PREVALENCE OF HUMAN PAPILLOMA VIRUS IN URINE AMONG PREGNANT WOMEN ATTENDING KENYATTA NATIONAL HOSPITAL

BY

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DECLARATION

I declare that this dissertation is original work developed 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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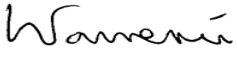

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DEDICATION

I dedicate this work to family, especially my late grandmother, who looked forward to seeing the fruits of my hard work by providing her spiritual, emotional, and financial support.

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LIST OF ABBREVIATIONS

ACCP	Alliance for Cervical Cancer Prevention
ANC	Antenatal Clinic
AUC	Atypical Urothelial Cells
D.P.X	Distyrene Plasticizer Xylene
DNA	Deoxyribonucleic Acid
EA-50	Eosin Azure-50
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
HC2	Hybrid Capture 2 assay
HGUC	High-Grade Urothelial Carcinoma
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
KAVI	Kenya Aids Vaccine Initiative
KNH	Kenyatta National Hospital
LBC	Liquid Based Cytology
LCR	Long Control Region
LGUN	Low-Grade Urothelial Neoplasia
NHGUC	Negative for High-Grade Urothelial Carcinoma
OG-6	Orange Green 6
p53	Protein 53

PCR	Polymerase Chain Reaction
pRb	Retinoblastoma protein
RLU	Relative Light Unit
RNA	Ribonucleic Acid
SHGUC	Suspicious for High-Grade Urothelial
SOP	Standard Operating Procedure
SPSS	Statistical Package for the Social Sciences
STI	Sexually Transmitted Diseases
URR	Upstream Regulatory Region
VIA	Visual Inspection using Acetic acid

ABSTRACT

Background: The Human Papilloma Virus (HPV) is a highly prevalent, sexually transmitted infection, with a prevalence of approximately 70% among sexually active people globally. Close to 99.7% of invasive cervical cancers have HPV, a necessary cause of cervical cancer. It is thought that during pregnancy, HPV acquisition increases due to hormonal influences and immunological depression as the pregnancy progresses. HPV infection is also found in the placenta and is believed to be associated with probable placental dysfunction leading to preterm births and spontaneous abortions.

Broad Objective: To determine the prevalence of high-risk HPV in urine specimens among pregnant women attending the antenatal clinic in Kenyatta National Hospital.

Methodology: One hundred pregnant women aged 18 years and above provided self-collected urine samples. Each urine sample was processed using liquid-based cytology, and DNA was extracted for HPV detection and genotyping using Real-Time PCR. Statistical data analysis was done using R software version 4.0.3. The results are presented as tables and figures and in the form of bar graphs and pie charts.

Results: The majority (70%) of the participants were aged 26-35 years and slightly more than a half (51%) in their third trimester. The prevalence of high-risk HPV was 22%, with 13 (59.1%) having a single infection, 6 (27.3%) with two HPV types, and 3(13.6%) having multiple infections. Most of the urine smears (93%) were Negative for High-Grade Urothelial Carcinoma (UCC), while one smear had Low-Grade Urothelial Neoplasia. The urogenital infections identified in smears included bacterial infection (21%), candida (9%), and trichomonas infection (1%).

Conclusion: The prevalence of high-risk HPV infection among this cohort of pregnant women is relatively high in urine samples. However, the prevalence of HPV-associated urothelial neoplasia was very low, with only one case reported.

Recommendations: Larger studies with paired samples be conducted to validate urine as an alternative screening sample for genitourinary HPV-associated neoplasia, especially during pregnancy when a cervico-vaginal sample is undesirable.

1.0 INTRODUCTION

Cervical cancer is the fourth most commonly diagnosed cancer amongst females [1], yet accounting for the most cancer deaths. The highest incidence and mortality rates are reported in Southern Africa, sub-Saharan Africa, and West Africa. Globally, an estimate of 570,000 new cases annually and 311,000 deaths have been documented [2].

Cervical cancer accounts for 22.2% of all cancers among the female population in sub-Saharan Africa [3]. The incidence is approximately 58.0 per 100,000 in Zambia and 44.4 per 100,000 in Uganda [4]. Cervical cancer incidence in pregnancy is higher than for any other cancer. About 3% of cervical cancer cases are identified during pregnancy, with an estimated frequency of one case for every 1000 to 5000 pregnancies [5].

HPV has been identified in about 99.7% of all invasive cervical cancers [6]. Human Papilloma Virus is categorized into Low-Risk types, which cause genital warts, and the High-Risk oncogenic types linked to cancer of the cervix and other genital and non-genital carcinomas [7].

In a meta-analysis, the highest HPV prevalence amongst females with normal cytological findings was 24.0% in sub-Saharan African,16.1% in Latin America, 21.4% in Eastern Europe, and 14.0% in Southeastern Asia [8]. In Africa, the HPV prevalence rates were 10.7% in Ghana [9], 23.7% in Morocco [10], and 21.6% in Nigeria [11]. In Japan, a prevalence of 35% was found in pregnant women [12], while the prevalence of 34% was demonstrated in Tanzania [13].

Human Papilloma Virus is commonly detected using vaginal and cervical specimens. Urine sampling, a non-invasive, acceptable, quick, reliable, and relatively inexpensive method, is appropriate during pregnancy when most clinicians and patients are hesitant to do pap smears [14]. In one study, HPV genotyping in urine specimens revealed actual infections in the cervix suggestive good agreement between urine and cervical samples [15]. Therefore, urine sampling can be adapted as a screening tool for HPV testing in pregnancy at the antenatal clinics including in KNH.

1.1 Rationale

In Kenya, cancer of the cervix is among the most common cancers in females, with 5250 new cases and 3286 deaths annually [2]. Currently, the coverage of cervical cancer screening in Kenya is approximately 14% [16]. There are few organized and sustained screening programs in most parts of the country. Low cervical cancer screening coverage is also due to a lack of financial resources, inadequate and untrained staff, shortage of medical equipment and infrastructure [17]. Asymptomatic women do not find it necessary to attend screening programs due to a lack of knowledge about cervical cancer. The reluctance to having a pelvic examination is due to cultural and religious practices or beliefs, which leads to low uptake of screening [18].

