proteins. This process coincides in deregulation of cell cycle progression, and seems to be critical for the spread of cervical cancers.[24] The high-oncogenic-risk HPV types related with infiltrative cervical cancer generate two oncoproteins deputed E6 and E7. These react with endogenous cell cycle regulatory proteins, which include p53 and Rb (Retinoblastoma) genes.[24]

Out of every 10 women, 7 to 8 manifest symptoms of HPV infection at some phase in their lifetime. The infection often carries on for few months and is frequently cleared within twelve to twenty four months by the woman's own immune system.[3] However, there are few exceptional cases that serve as outliers where the virus perdures for long with high propensity of evolving into Cervical Intraepithelial Neoplasm (CIN) and eventually cervical malignancy.[3,26,38,44]

EBV belongs to the herpes family and is a DNA virus which is connected with infectious mononucleosis. EBV infection may also result in malignant metamorphosis and carcinogenesis . For instance, Burkitt's lymphoma (BL) is a cancer correlated with EBV that is common to central Africa and New Guinea with a prevalence of 6 to 7 cases per 100 000. The primary site of this virus is the oral pharyngeal mucosa. Children and adolescents are grievously affected especially following oral contact, hence the name "kissing disease." With reference to serology, 95% of the world's grown-up population is infected with EBV and will remain so throughout their lifetime. This virus infects quiescent epithelial cells and human B-lymphocytes .The virus multiplies in the former and initiates latent infection in memory B-lymphocytes.

Current research has exhibited that EBV is correlated with B-cell malignancies like Hodgkin's Lymphoma (HL) and lymphoproliferative disease in immunosuppressed patients, and also some T-cell lymphomas and epithelial tumors like gastric cancers.[44] These cancers are distinguished by the existence of multiple extrachromosomal replicas of the viral genome in cancer cells and the expression of part of the EBV genome.

EBV affects as high as 90% of the population. Considering this demographic spread within the population and the molecular characteristics of the virus itself,helps us to summise that cultural norms especially those that govern the acceptable forms of sexual behaviour all play a major role in the prevalence and spread of the virus. We can therefore validly hypothesize that the various ways of intimacy such as oral sex predisposes men and women harboring EBV in the oral cavity to rapid sexual organ infection during the process. This necessitates the study of HPV/EBV co-infection because the two viruses have been shown to co-exist.[4,11,21]And more so,any such study should be done hand in hand with a close inquiry into the social and cultural context within which people live as the viruses and societies have also been shown to co-exist.

Other risk factors that contribute to carcinogenesis include the use of tobacco, unhealthy diet, physical dormancy as well as excessive use of alcohol.[43,44] Besides, HIV, malaria[33], cigarette smoking and infection with human papilloma virus, herpes simplex virus, Trichomonas vaginalis, cytomegalovirus , Chlamydia trachomatis, Neisseria gonorrhoeae, etc in the immunocompromised have been correlated with a high risk for CIN and ICC.[2,3,33,44]

Globally, adult females of low socio-economic level have increased risk of cervical cancer. Hence, the infection is sometimes called "disease of poverty"[31] or disease of poor women.[14,44] In a research done in Mali, West Africa, a population broadly infected with HPV, the findings presented: poor social conditions, high parity and poor hygienic conditions as the principal co-factors for cervical cancer.[2,3,6] Poverty is not only a major causative factor in the prevalence and spread of the disease but is also a critical

barrier to the prevention and treatment of cervical cancer.[3]

Furthermore, oral contraceptives are believed to elevate the risk of developing both cervical and breast cancers due to hormonal influence.[20,35,39,44,44] For instance, women who or whose mothers laid hold of the drug diethylstilbestrol (DES) in the course of pregnancy between 1940 and 1971 to avert miscarriages, premature labour and associated problems were more at risk of developing cervical cancer.[16,44] DES is an artificial form of the female hormone, oestrogen, and it increases the risk of some women developing clear cell adenocarcinoma.[16,20] In cases of clear cell adenocarcinoma, the abnormal cells resemble cancer but do not invade nearby healthy tissues. However, if untreated they become cancerous, leading to non-invasive glandular neoplasia.[16]

2.0.3 Clinical Presentation of Cervical Cancer

Early cervical cancer is asymptomatic in most cases. However, the symptoms that manifest include unusual vaginal bleeding between menstrual periods, coitus, or bleeding after menopause, continuous vaginal discharge which could be pale, pink, watery, bloody, brown, or foul-smelling; and with menses becoming heavier and lasting longer than usual.[28,44]

Cervical cancer can infiltrate adjacent organs like urinary bladder and the rectum/colon and metastasize to remote sites such as lungs and liver.[26] Symptoms of full blown cervical cancer may involve backache, osteodynia or fractures, exhaustion, urine or faecal incontinence from the vagina,pelvic pain, anorexia, single swollen leg, leg pain and weight loss.[28,28,44]

2.0.4 Epidemiology of Cervical Cancer

Around the world, approximately 25 million people are living with cancer.[26] In 2007, about 11 million fresh cases were diagnosed worldwide, and around 8 million individuals died from cancer.This accounted for 13% of all deaths in that year.[26] These numbers are however, expected to double by the year 2030 if adequate preventive, screening and treatment measures are not put in place.[9,41]

The fifth most prevalent cancer in women globally is cervical cancer which accounts for about 471,000 new infections annually.[26] It is also the second most prevalent cancer in adult females living in under-developed nations and accounts for up to 300,000 deaths annually.[2,26,38] About 80% of these cases occur in low or middle-income nations.[26,38] Cervical cancer affects women between the ages of 30 and 55 years.

Statistics from Mbuomba et al, shows that globally every two minutes, a woman dies from cervical cancer.[26] In Africa, a woman dies from cervical cancer every 10 minutes. Each year, 274,000 women die from cervical cancer and 85% of them are in third world nations.[26,37]

For every one case of cervical cancer in the United states, Africa has nine. Moreover, it has 24 times the mortality.[26] In Africa, SCC incidence is the highest in the world, with an age-standardized incidence rate of 31.0 per 100 000 women.[3,25,29] The prohibitive cost of prevention, screening and treatment has been one of the central barriers to addressing cervical cancer in Africa. A lot of adult females in Africa usually lack access to cervical screening and prevention, even though these technologies have existed for more than half a century.[2,3,36,43] As a consequence, at the time of diagnosis, most of the cancers are already at advanced stages.[26] Wrong beliefs, unavailability and inaccessibility of cervical cancer screening services in sub-Saharan Africa are responsible for the low number of women being screened. A large number of these are sited within secondary or tertiary health care facilities located in urban settings.[2,3]

2.0.5 Laboratory Screening/ Diagnosis

The known methods available for cervical abnormality screening include: conventional Pap smear cytology or lbc), visual inspection with acetic acid or Lugol's iodine and HrHPV DNA testing.[38] The Papanicolaou test, also called Pap smear, is the most commonly used test in cervical cancer screening.[38,44] For this method, cervical cells are collected from the transformation zone and prepared for cytology. After staining with Papanicolaou stain, slides are examined under the microscope to establish if the cells are normal or abnormal and to rank them systematically using the Bethesda System of reporting cervico-vaginal cytology.[38,44]

Over the years, alternative tests such as LBC have been introduced in order to raise screening effectiveness . In the LBC test, the cervical specimen is collected using a cervical brush and then transferred into a preservative liquid before it is dispatched to the laboratory for smear preparation. This increases the specificity, and more cells are harvested leading to ease of interpretation of results as compared to a conventional Pap smear. However, this requires additional training for the technicians.[38] Visual inspection technique can be executed by utilizing acetic acid (VIA) or Lugol's Iodine (VILI). Both methods provide screening options in poor nations that are not engaged in the use of laboratory services.