Cervical cytology has been the mainstay of cervical cancer screening, which involves a speculum examination to obtain a sample. Whereas this has been one of the most successful tool in cancer prevention of all time, it is intrusive and not always acceptable. The discovery of HPV as the necessary cause of cervical cancer enabled the development of molecular screening with HPV DNA or RNA testing and even self-sampling. However, HPV screening is still not widely available, and there is still scanty knowledge among the public and health care providers about HPV testing and its benefits and advantages. Urine sampling for HPV screening is, in essence, self-sampling as other than providing a container and instructions, the provider is not involved. This removes the many disadvantages of health provider sample collection that beset cervical cytology sampling for cancer screening.

Pregnant women have a high risk of acquiring HPV [19]. HPV is usually transient, but the high-risk types are more likely to persist [20]. Blood estrogen and progesterone levels rise during pregnancy, indirectly activating the expression of early HPV oncogenes [21]. There is evidence of HPV infection in the placenta that may cause placental dysfunction leading to preterm births and spontaneous abortions [22]. Traditional HPV testing requires re-visits that emerge as barriers to cervical cancer screening and disease prevention.

In Kenyan health facilities, as elsewhere in low-income countries, pregnant women are not screened for HPV infection as they are for syphilis and HIV. Therefore, this makes it hard to determine the HPV infection prevalence in this population. HPV infection among pregnant women has not been studied in Kenya or at Kenyatta National Hospital to establish the prevalence and high-risk genotypes and any associated cytological abnormalities in urine samples. Pap smears and

HPV testing are both invasive and expensive screening tests compared to the use of urine that is easier to obtain and acceptable to women and their physicians, especially during pregnancy.

Pregnant women generally seek regular medical attention providing an opportunity for cervical cancer screening. In Kenya, cervical cancer screening in pregnant women is acceptable up to twenty weeks of gestation using a pap smear. This study used non-invasive urine samples, an acceptable method that encouraged more pregnant women during all three trimesters to participate.

Therefore, this information will help health practitioners prevent and manage genitourinary HPV-related disease among pregnant women.

1.2 Research Questions

1. What is the prevalence of HPV in urine specimens of pregnant women attending the antenatal care clinic at KNH?
2. What are the high-risk HPV types prevalent in urine specimens of pregnant women attending the antenatal care clinic at KNH?

1.3 Objectives

1.3.1 Broad objective

To determine the prevalence of high-risk HPV types in urine specimens among pregnant women in KNH.

1.3.2 Specific objectives

1. To detect HPV infection in urine among pregnant women in KNH.
2. To identify the high-risk HPV types in urine among pregnant women in KNH.
3. To determine the distribution of high-risk HPV types among pregnant women in KNH.

1.3.3 Secondary objective

To describe the cytomorphological features in urine smears among pregnant women in KNH.

2.0 LITERATURE REVIEW

2.1 Background

HPV is known as the most prevalent sexually transmitted infection worldwide. Globally, HPV infection amongst the sexually active population is about 70% [23]. A systematic review of 194 studies showed a prevalence of 11.7% of HPV infections among women globally [7]. The HPV prevalence in a study in the United States among females was 26.8% [24]. In West Africa, the prevalence of HPV was 26.3% in Ibadan, Nigeria [25], and 44.3% among Kenyan women [26].

Studies in pregnancy show that the prevalence of HPV ranges between 5.5% to 65% [23]. In Turkey, the HPV prevalence amongst pregnant and non-pregnant females has been found to be 29.2% and 19.6%, respectively. The high-risk HPV prevalence infection was 14.6% and 9.8% in pregnant and non-pregnant women in Turkey [6]. A study in Thailand indicated a prevalence of 35.5% among HIV-infected pregnant women. Among these women, almost half of the HPV infections are multiple [27].

A recent study in Nigeria among pregnant women indicates an HPV prevalence of 24.5%. In the first trimester, women have a prevalence of 6.74%, in the second trimester, 8.51%, and the third trimester having the highest prevalence of 9.22%. Hence this difference in the HPV prevalence suggests that hormonal and immunological factors activate the virus as the pregnancy progresses [28]. A study in Uganda among young primigravida pregnant women showed an HPV prevalence of 60%, and 7.3% of whom were HIV-positive. A similar study in Uganda demonstrated an HPV prevalence of 74.6% and 8.6% among HIV-positive women, slightly more significant than the survey amongst pregnant women [29]. A study in Tanzania showed an HPV prevalence infection of 34% amongst pregnant females [13].

Looking at the three trimesters, in a meta-analysis, HPV prevalence was 18.20%, 14.38%, and 19.32%, respectively. The commonly identified High-risk types in the study were 16 and 18 [19]. A recent study in Japan shows that subtypes 16 and 52 are the most shared in pregnancy [12].

2.2 Papillomaviruses classification

Papillomavirus is ubiquitous and seen in mammals and birds [30]. The HPV genotypes belong to three genera: Alpha papillomavirus found in genital lesions. The other two genera, gamma and beta papillomaviruses, originate in the cutaneous lesions [31]. Alpha papillomavirus is an important genera in benign lesions of the oral or genital mucosa. This genus is also associated with common warts. Beta papillomavirus is associated with viruses such as HPV 5, which causes cutaneous lesions. Gamma papillomaviruses such as HPV 4, HPV 48, HPV 50, HPV 60, and HPV88 cause cutaneous lesions [32].

2.3 HPV Genotypes

One hundred seventy human papillomavirus types have been detected in humans [33]. HPV comprises high-risk and low-risk types. There are 15 types recognized as high risk which include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73,and 82. There are HPV genotypes that are probably high risks, such as 26, 53, and 66 [34]. Twelve low-risk HPV genotypes include:6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108, are associated with the growth of genital warts. HPV 34,57 and 83 also associate with undetermined risk [35].