These techniques are usually used as primary screening tools and in instances where atypical lesions have been spotted, they can be used for treatment.[32,38] These alternative methods can pick up pre-malignant lesions and allow treatment as well as prevention of cervical cancer progression .[26,44] In the recent past, there has been incredible scientific and technological advances that have led to the creation of more effective and cheaper testing methods for screening for the HPV .[26] The presence of HPV DNA in the sample simply confirms the existence of HPV infection . The patient is then followed up more closely and regularly to monitor and observe for the development of pre-malignant lesions.[44]

Recent studies suggest that the risk of cervical cancer can be lessened by up to a third if adult females in emergent nations get access to at least one cervical screening in their lifetime.[26,44] Research has also associated STIs, other than HPV with cervical cancer.[2] The STIs elicit a chronic inflammatory feedback that is a source of free radicals which are known to play a major role in carcinogenesis and cancer progression.[2,3] A lot of women with these infections, unfortunately, receive inadequate treatment, because of inaccessible (financial or geographical) quality health care, hence resulting in common chronic and persistent infections.[17]

Cervical screening coverage is still a challenge in numerous areas of Africa. Currently, the screening coverage ranges from 2.0% to 20.2% in city places and 0.4% to 14.0% in rural settings.[26] Worse still, at the level of public health, there are hardly any large scale initiatives at hand to introduce community-based screening using cytology, visual inspection or HPV testing. In this study therefore, Hybridization or Hybrid Capture was used because of the fact that it is a gold standard for HPV detection. For HPV DNA detection, Hybrid Capture 2 methodology provides an inexpensive,accurate, user-friendly signal amplification suitable for diagnosis and management of infectious diseases. It has excellent result reproducibility with minimal cross contamination due to signal instead of target amplification. Furthermore, the method is less complicated than target amplification technique.

2.0.6 Prognosis of Cervical Cancer

Cervical cell changes can be detected before their metamorphosis into malignancy, consequently contributing to a window for early and added effective treatment. It is generally more difficult to treat adult females who hardly go through periodic check-ups, and they tend to have an increased risk of progressing to frank cervical cancer.

Early and routine screening for cervical cancer using the Pap test has exhibited a reduction in deaths from the disease by as much as 74% .[27]. This test demands observing cells from a cervical specimen under the microscope checking for the presence of any atypical cells. Apart from finding the abnormal cervical cells, the Pap test also

helps to identify cells that have the propensity to become cancerous in future. HPV, is a known causative agents of cervical cancer. However when in co-existence with EBV, cancinogenesis is not only aided, but 'catalyzed' and progressed because the two viruses co-exist and act synergistically.

2.0.7 Treatment of Cervical Cancer

Cervical cancer treatment is dependant upon four conditions which are as follows: the size and structure of the tumour, cancer stage, the patient's age, the woman's wish to reproduce in future and general health condition.[3] Pre-cancerous lesions and early cancer can be prevented by eliminating or destroying the pre-malignant and malignant tissue respectively. There are several surgical procedures of performing cancer cell destruction while avoiding uterus removal or damage to the cervix and at the same time preserving the woman's reproductive capacity. The procedures include: Cryotherapy which involves the freezing of abnormal cells identified as cancerous or precancerous after colposcopy, Loop Electrosurgical Excision Procedure (LEEP), which utilizes electricity to eliminate cancerous tissue; and Laser Therapy which uses light to do the same. Removal of the uterus but not the ovaries (hysterectomy) is not usually done for cervical cancer that has not invaded other tissues. This procedure however, can be performed on cases of women who have had repeated LEEPs.[2,44]

Treatment for advanced cervical cancer can include: radical hysterectomy, which involves uterus removal as well as areas of the surrounding tissues.The surrounding tissues include the lymph nodes, upper vagina parts, and pelvic exenteration, which is an approach to the farthest limit of surgery that involves removal of all organs of the pelvis, bladder and rectum.[28,44] To treat cancer which has infiltrated far off the pelvis, or malignancy that has re-occurred, radiation can be used. Radiation therapy is classified into external beam and internal one. Internal radiation therapy utilizes an equipment packed with radioactive material, and is placed inside the woman's vagina close to the cervical lesion. This tool is removed before a woman returns home. External therapy, beams radiation from a large machine onto the body where the lesion is located. It is indistinguishable

to an x-ray.[44] Chemotherapy utilizes medication that destroys cancerous cells. Some drugs used for cervical cancer chemotherapy include 5-fluorouracil (5-FU),carboplatin, cisplatin, ifosfamide, paclitaxel, and cyclophosphamide. In some cases, chemotherapy and radiation are put in use before or after surgery.[28,44]

2.0.8 Primary Prevention of Cervical Cancer

Vaccinating young girls aged 10 to 14 years in a two or three dose schedule as per recommended guidelines in six months with HPV vaccine is now proven as a primary prevention strategy for cervical cancer.[43] This poses a distinct challenge in Africa.This is because girls of this age might not be in school in which immunization is easiest to administer.[38] However, successful programmes have been rolled out in a few countries such as Rwanda.

The cost of HPV vaccine differs depending on the vaccine type and where it is being obtained from.The cost ranges from 130 dollars to 170 dollars per shot,which totals to 390 dollars to 500 dollars per course. Therefore,one course of a vaccine injection costs approximately 400 dollars per girl and it is way past the budget in many African administrations.[26,36,41]

This notwithstanding, GAVI/UNICEF program approved to support 20 African countries and launched HPV vaccine immunization in 8 countries (Sierra Leone, Ghana, Niger, Kenya, Tanzania, Malawi, Madagascar and Lao PDR) since 2013. One of the most commonly used vaccine, Gardasil manufactured by Merck guards against most genital warts, as well as some malignancies (HPV types 6, 11, 16 and 18) of the vulva, vagina, and anus.[26,38,44] It has also been shown to have modest effects on other HPV types such like 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59.

This indicates that Gardasil may be capable of preventing more cervical cancer incidences, and provide extensive protection than originally projected.[10,38] Cervarix, manufactured by GlaxoSmithKline, is a bivalent vaccine that is directed against HPV strains 16 and 18.[26,38] A few years ago, a more advanced version, Gardasil 9 was

introduced. This is a nano-valent vaccine that is targeted against 9 types of High Risk HPV, namely HPV types 6, 11,16,18,31,33,45,52 and 58 (NIH 2015).

In Kenya, a pilot study of HPV vaccination for young girls was introduced for a first time in May, 2012 and ended in 2013.[27] This pilot HPV Vaccination study targeted girls in grade 4 (average age 9 to 14 years). The first phase involved girls from 10 different randomly selected public schools in Kitui County. However, the second phase included girls from the wider community through community-based strategies. Out of the 3000 girls enrolled in the first phase, 2500 were fully vaccinated at the end of the program. The vaccination was aimed at reducing the prevalence of cervical cancer in women.[18,42,43]

The HPV Vaccine, Gardasil, worth US$11 million and prevents HPV types 16 and 18 was acquired by the Kenyan government in collaboration with some private health sectors in the struggle to rescue the next generation of adult females from cervical cancer. The vaccine is administered through an injection on the upper arm. For full protection of a girl, three (3) doses of HPV vaccine should be administered. The 2nd dose should be administered 2 months following the 1st dose; and 3rd dose must be given 4 months subsequent to the 2nd dose. Some Kenyan girls received their second dose in 2013.[41] (WHO now recommends only TWO doses for the sexually naïve, and three for those sexually active or previously exposed).

2.1 Statement of the Problem

Despite the fact that cervical cancer is the second most common malignancy following breast cancer in Kenya, and is a dominant cause of death, averaging about 2000 deaths per year, not much progress has been made in terms of tackling it head-on.[23] This lacuna has been attributed to a plethora of factors, among which include: inadequate access to cervical cancer screening and treatment, secondary to low economic status, poor knowledge, awareness and research on the disease, excessive tobacco use, sexually transmitted infections, the practice of polygamy and the challenges of some traditional

beliefs especially around sexuality[3,9,44] and increased cost of HPV vaccines among others.

The challenge is further exacerbated by a more daunting deficit in medical expertise. Few trained cytologists and pathologists and inadequate infrastructure is still a huge challenge for the population of women at risk.[2,3] HPV and EBV infection co-exist in the cervix and work synergistically to promote tumorigenesis. This is known to have been studied in few countries like Algeria. Kenya has not also experienced similar extensive study, even though she has a high prevalence.This therefore makes it a rich site for such a research, with a view to formulating ideal policies. There has also been no study conducted in Kenya at Kenyatta National Hospital to establish the frequency of HPV and EBV Co-infection and its correlation with cytological abnormalities in the cervix even though cervical cancer is commonest as it is a referral and national hospital.

2.2 Justification

KNH receives patients from all parts of the country. She also receives quite a number of cervical smear specimens from some private health facilities. The high number of cervical cancer cases being diagnosed has elicited the need to determine the prevalence of the co-infection of HPV and EBV, and their synergic effect in cervical carcinogenesis. Whereas, effective HPV vaccines exist and programmes have been rolled out across the globe, and in some African countries, including Kenya, yet EBV still has no licensed vaccine.

EBV is linked with 200,000 new malignancies per year around the world. Several candidate EBV vaccines, targeting the viral glycoproteins implicated in viral entry, gp350/220 and gH/gL are undergoing pre-clinical testing to determine their ability to elicit neutralizing antibody responses. Demonstration of the influence of HPV and EBV co-infection in cervical neoplasia may very well cause a paradigm shift in screening strategies for cervical cancer prevention.

2.3 Hypothesis

HPVand EBV co-existence and cervical cytological abnormalities among women attending Kenyatta National Hospital.

2.4 Research Questions

1. Does HPV and EBV co-infection co-exist in cervical samples?
2. How does the co-infection of HPV and EBV correlate with cervical cytological findings?

2.5 Objectives

2.5.1 Broad objective

To ascertain the prevalence of Human Papilloma virus and Epstein Barr Virus co-infection and cervical cytological abnormalities among women attending Kenyatta National Hospital clinic, Nairobi.

2.5.2 Specific Objectives

1. To screen women attending a gynecology clinic at KNH for cervical lesions using Liquid-Based Cytology (LBC).
2. To establish the prevalence of HPV and EBV co-infection in cervical samples of women attending the Gynecology clinic in KNH.
3. To determine the prevalence of HPV and EBV co-infection in cervical samples of women attending the Gynaecology clinic in KNH.
4. To find out if there is association between HPV and EBV co-infection and the cytological findings of the cervical samples.

Chapter 3

Materials and Methods

3.1 Study Design

This was a descriptive cross-sectional study in which data and specimens were systematically collected from female patients/women at Kenyatta National Hospital in Nairobi. The women were enlightened about the study and gave consent to participate using a consent form by signing or thumb printing. A questionnaire was administered and a study identification number was allocated specific to each participant as names were not used to maintain confidentiality and ease traceability respectively. Clinical records and national id's as well as request forms were used to confirm patients' details.

3.2 Study Population

The study analysis comprised of females aged 18 years and above attending cervical cancer screening in Clinic 66 of Kenyatta National Hospital who volunteered to participate in the study and who met the criteria for cervical cancer screening.

3.3 Study Site

The study was conducted in Kenyatta National Hospital in Nairobi, Kenya. The hospital is a tertiary, national referral hospital, that serves about 32 000 people every year. It has a bed capacity of 2000 patients. The study was conducted in Clinic 66 where women go for cervical cancer screening. The women received routine information about cervical cancer and each one of them was counselled in a private room and a cervical specimen were obtained by a registered competent nurse.

3.4 Inclusion and exclusion criteria

3.4.1 Inclusion Criteria

All cases of women from 18 years (World Health Organization (WHO) Recommendation/ Kenya National Cancer Treatment Guidelines) who were sexually active presenting for the first time to the clinic for cervical screening or routine follow up after previous negative results as well as review due to previous abnormal smears (pre-cancerous lesions) were incorporated in the study.

3.4.2 Exclusion Criteria

1. Clients younger than 18 years and older than 50
2. Women on chemotherapy/Radiotherapy for cervical cancer related treatment
3. Women who were pregnant were excluded because pre-malignant lesion would not be treated until after delivery and a cervical brush could be used on them.
4. Women who had had previous ablation for cervical lesions

3.5 Sample Size

Prevalence of HPV and EBV co-infection in Kenya at large and at Kenyatta National Hospital more specifically, is unknown. Since the it is unknown, a conservative estimate of 50% was utilized. In order to for an estimate within measures of 5% (or 0.05) prevalence as well as taking into consideration a confidence level of 95% , a minimum specimen size of 109 was calculated, as shown below:

$$n = \frac{p(1-p)}{e^2}$$

where $p = 50\%$ (or 0.5) is the specimen percentage and

$$e^2 = \frac{0.05^2}{1.96} \approx 0.000625$$

N = 400. The cases to be analysed were selected by random sampling. Firstly, a list of all cervical cancer cases was made. The kth number in the sampling frame was divided by 400. The total number of cases (K) will be done using simple random sampling. Then every kth case was selected 48. Hence, calculation of the new sample size (new n) was shown below;

$$\text{New N} = \frac{n}{1 + \frac{n-1}{N}}$$

$N =$ The total number of cervical cancer samples per month was estimated as 150

$$\text{New N} = \frac{150}{1 + \frac{150-1}{400}} \approx 109$$

Therefore, the calculated sample size (n) was 109. (Applying correction for finite population size formula)[14]

3.6 Recruitment Procedure

The women were well educated on the study and gave consent to participate using consent forms by signing or thumb printing.A questionnaire was administered and a study identification number was allocated specific to each participant.The participants' names were not used in order to maintain confidentiality and ease traceability. Clinical records and national identifications as well as request forms were used to confirm patients' details.Each woman had her vagina/cervix physically examined for any infection that would obscure cells during microscopy. The women who met the criteria for research had their samples collected and study protocol was followed as illustrated in the flow chat below.