2.4 HPV Genome

This virus is a non-enveloped circular double-stranded DNA genome. The virion is 52-55nm in diameter, where 8000 base pairs bind together with cellular histones [30]. HPV genome comprises three regions. First, the LCR has a regulatory role for DNA replication and transcription of E6 and E7 genes. The second early region consists of E1, E2, E4, E5, E6, and E7 genes that code for structural proteins essential for replication and transcription. The Late region consists of structural proteins; L1 contains type-specific protein domains, and L2 encodes for specific epitopes [29,34].

2.5 HPV infection burden in pregnancy

In pregnancy, the prevalence of HPV ranges from 5.5% to 65% [23]. A meta-analysis of 28 studies suggests that the HPV prevalence amongst pregnant women increased by 1.42% [19]. In addition, some studies report an increased prevalence of HPV infections during pregnancy compared to non-pregnant women [14,35]. According to age, it is clear that women under the age of 25 have a higher chance of acquiring HPV. This risk could be because they are sexually more active than older women [19]. During pregnancy, the prevalence of HPV infection mostly rises during the second

and third trimesters [19,36]. Studies report two factors that raise the risk of HPV during pregnancy. First, hormonal influences and immunological depression during pregnancy may favor the presence and persistence of HPV [37]. Second, high levels of estrogen and progesterone lower the cell-mediated immunity that significantly affects the ability to clear the HPV infection [29].

2.6 Transmission and clinical manifestation

2.6.1 Transmission

HPV affects the skin, mucous membranes, and also through direct skin-to-skin sexual contact. The virus is transmitted both sexually and non-sexually [38]. HPV found in amniotic fluid and fetal membranes can be transmitted to the fetus [39]. Current studies demonstrate the presence of HPV in the foreskin of newborns and the oral mucosa of healthy preschool children [40].

Transmission of HPV via the oral mucosa from an infected person is due to the presence of micro lesions [41]. A study reported the presence of low-risk HPV on fingers of individuals with genital warts showing non-sexual transmission [42].

2.6.2 Clinical manifestation

Human Papillomavirus infection lesions range from benign lesions to cancer. This viral infection can cause warts of the cutaneous epithelium common on hands and feet. Warts have hypertrophied layers of the epidermis, forming a thickening, folding, and hyperkeratosis, which often presents with abnormal keratohyalin granules. These can resolve within 1 to 5years. Some types colonize the face and cause skin cancer. Others affect the mouth, forming lesser prominent swellings that are precursor lesions to squamous cell cancers [43]. HPV genital infections result in anogenital warts forming cauliflower lesions [44]. High-risk HPV types cause cellular variations resulting in vaginal, vulvar, urethral, skin, and cervical neoplasia [45].

2.7 Pathogenesis of HPV

2.7.1 Life cycle

The virus infects keratinocytes of the basal layer epithelium through micro-wounds [46]. The virions attach to the basal membrane via the heparan sulfate proteoglycan. These will move to the receptor expressed on the keratinocytes and enter the cells [47]. On infection, the genomic DNA in the nucleus maintains low copy numbers, and the life cycle of the virus ceases until the host cell begins to differentiate [46].

During a cell division, one daughter cell moves and differentiates, activating the productivity of the viral life cycle [48]. As a result, there is suppression replication of DNA among the existing differentiated cells from the cell cycle. During reactivation in cell division, the differentiated inhibited cells will ensure that the viral genome undergoes replication [49].

The differentiated cells then move into the S phase, allowing amplification of the viral genome. The late proteins then enclose the viral genomes together with the virions and leave the upper section of the epithelium in the squamous cells [48].

2.7.2 Molecular biology of HPV

HPV infection leads to malignancy, best explained using cervical cancer. Oncogenic HPV infection develops following molecular mechanisms(Figure 1) [50]. Incorporating the virus in the host genome interrupts E2 function, which then losses an expression [51]. The disruption of the role of E2 amplifies the manifestation of the E6 and E7 genes.

During HPV infection, the E6 and E7 gene products disrupt the cell cycle forming complexes that deactivate p53 and pRb. The first complex, E6-p53, inhibits growth arrest and apoptosis [52]. The second complex, E7-pRb, interrupts the complex between pRb and the cellular transcription factor E2F-1, releasing the E2F-1. This factor permits transcription of the genes whose products are necessary for the cell to move in the S-phase [53].

The association of the E7 gene product and cyclin E stimulates DNA synthesis and cell multiplication. On the other hand, the E5 gene product increases the mitogen-activated protein kinase activity, amplifying cellular reactions to growth and differentiation, resulting in endless

proliferation and hindered differentiation of the host cell [54]. Inactivation of p53 and pRb proteins result in a high proliferation rate and genomic unsteadiness. Damaged DNA increases, which is unrepairable, forming transformed cancerous cells [55].

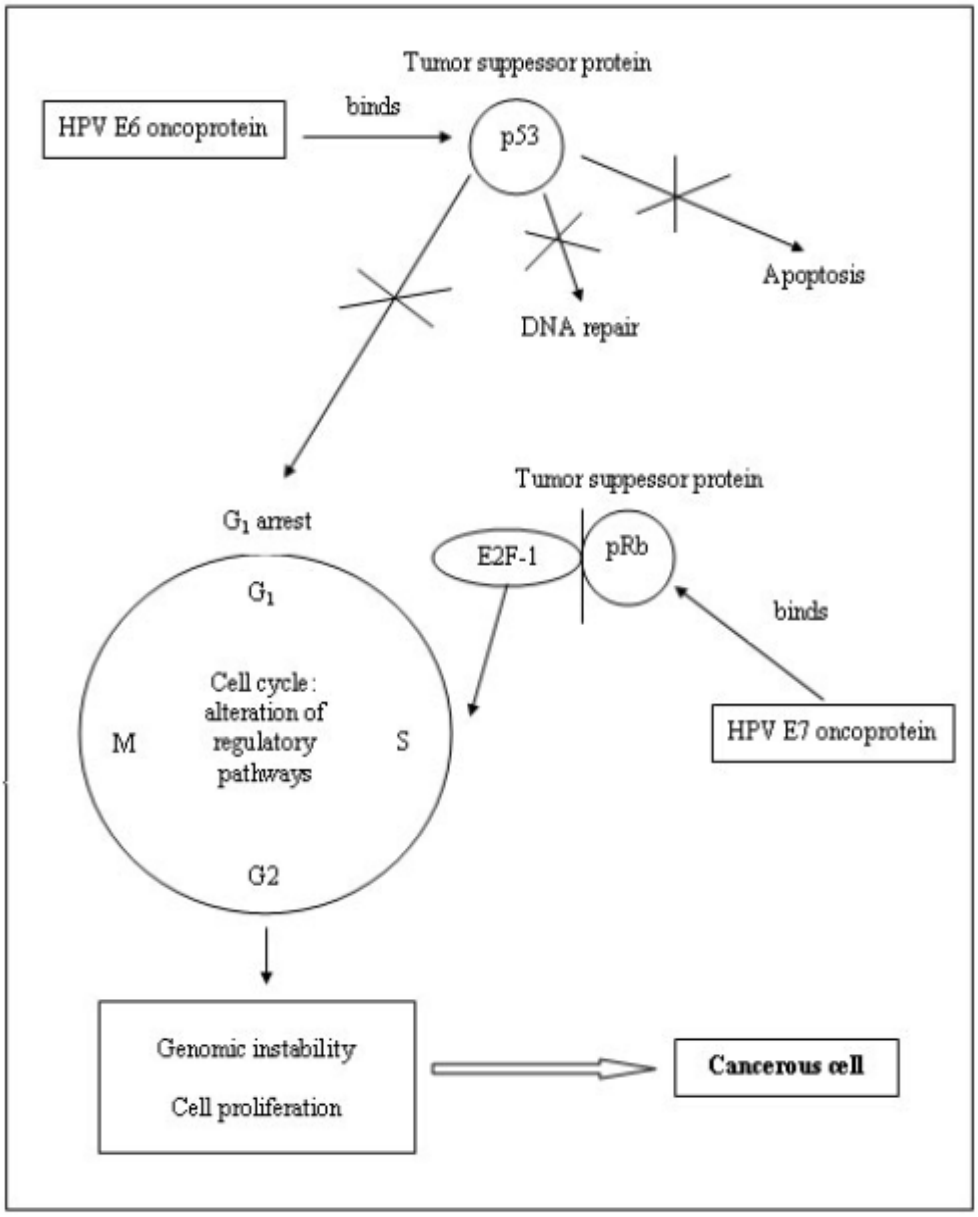

Figure 1: Molecular mechanisms of oncogenic HPV infection [50].

2.8 Molecular methods for the detection of HPV

HPV centers are recognized when viral nucleic acids are discovered, especially viral DNA [56]. A recent study has shown that HPV can also be detected using E7/E7 messenger RNA [57]. The molecular techniques applied in HPV detection include direct nucleic acid probe, signal amplification, and target amplification methods [58].

2.8.1 Direct nucleic acid probe

In HPV genome analysis, Southern blot and Northern blot are essential in analyzing DNA and RNA molecules [59]. However, the hindrances of this probe include low sensitivity, time-consuming techniques, and the need for probably copious quantities of substantially purified DNA. In addition, the degradation of DNA in fixed tissue does not favor the use of southern blot hybridization [58]. However, these tissues can then be processed using In-situ hybridization to allow localization of target genomes [59].

2.8.2 Signal amplification

Digene's HC2 test and branched DNA assay are examples of the amplification method [58]. The Hybrid Capture assay centers on the hybridization of RNA probes which are complementary to the genomic sequence of 13 high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and five low-risk (6, 11, 42, 43, 44) HPV types. The "A" probe cocktail has RNA probes that detect low-risk types of HPV, and the "B" probe cocktail has RNA probes that detect the high-risk types of HPV in separate reactions [60]. The new Hybrid Capture 3 assay uses probes as the HC2 tests and is essential for DNA or RNA targets [60].

2.8.3 Target amplification

A polymerase chain reaction is an example of target amplification. Amplification of HPV DNA occurs in a series of reactions leading to high viral sequences. The amplified products can be analyzed using gel electrophoresis and DNA sequencing [60].

Target amplification is very flexible and has a very high sensitivity. PCR also allows the identification of different HPV types. One disadvantage of PCR is that previously amplified leads to false positives due to contamination [58].

2.9 Risk elements for HPV

Risk causes for HPV infection include the number of sexual partners, STIs, smoking, parity, and immunosuppressive illnesses [61]. The use of oral contraceptives for five years, having more than five pregnancies, and exposure to HIV raise the risk for HPV persistence and progression to HPV lesions [62]. Early initiation of sexual intercourse at a young age increases the risk of acquiring HPV because of the behaviors and partner characteristics [63]. Condom usage also exposes one to HPV since transmission is through contact with diseased labial and anal tissues, which the condom does not cover [43].

2.10 Treatment and Prevention of HPV

2.10.1 Treatment

Topical application of cytotoxic substances, intralesional injections of infernos, cryosurgery, excision, electro, and laser surgery are essential in the treatment of genital warts [64]. In 2011 ACCP found that cervical cancer precursors are identified and treated using VIA or HPV- DNA testing in low-resource locations, together with cryotherapy [65]. Colposcopy and biopsy is the best recommendation for pregnant women during the postpartum period between 6-12weeks [66].

2.10.2 Prevention

The actions taken up for the prevention of HPV infection include risk decline plus HPV vaccines. HPV transmission decreases with the use of physical barriers like consistent usage of latex condoms and spermicides, delay in age of initiation of sexual intercourse, and reduction from sexual behavior [43]. There are 3 vaccines directed against HPV that been approved by the FDA: Gardasil 9 targets HPV types 6,11,16,18,31,33,45,52 and 58: the bivalent vaccine Cervarix (GlaxoSmithKline) targets HPV 16 and 18: and the quadrivalent HPV vaccine (Gardasil) targets HPV 16,18,6 and 11. HPV vaccination starts at age 11 or 12 and also as early as age 9.

The 2^{nd} dose is suitable for the 9-valent vaccine early the age of 15. This 2^{nd} dose is given 6 to 12 months after the first. For those who initiate HPV vaccination between ages 15 and 45, the 3^{rd} dose at 0,1, 2, and 6 months is effective [67].