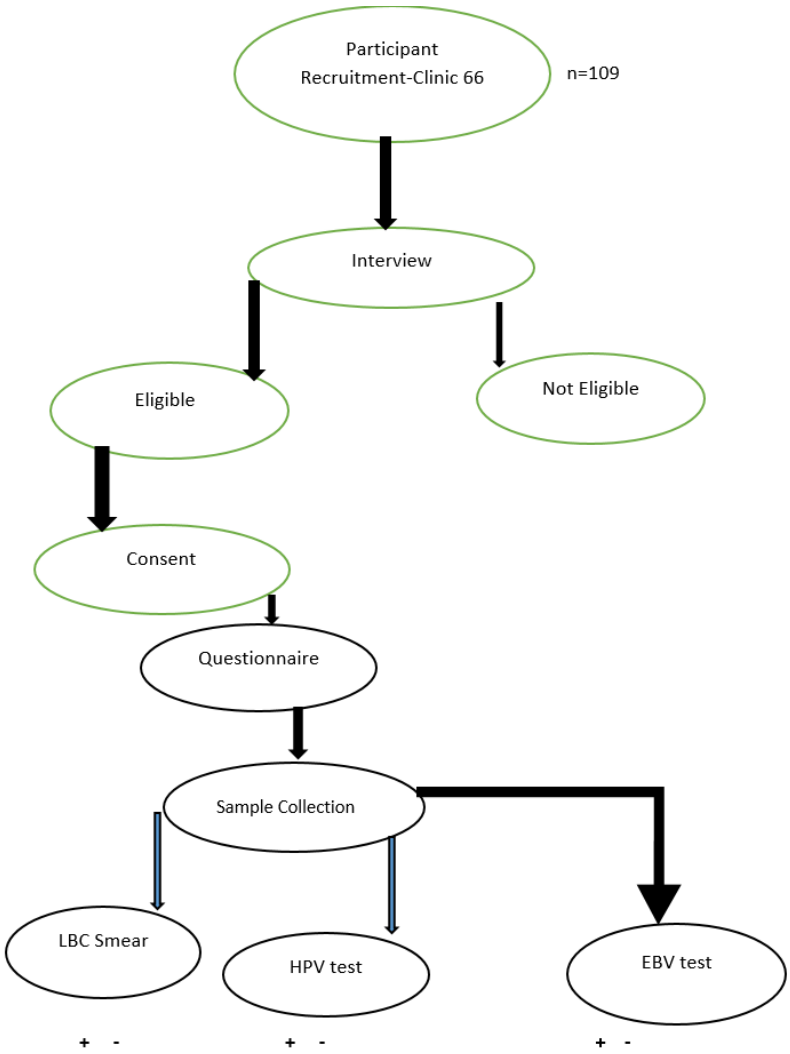

Figure 3.1: Study Selection Flow Chart

3.7 Detection of HPV/EBV using molecular techniques

3.7.1 Specimen Collection

Cervical samples (n=114) were collected by qualified health care personnel. Collection was performed in DNA-Pap cervical samplers which contained a transport medium that preserves the cells, organisms as well as the Deoxyribonucleic Acid (DNA) for Molecular tests and LBC. The samples were transported to KAVI-Molecular laboratory where reception, recording and storage by refrigeration were done. Two hundred $(200)\mu l$ of the specimen was obtained from the transport media vial into well labelled vials and refrigerated at $2-8°C$. Samples tested after week of collection were frozen at $-20°C$ for less than 3 months. Hybrid capture 2 and PCR were used for molecular testing of HPV and EBV while LBC was used to interpret cervical cell morphology in the Cytology Laboratory.

The specimens were spun in a cyto-centrifuge and samples transferred onto the slides by centrifugal force which were later stained by Papanicolaou method and results interpreted under a microscope. If not immediately processed, the specimen were to be stored at $2-8°C$.

Hybrid Capture 2 for Hr HPV DNA was utized with a basic solution which disintegrates the cells to expose the virus. Epstein Barr Virus in the biological material was also treated the same way but with different salt concentrations and ethanol.

DNA extraction was performed (Appendix A.9). The DNA was amplified and detected using chemiluminscence for EBV; Probes that contain fluorescent reporter dye that are specific for EBV DNA were used . Simultaneously, Internal Control (IC) and endogenous IC glob (β-globine gene) β-globin gene were also used in the DNA amplification. Considering that DNA is part of the human genome, it has sufficient amount of DNA acquired from the cells. There must be nothing less than 20 000 genomes per specimen

(DNA from 10 000 cells) for EBV. IC added in the course of specimen preparation from plasma, sputum, amniotic liquid, bronchial lavage,liquor and other cell free or low DNA content materials, acts as an amplification control for each specific processed sample. Additionally,this identifies possible reaction inhibition, while endogenous IC β-globine gene), present in all specimens obtained from cells (whole blood, biopsy and autopsy material,white blood cells, swabs and saliva,) provides the possibility to control analysis steps and evaluate specimen handling and storage.

EBV Latent Membrane Protein (LMP)-gene DNA expansion is recognised on JOE (Yellow)/HEX/Cy3 channel, the IC glob (β-globin gene) DNA amplification is signaled on FAM (Green) channel and exogenous IC is detected on Rox (Orange)/TexasRed channel.

3.8 Hybrid Capture Protocol for HPV detection

Preparation of clinical specimens was performed by adding a base solution to the cervical samples, which disintegrates the virus and releases target DNA. RNA probe was hybridized with the target DNA. Formation of RNA:DNA hybrids were created as the target DNA combined with specific RNA probes. The RNA:DNA hybrids were then captured onto a solidified medium coated with universal capture antibodies specific for RNA:DNA hybrids. To increase the signal, captured RNA:DNA hybrid was found to be present with multiple antibodies conjugated to alkaline phosphatase. Resulting signal was amplified to at least 3000-fold. Through chemiluminescence, the resulting signal was read and interpreted (Hybrid Capture 2-Assay Manual).

The digene HC2 High-Risk HPV DNA test Cut Off of 1pg/ml is equivalent to 100,000 HPV copies/ml or 5,000 HPV copies per assay. Therefore samples with a RLU/CO value ≥ 1.0 are regarded “positive” for one or more of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. STM samples with a RLU/CO value below 1.0 are considered “negative” or “no HPV DNA detected” for the 13 HPV types tested. High-risk HPV DNA sequences are either absent or exist below the detection limit of

the test.

3.9 Polymerase Chain Reaction for EBV Detection

3.9.1 Protocol for EBV Real Time PCR

The required number of tubes or PCR plate were prepared. For each specimen, a new sterile tube containing $10^{*}N\mu l$ of PCR-mix-1, $5^{*}N\mu l$ of PCR-mix-2 buffer and $0.5^{*}N\mu l$ of Hot Start DNA Polymerase were prepared. $15\mu l$ of Reaction Mix into each tube was added. Thereafter, $10\mu l$ of the DNA sample extracted from the initial specimen was appropriately added to the Reaction Mix containers. A positive control and one negative control were prepared for a qualitative run.
To the tube labeled Cpos, $10\mu l$ of QS2 was added. To the second tube labeled Cneg, $10\mu l$ of TE-buffer was added. For quantitative analysis 4 tubes were prepared and QS1 and QS2 standards were performed twice. The tubes were closed and transferred into the apparatus in this sequence: samples, negative controls, positive control, and standards (EBV PCR Manual). The results were read and interpreted.(EBV Real TM PCR Quant.Handbook,2013)

3.10 Determination of Cytological finding

3.10.1 Liquid Based Cytology

Liquid Based Cytology was performed by collecting sample with a cervical brush into a bottled liquid preservative. The preservative liquid removed elements such as mucus and lysed the red blood cells before a cell layer was placed on a glass slide. The head of the cervix brush with the collected cervical sample was placed in the container with the preservative fluid (commercially prep from ThermoShandon) which was centrifuged to concentrate the cells and at the same time deposit them on a slide. Then after, Pap

stain was performed as demonstrated in Appendix A.6.

3.11 Quality Assurance

A paper presentation was made by the research investigator to the gynea department where this research was carried out.The presentation participants comprised specifically of trained and competent nurses involved in various aspects of the research.

The critical points in the work flow from recruitment to result generation were highlighted with emphasis. All the samples and reagents were obtained and used according to the set standards and in line with the Standard Operating Procedures (SOPs) as well as Manufacturer's instructions respectively.

All the specimens were collected in uncontaminated sterile containers with appropriate transport media.The molecular Specialist and Consultant Pathologist were involved in Quality Control of all the specimen results as well as in ensuring Good Clinical Laboratory Practice (GCLP). Samples for Liquid Based Cytology was fixed immediately after collection and the samples for molecular tests which were not tested after one week were stored at 2-8 degrees celsius.

3.11.1 Data Management

Collected data was stored under lock and key and two note books were safely kept-one in clinic 66 and the other with the principal investigator.Unique identifying numbers were used for each participant in order to maintain confidentiality.This unique identifier linked each participant on a password soft copy for ease of traceability; which conforms to Good Clinical Laboratory Practices.Data collected is to be stored for half a decade.

3.11.2 Data Analysis

Data was recorded, analysed and validated with the help of Stata 11.2 and R-software.The prevalence and odds ratio to compare the variables namely women with HPV/EBV co-infection, patients' ages distribution and clinical stages or grades of cervical lesions using Fisher's exact test where appropriate were used.

Descriptive statistics for continuous outcomes including means or medians as well as the corresponding standard deviation or interquartile ranges were appropriately calculated.Categorical outcomes such as age group was presented using counts and respective proportions or percentages.The molecular tests (HC-2 and PCR) and Liquid Based Cytology for cell morphology in the absence of a reference standard were analysed. This software also calculated the p-value to answer the null hypothesis.The P-value of 0.05 was used to show statistical significance.The results were appropriately presented in tables,charts or graphs using Latex software.