2.11 Urine cytology in pregnancy

Currently, there are no studies examining urine cytology for cervical cancer. However, the elevation of hormones during pregnancy increases the risk of infections and HPV acquisition [21], a leading cause of cervical cancer. In addition, HPV is a genito-urinary infection, and detection in urine samples highly correlates with cervico-vaginal samples [15]. Therefore, a urine sample that is easier to obtain would suggest that it would be a helpful screening sample similar to cervico-vaginal samples and appropriate during pregnancy, where most clinicians and patients are hesitant to do a pap smear [14]. Therefore urine cytology will help assess the genitourinary HPV-associated neoplasia in pregnancy.

3.0 METHODOLOGY

3.1 Study design

The study was a cross-sectional descriptive survey.

3.2 Study area

The study area was the ANC Clinic 18 in Kenyatta National Hospital, which serves the population within and around Nairobi. Several pregnant women were initially seen at other hospitals and the Nairobi city's periphery and referred to KNH. The antenatal clinic runs from Monday to Thursday, and about 30 to 50 new mothers are attended to every week. Pregnant women with no pregnancy-associated complications usually have at least four antenatal visits.

3.3 Study population

The study consisted of pregnant women aged 18 and over in the ANC Clinic at KNH willing to partake in the survey.

3.4 Selection criteria

3.4.1 Inclusion criteria

Pregnant women aged 18 years and over who consented to partake in the study.

3.4.2 Exclusion criteria

Pregnant women who developed obstetric and gynecological emergencies during the current pregnancy, for example, vaginal bleeding.

3.5 Sample size determination

The prevalence of HPV among pregnant women both in Kenyatta National Hospital and Kenyan is unknown. A conservative estimate of 50%, considering a confidence interval of 95%, was used to estimate the prevalence [68]. The sample size was calculated as below:

$$n_1 = \frac{Z^2 p(1-p)}{D^2} = \frac{1.96^2 \times 0.5(1-0.5)}{(0.05)^2} = 384.2$$

n_1 = Required sample size

Z = Normal deviation at the desired confidence interval 95%, Z value is 1.96

P = Expected prevalence of proportion expected with desired characteristics = 50%

D = Degree of precision, 0.05%

The value (n_1) was then adjusted to account for the size of the target population. As a result, the new sample size(n_2) is calculated below:

$$\text{New}(n_2) = n_1 \left[\frac{N}{N + n_1} \right]$$

N= The target population of pregnant women seeking health care in the antenatal clinic is approximately 120

$$\text{New}(n_2) = 384.16 \left[\frac{120}{120 + 384.16} \right]$$

The new sample size(n_2) = 91

The first nine samples were a pilot for testing procedures used to analyze the samples making 100 samples.

3.6 Sampling procedure

Participants were selected using systematic sampling. The target population size in two months was approximately 240, divided by the sample size of 91. The obtained value of 3 was considered as the sampling interval. The first 3 participants were used to select one at random. Then, the second participant was selected, and the following 5^{th}, 8^{th}, 11^{th}, respectively.

3.7 Recruitment and consenting

Pregnant women were informed about the study and recruited using a screening questionnaire (See Appendix 2). The research assistant explained to the eligible participants about their participation's purpose, benefits, and risks. Next, the potential participants received the consent form, read it, and signed it. The research assistant guided the participants who could not read and sign the consent form and administered a structured questionnaire to all the participants (See Appendix 2). The urine samples were collected and processed following the study work plan, as shown in figure 2.

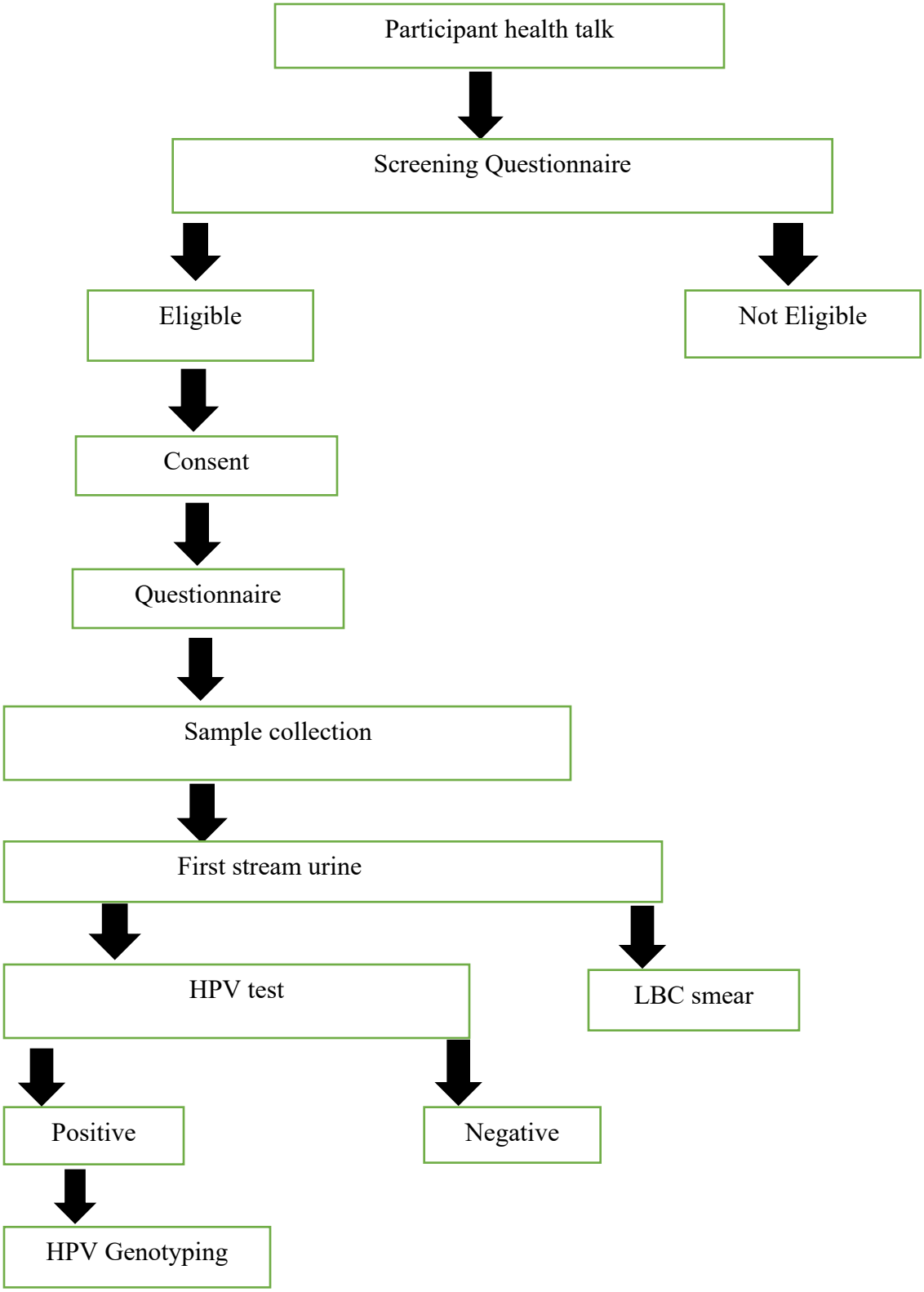

Figure 2: Work plan of the study

3.8 Sample collection

3.8.1 Instruction in sample collection

The eligible participants were each given one well-labeled sterile urine collection cup of 50ml. They were asked not to fill urine up to the rim to decrease the risk of leakage, and hence contamination. The participants were instructed to collect the urine as follows:

1. Wash hands before getting to the toilet
2. Dry the hands completely
3. Collect the first stream urine sample into a well-labeled sterile urine cup about three quarters and avoid contamination.
4. Then place the screw top onto the urine cup and close tightly and wipe the outside of the cup dry.

3.8.2 Sample transportation

The research assistant transported the samples collected to KAVI-Molecular Laboratory. Each sample was divided equally into two, where one remained in the KAVI-Molecular Laboratory for HPV testing. The second sample was transported to the Cytology laboratory for LBC, where they were received and recorded.

3.8.3 Sample storage

The research assistant preserved the samples designated for HPV-DNA analysis at freezing temperatures of -80^0 Celsius until testing commenced.

For the second urine sample, 3ml of the urine was pipetted and placed in a different tube, stored at room temperature for 2-3hours, and later processed.

3.9 Specimen analysis

3.9.1 Extraction of DNA

DNA was extracted using the QIAamp DNA mini kit. After thawing, 200µl of each sample was vortexed and added to a 1.5ml microcentrifuge containing proteinase k. The addition of proteinase K and lysis buffer helped in the isolation of DNA from the cells. DNA was then concentrated by ethanol precipitation and later preserved at -80^0 Celsius for further testing.

3.9.2 The detection and genotyping of HPV

The extracted DNA samples were first thawed, and then the required number of PCR tubes were prepared. A mix for 132 reactions was prepared by adding PCR-buffer-FRT 60 and Hot Start DNA Polymerase into a sterile tube. For every PCR-mix-1, a sterile tube was designed, then 10*N+3 µl of PCR-mix-1 and 5*N+3 µl of the mix was added, forming a reaction mix. Thus, four tubes were selected for every sample, and 15 µl of reaction mix was added.

Furthermore, the addition of the following reagents was done, in the first tubes, 15 µl of mix '16,18,31, IC', in the second 15 µl of mix '39,45,59, IC', in the third 15 µl of mix '33,35,56,68' and lastly in the fourth 15 µl of mix '51,52,58,66' respectively. In addition, 10 µl of the extracted DNA sample was added. For each panel, controls and standards were prepared as follows: to the four tubes of negative control (half strip), 10 µl of DNA extracted from negative control was added, and to the four tubes of standard K2 (half strip) 10 µl of each standard was added. The tubes were transferred and processed in a Real-Time PCR.

The results of HPV DNA detection were negative on identifying the Internal Control in the first two tubes for the sample. However, the sample was positive in FAM/Green, JOE/Yellow/HEX, ROX/Orange, Cy5/Red channels after identifying a signal in at least one of the four tubes. Upon genotyping HPV on the different channels, the following genotypes were identified; 16, 39, 33, 58 on FAM/Green channel, on JOE/Yellow/HEX channel 31, 45, 35, 52, on the ROX/Orange channel 18,59,68,66 and the Cy5/Red channel had genotypes 56 and 51. These results were interpreted using the HPV Genotypes 14 Real-TM Quant handbook [69].

3.9.3 Sample preparation for cytomorphology

Liquid-based cytology was performed immediately after sample collection by pipetting 3ml of urine into a cytology funnel. The sample was centrifuged at 1500rpm for 6 minutes, and a thin deposit of cells was automatically deposited on the slide. The slide was immediately immersed in a fixative of 95% ethanol. Routine Papanicolaou staining was performed.

3.10 Quality assurance

The urine samples were collected in well-labeled sterile urine cups with screw-tops and transported immediately on collection to the KAVI-Molecular laboratory. All reagents were prepared and

stored according to the standard operating procedures(SOPs) as per the manufacturer's instruction manual.

The molecular procedures were performed by a well-trained laboratory scientist and the principal investigator. The research assistant incorporated the positive and negative controls into the procedure. In Liquid-based cytology, the urine samples were immediately processed to prevent the degeneration of cells. The slides were stained using Papanicolaou stain following the SOPs in the laboratory (See Appendix 6).

The principal investigator screened the urine samples for cytology. These smears were counter-checked by the pathologists/supervisors using the Paris system for reporting urinary cytology.

3.11 Data management

The samples were allocated unique study numbers for identification and further linkage with the patient's socio-demographic and other characteristics. The results were recorded and stored in hardcopy books, on report forms, and in a Microsoft Excel workbook protected by a strong password.

3.12 Data analysis

The patient socio-demographic, behavioral, and clinical characteristics were collected using structured questionnaires. These were digitized using KoBoToolbox and linked with cytology results using patient unique study numbers. Statistical data analysis of the results was done using R software version 4.0.3. The descriptive statistics were presented as tables and figures in the form of bar graphs and pie charts.