3.11.3 Ethical Consideration

This was a laboratory based study.Permission to obtain samples from human subjects and records from consenting women were sought from the KNH.The University of Nairobi Research,KNH and Ethics Research Committee authorised and gave permission for this research to be carried out on human subjects.To ensure patient safety and confidentiality,unique study identification numbers were assigned to the study cases to avoid obtaining patient details such as names or addresses.Specimens were collected by trained and competent nurses.Moreover,the ethics committee secretary,the principal supervisor and investigators' phone numbers were given to every participant for any arising queries.

Chapter 4

Results, Analysis and Interpretation

4.1 Socio-Demography and Clinical Information

Table 4.1: Socio-demographic and clinical information

Characteristic	**N = 114**
Age (years)	
Range	20 – 59 years
Mean SD	39.8 ± 8.9 years
Occupation, n(%)	
Self-employed	95 (83.3%)
Employed	1 (0.9%)
Other	18 (15.8%)
Contraceptive use, n(%)	
None	42 (36.8%)
IUCD	25 (21.9%)
Implant	14 (12.3%)
Depo	7 (6.1%)
Pill	10 (8.8%)
Condoms	8 (7.0%)
BTL	7 (6.1%)
Other	1 (0.9%)
HIV Status, n(%)	
Unknown	2 (1.8%)
Negative	95 (83.3%)
Positive	17 (14.9%)

Table 4.1 shows 114 samples that were collected from the research participants.

The Age ranged from 20-59 years with the standard deviation of 39.8 ± 8.9 *years. Most participants were self- employed 95(83.3%); and 71(57.1%) were on different contraceptives, 36.8% were not on contraception while 0.9% were on none; 95(83.3%) were HIV negative, 17(14.9%) were HIV positive and 2(1.8%) had unknown HIV status.*

4.2 Cervical lesions using LBC

(Objective 1)

Table 4.2: Cervical cytomorphological findings

Cytomorphological findings (N = 114)	**n (%)**
Cervical Lesions	
NILM	99 (86.8%)
LSIL	2 (1.8%)
ASC-US	3 (2.6%)
ASC-H	2 (1.8%)
No sample/Insufficient sample	8 (7%)
Non neoplastic lesions	
Negative	75 (65.8%)
Inflammatory	27 (23.7%)
Atrophic	6 (5.3%)
Atrophic/Inflammatory	3 (2.6%)
Insufficient	3 (2.6%)
Organisms	
Negative	103 (90.4%)
Candida	4 (3.5%)
Bacterial Vaginosis	7 (6.1%)

Table 4.2 shows a total of One hundred and fourteen (114) samples which were prepared using Liquid Based Cytology.The specimens were stained with Papanicolaou stain and microscopy was reported using the Bethesda Reporting System as a guide. Eight (7%) samples were insufficient for evaluation, ninety nine 99(86.8%) were negative for intraepithelial lesions or malignancy, 3(2.6%) had ASC-US, 2(1.8%) had ASC-H and 2(1.8%) had LSIL totaling up to 7(6%) of samples with lesions. Furthermore, a total of 11(9.6%) samples were reported to have micro-organisms; 4 had Candida species and 7 had clue cells morphologically consistent with Bacterial vaginosis.

Figure 4.1: Photomicrograph showing Cervical lesions and microorganism under the microscope.

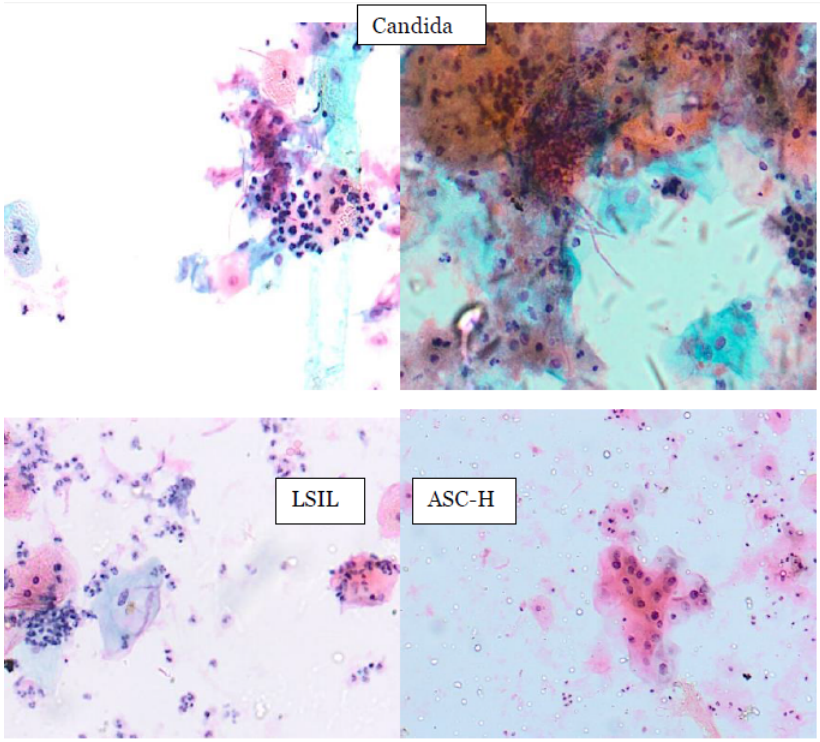

Figure 4.1 shows photomicrographs of the cervical smears observed under the microscope. The upper right and left photomicrographs demonstrate candida species with pseudohyphae and budding in inflammatory background. The bottom left and right show Low-Grade Squamous Intraepithelial Lesion (LGSIL) (with intermediate epithelial cells that exhibit halo formation of cytoplasmic clearing known as koilocytosis) and Atypical Squamous Cell cannot exclude High grade (ASC-H) (which demonstrate hyperchromasia in metaplastic cells with enlarged nuclei to cytoplasmic ratio, irregular nuclear margins and pleomorphism) respectively.

4.3 Prevalence of HPV and EBV infections in cervical samples using molecular techniques.

(Obective 2-3)

Table 4.3: Prevalence of HPV and EBV

N = 114	**n (%)**
HPV	
Positive	32 (28.1%)
Negative	82 (71.9%)
EBV	
Positive	17 (14.9%)
Negative	97 (85.1%)
HPV and EBV Co-infection	7 (6.1%)

In the Table 4.3, 114 samples were tested using Hybrid Capture 2 for HPV and PCR for EBV. Out of 114 samples, 32 (28.1%) were reactive (positive) for HPV and 17 (14.9%) were reactive (positive) for EBV. A total of 7 samples (6.1%) were found to have the HPV/EBV co-infection.

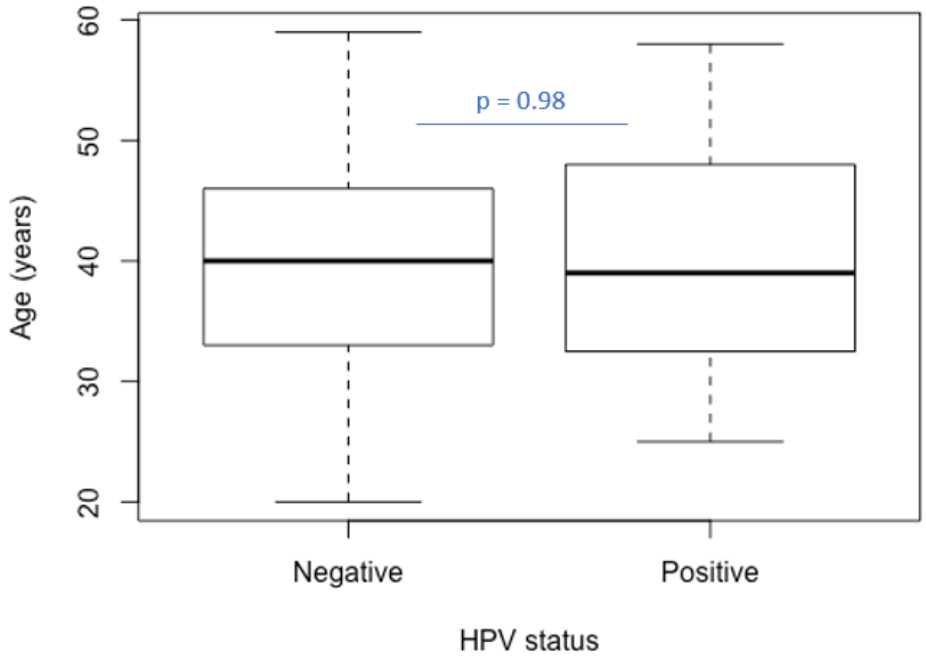

Figure 4.2: Box plots showing Age vs. HPV status

Figure 4.2 shows box plots for age against HPV status for the women in the population under study. The Negative patients had a median age of 40 years which was slightly higher than that of the HPV positive patients. The age of most of the participants ranged from 30 and 50 years and were also the most affected group with HPV. The whisker for the HPV negative women show the minimum age at 20 years and 59 as the maximum age. However, the whisker for the HPV positive patients shows a minimum age at 24 years and miximum at 58 years. The box plot for the HPV positive women also shows a slightly skewed chart. However,both box plots have no outliers.