3.13 Ethical consideration

The Kenyatta National Hospital/University of Nairobi/Ethics Research Committee gave ethical approval for this study. The participants received urine examination procedures at no cost. Each consented participant attained a unique code for confidentiality. The participants' information was stored in soft copies, protected from unauthorized persons using a strong password, and kept in a securely sealed cabinet. The participants were informed to contact the principal investigator, supervisors, and secretary of the ethics committee for any concerns regarding this research.

3.14 Variables

3.14.1 Independent variables

The social demographic data encompassed age, marital status, occupation, and educational level. The behavioral characteristics included the age of sexual debut and the number of sexual partners in the past year. The clinical information included HIV status, method of contraception, parity, pregnancy complication history, and the gestation period.

The urine cytomorphological interpretations, also an independent variable, included NHGUC, LGUN, and infections.

3.14.2 Dependent variables

High-risk HPV types.

4.0 RESULTS

4.1 Socio-demographic, behavioral, and clinical characteristics of the participants

4.1.1 Age of the participants

A total of 100 participants were enrolled in the study. A third (38%) of the participants were aged between 26-30 years. Thirty-two (32%) of the participants were aged between 31-35 years, while only one was in the age bracket of 18-20 years (Figure 3).

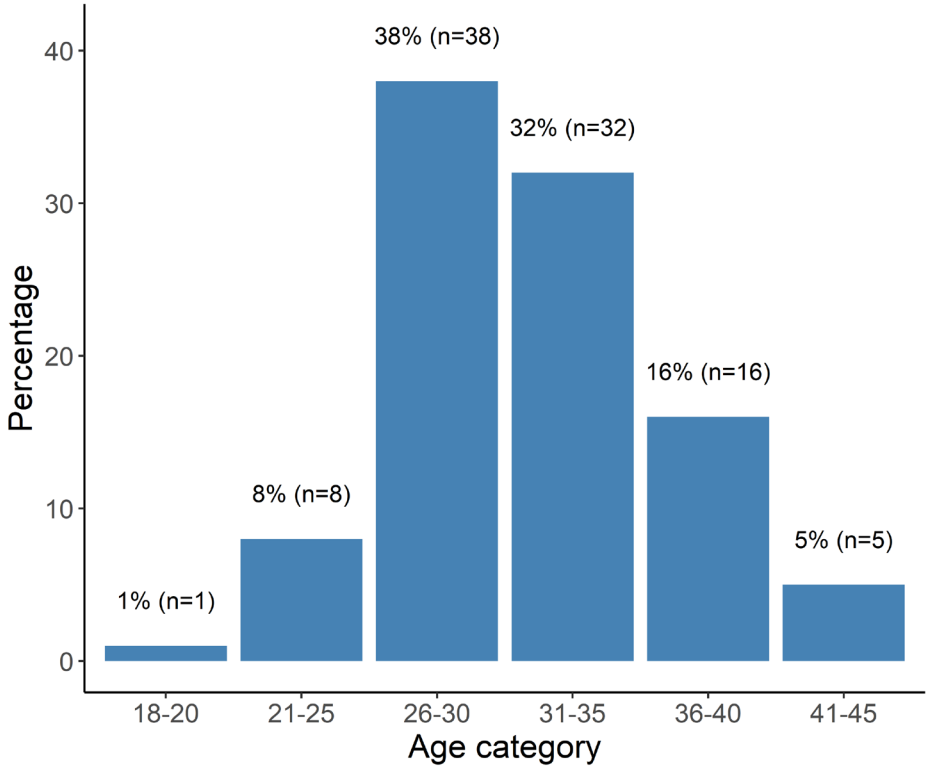

Figure 3: Frequency distribution of the age of the participants

4.1.2 Gestation/trimester of the participants

The data indicates that slightly more than half of the participants (n=51) were in their third trimester. Thirty-eight participants were in their second while 11 were in their first trimester.

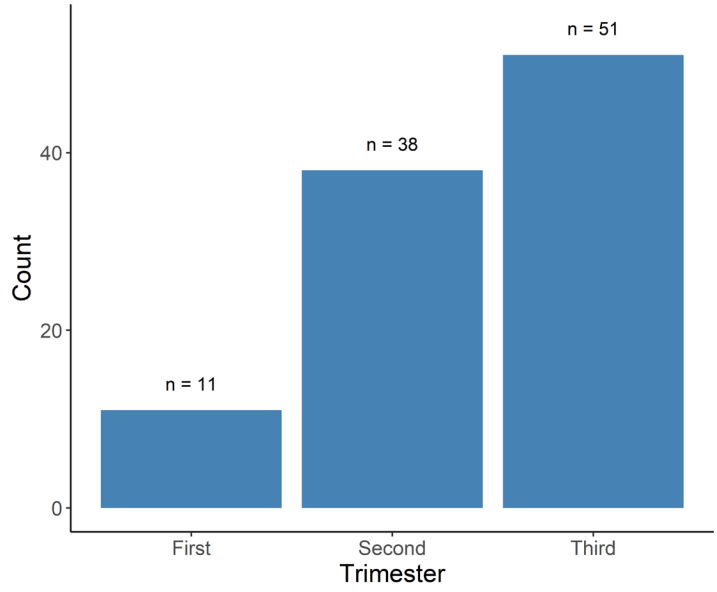

Figure 4: Distribution of the gestation period/trimester of the pregnancy

4.1.3 Distribution of other key variables

The study showed that most of the study participants were married (93%), and slightly more than half had attained university (51%). In addition, just under half of the participants (49%) were self-employed. Fifty-two percent (52%) had their sexual debut after the age of 20 years, and 95% had had only one sexual partner in the past year. A majority (68%) of the participants had 1-2 children. Nine (9%) gave an HIV-positive medical history. More than three-quarters (76%) had a history of using contraceptives, with 30% having used oral contraceptive pills, followed by condoms and IUCDs with 17% and 16%, respectively. In comparison, the least number (4%) had used depo. Twenty-four percent gave a history of not having used contraception. The study showed that 24% of the participants had a history of pregnancy loss, 8% had stillbirths. Only 4% of the participants had a history of miscarriage and stillbirth, while most (64%) had had uncomplicated livebirths, as shown in the table below.