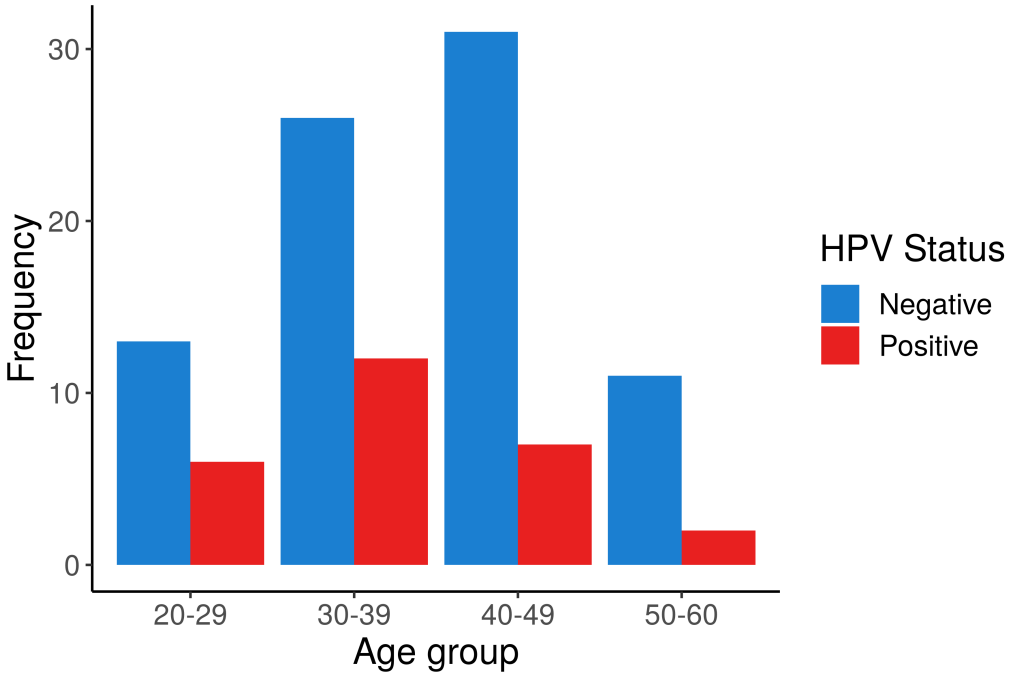

Figure 4.3: HPV vs Age

Figure 4.3 is a bar chart showing the distribution of HPV status against age of the women in the population under study as explained in the plot boxes. Those mostly affected are women between 30-39 years of age (>10), followed by those who are 40-49 years (>5). The least affected are older than 50 years.A higher percentage of women 82(72%) were negative for HPV and 32(28%) were positive for Hr HPV.

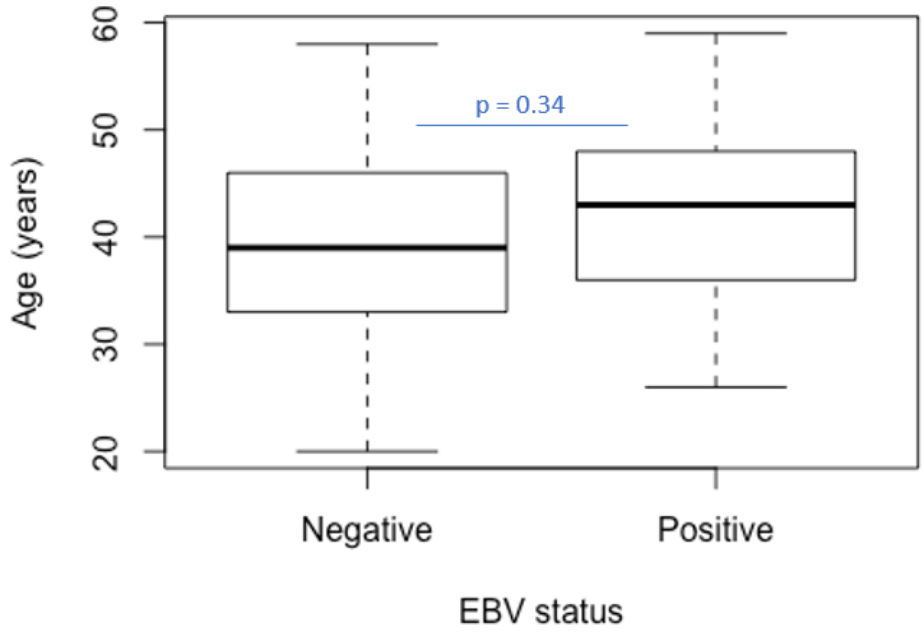

Figure 4.4: Box plots showing Age vs. EBV status

Figure 4.4 are box plots showing the EBV status against age of the women in the population under study. From the box plots, we notice that median age for the EBV positive women is slightly higher than 40 years of age , yet the negatives ones are below 40 years. The minimum and maximum ages for both negative and positive women is almost the same as in the HPV box and whisker charts. The boxes in this study illustrate the bottom part as the First Quartile and the upper part as the Third Quartile chart.This therefore shows that the box plot of the EBV positive patients is skewed to the right. However, both box plots have no outliers.

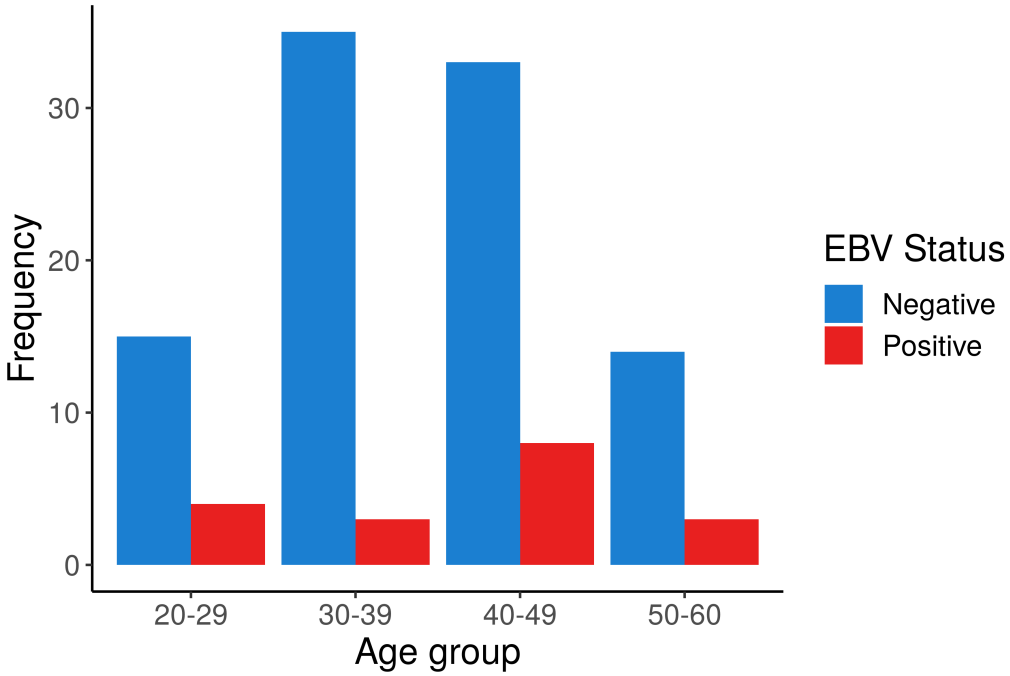

Figure 4.5: EBV vs Age

The EBV infection is 15% and the age ranges mostly affected are between 40-49 years of age, followed by 20-29 years. The least age affected is between 50-60 years.

4.4 Prevalence of HPV and EBV Co-infection in Cervical samples

(Objective 4)

Figure 4.6: Percentages of HPV in Cervical Samples

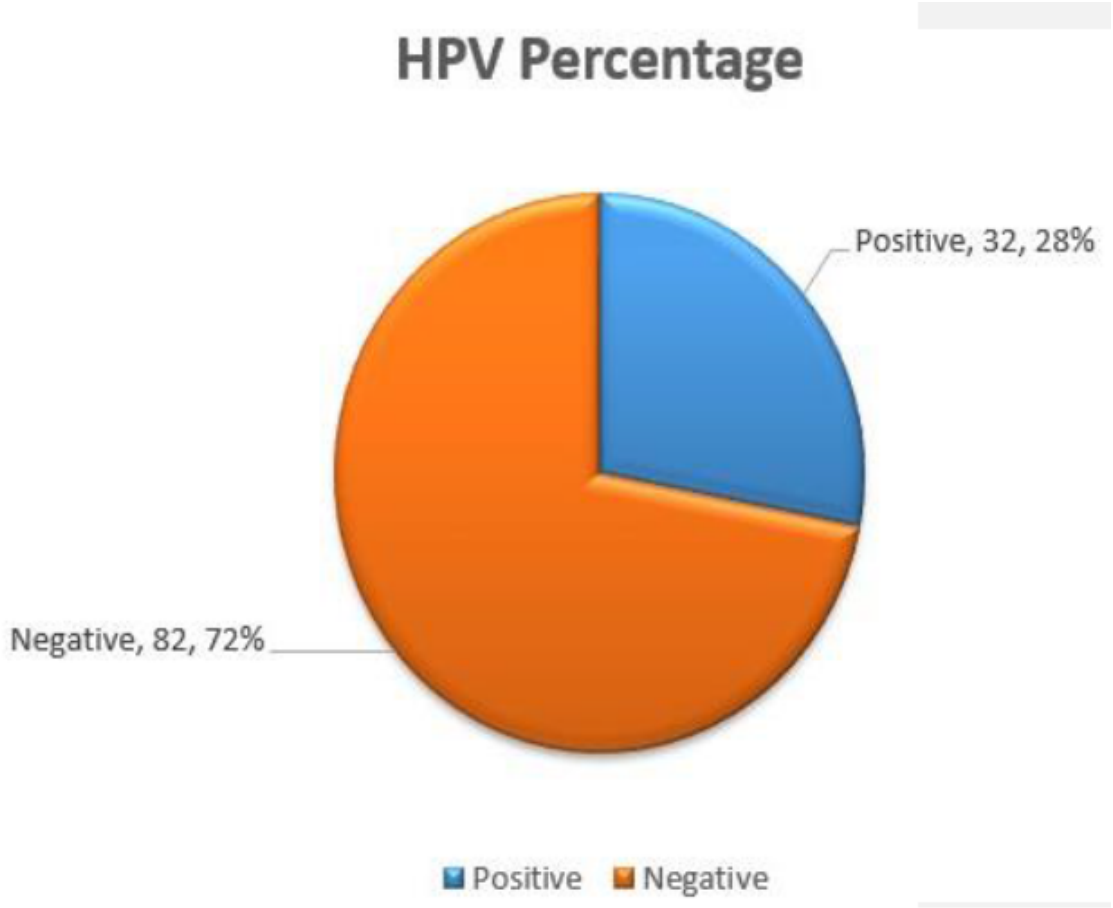

Figure 4.6 is a pie chart showing the Prevalence of Hr HPV in women from this study. A higher percentage of women 82(72%) were negative for HPV and 32(28%) were positive for Hr HPV.

Figure 4.7: Percentages of EBV in Cervical Samples

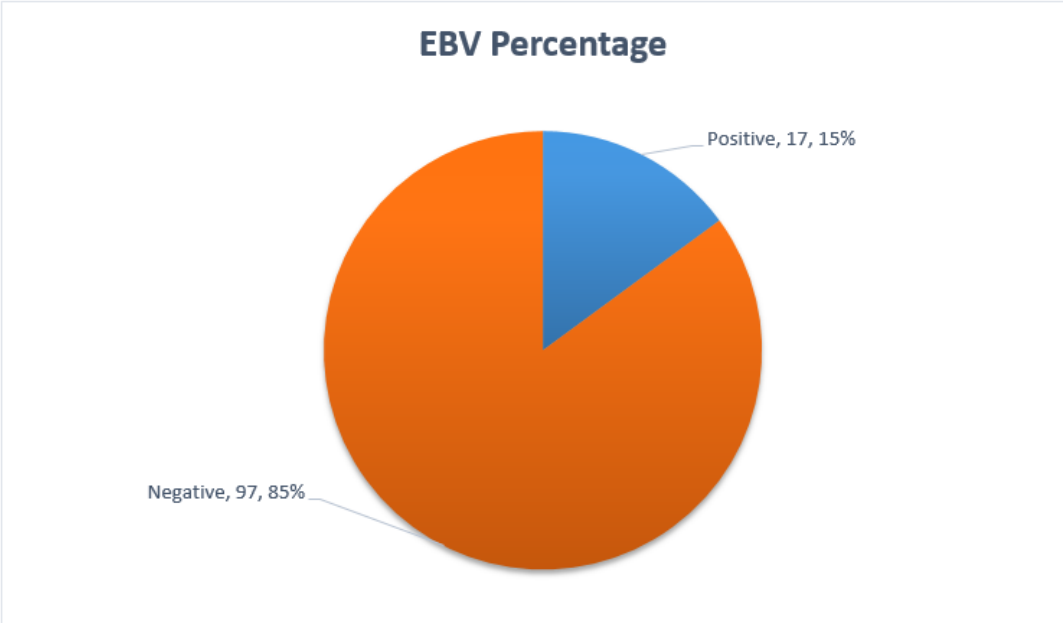

Figure 4.7 shows a pie chart illustrating the EBV prevalence in the cervical samples of women in this study. 14.9% of women were positive for EBV. The above pie chart shows the EBV distribution among women participants within the study being 17(15%) and the rest are negative for EBV.

4.5 Correlation of the HPV/EBV co-infection with Cytological findings.

Table 4.4: This table shows HPV/EBV Coinfection and cytomorphological lesions

	Cervical lesions on cytomorphology		
Coinfection	Lesion present	Lesion Absent	**Total**
HPV & EBV Present	2	5	7
No Coinfection	5	94	99
Total	7	99	106

Table 4.5 demonstrates HPV/EBV co-infection and cytological findings which were correlated using Fishers Exact test and the results obtained were not sufficient to make a conclusion that EBV/HPV co-infection co-exists in cervical samples of women with lesions exist. Only 6.1% of those who had lesions also had the co-infection. A P-value of 0.06727 was determined which demonstrated that there was no correlation between exposure (HPV/EBV) and outcome (lesions). And from this we can surmise that the women in our study population had insignificant P-value to conclude that synergistic effects caused by co-existence of EBV and HPV in the cervix could accelerate cervical carcinogenesis.

Chapter 5

Discussion, Conclusion and Recommendation

5.1 Discussion

In our study, 114 samples were randomly chosen. Most of the participants were between 39-49 years old and majority of the women were self - employed. Additionally, more than 60% of these women were on contraception and IUCD was the highest in use. A total estimate of 14.9% of the participants were reported to be known HIV positive patients. Cervical lesions were identified microscopically using Liquid Based Cytology. The slides were prepared and stained with Pap stain. A higher percentage of these women (83.3%) were reported as Negative for Intraepithelial lesions and Malignancy, 7% were inadequate and/or unsatisfactory for evaluation according to Bethesda 2014 and 6.1% had cervical lesions. This entire picture is in conformity with similar results by Arul 2016, Chagwa et al 2015. Furthermore, the micro-organism detection rate was 10% in total which correlates with findings by Sulochana et al 2017.

HPV prevalence is reducing worldwide due to the availability of vaccines such as Cervarix, Gardasil and currently the new nona-valent Gardasil with 9 different HPV subtypes encompassing both low and high risk strains, namely: 6, 11, 16, 18, 31, 33, 45, 52 and 58. However, these vaccines are only fully accessible in developed countries and less so in developing ones such as Kenya. Notably, the vaccine was first introduced in Kenya in May, 2012 and March 2013, and only to selected school girls aged 9-13 years who were in primary school at the time the program was carried out. This goes without saying that in our targeted study population, none of the participants would have qualified to be vaccinated at the time, and the probability that all or any of these

women would have been from the selected schools where the vaccine was administered is too slim. Therefore, we could validly conjecture that none of these participants had received the vaccination, a hypothesis that the questionnaires would later validly confirm.

Analysis of our specimens show that 28.1% of the women had HrHPV. This demonstrates that developing countries still have an increased prevalence and danger of losing women to cervical cancer by the year 2030 because HrHPV is the 99.7% causative agent of the malignancy. Moreover, the common age of the women with HrHPV was in the age range 30-40 years followed by 40-50 years age range which shows that most of them are in the reproductive age. And those commonly found with EBV were 40-50 years followed by 20-30 years of age. Most women do not get access to tests for HrHPV DNA due to the high costs in developing countries as well as the paucity of facilities, infrastructure and experts who can carry out such tests.

EBV is one virus that was the earliest to be discovered in Africa among children with Burkitts Lymphoma. The virus-induced malignancies amount to 12%, while those due to HPV and EBV make up 38% of these lesions. For this reason, HPV/EBV co-existence had been studied in different organs such as the breast, nasopharyngeal area, anal regions, and the female reproductive organ are some of the most recent. In this study, women with EBV were estimated to be 14.9% and this could be attributed mostly to their sexual life style, for instance, the oral sex and lubrication of sexual organs using saliva have been shown to be correlated with the transmission of these infections. Considering the fact that this is a baseline study involving the coinfection of HPV/EBV in cervical lesions, women with healthy cervices (without precancerous lesions or cancer) were included in the study; and this accounts for the low HPV/EBV co-infection prevalence which amounted to 6.1%. This prevalence is exactly the same as that of HIV in Kenya; and these kinds of correlation must not be ignored, especially if it is the case that HPV/EBV dual-infection speeds up Carcinogenesis. Likewise, this prevalence rate is a call to urgent action. It calls for and even necessitates actions of mass sensitization and political action, especially considering the gendered nature

of the infections. Unlike HIV which affects both men and women, this is more of a woman's disease. And in a world which is politically a man's world, the likelihood of many women dying in silence and amidst general silence, without the political will being steered in their direction is indeed very high.

Of the 32 (28.1%) of women with HrHPV, only 5 had cervical lesions and 99% were over 30 years of age. Hence, a total of 25(%) with HrHPV Positive and normal cervices are also at risk of developing precancerous lesions. It is therefore, incumbent on them to pay attention to their health. This is because there are a plethora of factors such as prolonged exposure to the virus, nutritional and health status as well as molecular responses (for instance, viral interactions) which may increase the probability and rate of development of cancer.The 14.9% women in this study with EBV also are at risk of developing cancer such as cervical cancer and the other forms derived from it such as lymphoepithelioma like-carcinoma as argued by Vranic et al 2018.

And if EBV is in co-existence with HPV, tumorigenesis is very likely to ensue. Currently, EBV has no approved vaccine which makes it more difficult to eliminate or tackle. Interestingly, out of the 17 women with HIV, 3 had HPV only, 2 had EBV, 2 had both EBV and HPV and 1 had the coinfection and lesion. However, this does not correlate or qualify HIV to be confounded in cervical lesion causation as this gives indication that 8 out of the 17 with HIV had other virus(s) co-existing. This information is important in future research as it could provide us with information on the connection between the infection's existence and lesion development as well as on the survival rates of those infected by the virus(es),among others.

In this research, our main objective was to correlate HPV/EBV co-infection in cervical lesions. But due to the large population of normal cervices, the prevalence was too small to be generalized. However, it opens up room for more research. For instance, considering the 7 samples with lesions only (6.1%) and correlating them with the presence of the coinfection HPV/EBV, the discussion correlates with the recent meta-analysis by De Lime and his colleagues, in they which suggest that EBV/HPV co-infection rises in proportion to the lesion grade resulting unto high propensity tumorigenesis. The

samples with lesions were 3(42.9%) who had ASC-US, with 50% HPV positive and no EBV. Two (2 or 28.6%) had ASC-H with 100% HPV and 50% EBV and these rates applied to LSIL which had same values. This totally suggests that in cervical samples of women with lesions, HPV/EBV co-exist and hence conform to the research finding by Abudoukadeer et al 2015, Khenchouch 2013, de Lime 2018 which makes the case that "increasing rate of HPV/EBV dual infection is gradual to lesion class."

5.2 Conclusion

In conclusion, the co-infection of HPV/EBV in cervical samples exists in women attending KNH (6.1%). This co-infection increases as the grade of the lesion proportionally increases as well. This is therefore, an indication that a potential oncogenic interplay between HPV and EBV is possible. The paucity of baseline studies on HPV and EBV inter-action in the cervix also creates a gap for further research. And this commonly affects women between the age ranges of 40-49 years followed by 30-39 years respectively.

5.3 Recommendations

We highly recommend that women with positive HrHPV and negative Pap critically be followed up to avoid them presenting to the clinic only in the late cancer stage. This is also critical for the accumulation and documentation of scientific knowledge on the metamorphosis process from normal to abnormal cytology.

This research also encourages the KNH management as well as cancer health regulation bodies in Kenya, and also policy makers more broadly, to help consider changing cervical cancer regulations and the algorithm on how to proceed with the testing and management of women over 30 years of age, whose HrHPV DNA is positive but with negative cytology. Performing Colposcopy on HrHPV positive women and making follow ups can be ideal engendering a better individual,community and a productive healthy nation.Hence we recommend that these women be referred for Colposcopy.

Furthermore, we challenge scientists and researchers in formulating an ideal vaccine

for EBV considering that it is implicated in many cancers such as Burkitts lymphoma, Lymphoepithelioma-like carcinomas, Hodgkin's Lymphomas common in young adults, nasopharyngeal carcinomas in AIDS related or immuno-compromised people and other infections such as Infectious Mononucleosis (Kissing Disease). EBV infected patients more commonly than normal attract mosquito bites which is the predominant cause of the number one killer in Africa - "Malaria". As such, eliminating this virus from childhood using a vaccine would indeed create a herd effect or herd immunity, and in the long-run reduce mortality rates drastically.

5.4 Limitations

Having utilized a small sample population largely on account of the high cost of molecular tests,and one site for this research,it as difficult to generalise results.A robust area would be advantageous for this type of a baseline study.

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Appendix A

Appendices 1-8

A.1 Research Questionnaire

A.2 Consent forms

A.3 Cytology Request form

A.4 EBV Request and Results form

A.5 HPV DNA Request and Results form

A.6 Pap staining protocol

A.7 HPV HC-2 Results from Rotor Gene machine

A.8 EBV PCR Results from Rotor Gene machine