Table 1: Distribution of the key socio-demographic variables

Variable	n	Percentage (%)
Educational level		
Primary	14	14
Secondary	35	35
University/College	51	51
Occupation		
Unemployed	34	34
Employed	17	17
Self-employed	49	49
Marital status		
Single	7	7
Married	93	93
Age of sexual debut		
< 18	7	7
18-20	41	41
> 20	52	52
Sexual partners in the past year		
1	95	95
≥ 2	5	5
HIV Status		
Positive	9	9
Negative	91	91
Parity		
0	24	24
1	34	34
2	34	34
3	8	8
Method of contraception		
Oral Pills	30	30
Condoms	17	17
Intrauterine device	16	16
Implant	9	9
Depo	4	4
None	24	24
Pregnancy complication history		
Miscarriage	24	24
Stillbirth	8	8
Both	4	4
None	64	64

4.2 Prevalence of HPV

A total of 22 women (22%) tested positive for at least one HPV type in their urine (Figure 5).

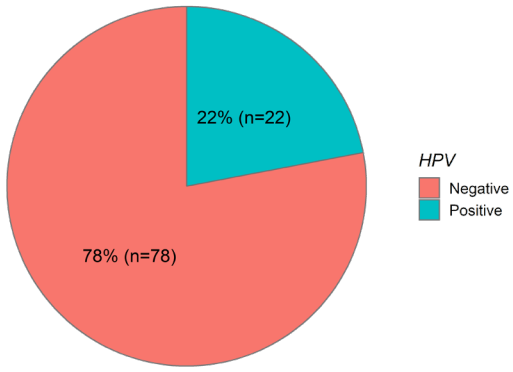

Figure 5: Prevalence of HPV

4.3 Distribution of high-risk HPV types

The results in Figure 6 show the distribution of high-risk HPV types, which included the following 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, and 68. As shown below, types 18 and 56 had the highest prevalence of (5/22) 22.7%, followed by types 31,35, 45, 51, 58, and 66 at (3/22) 14% each.

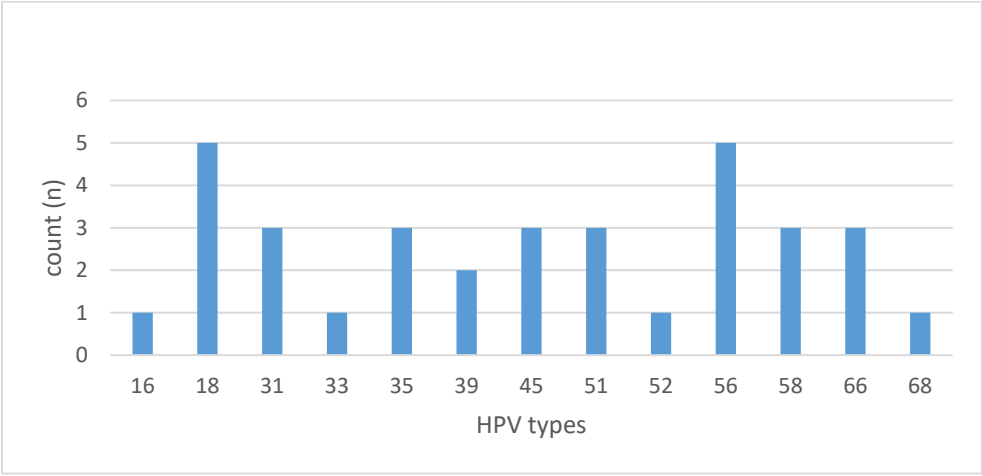

Figure 6: The frequency of high-risk HPV types

4.4 The cytomorphologic features in urine smears

The study also assessed the cytomorphologic features of urine smears which included evaluating the presence of urothelial cells and infections in the urine. The majority (n=93) of the smears showed urothelial cells Negative for High-Grade Carcinoma (NHGUC). Six (n=6) smears had inadequate (unsatisfactory) cells for diagnosis, while only one (1) showed Low-Grade Urothelial Neoplasia (LGUN). The most common infection was bacterial infection, 21%, while trichomonas infection was the least at 1%.

Table 2: The cytomorphologic features in urine smears

Cytomorphologic features	**n**	**Percentage%**
Unsatisfactory (no cells)	6	6
NHGUC	93	93
AUC	0	0
SHGUC	0	0
HGUC	0	0
LGUN	1	1
Total	100	100
Infections		
Bacterial infection	21	21
Bacterial infection and candida	4	4
Candida	9	9
Trichomonas infection	1	1
None	65	65
Total	100	100

4.5 Comparing positive high-risk HPV samples with cytomorphological features

Of the 22 participants who tested positive for high-risk HPV, 13 (59.1%) had a single infection, 6 (27.3%) had two HPV types, while 3 (13.6%) had multiple infections. In addition, according to trimesters, 3 (13.6%) were in the first trimester, 9 (40.9%) second trimester, and 10 (45.5%) in the third trimester of pregnancy. A majority (86.4%) of the participants were negative for a urothelial

cell abnormality, and 10 (45.5%) had an infection. The only study participant who had a cell abnormality (Low-Grade Urothelial Neoplasia) had HPV type 66, as shown in the table below.

Table 3: HPV genotypes, cytomorphology, and infections in HPV positive participants

Trimester	Genotypes	Cytomorphology	Infection
First	35	Unsatisfactory	None
	56	Negative	None
	31, 58	Negative	None
Second	45	Negative	Bacterial infection
	31	Negative	Bacterial infection
	35	Negative	None
	56	Negative	None
	18, 39	Negative	Candida
	18,52	Negative	Bacterial infection
	45, 58	Negative	None
	56, 51	Unsatisfactory	None
	45, 35, 68	Negative	None
Third	18	Negative	Candida
	18	Negative	None
	31	Negative	None
	33	Negative	Bacterial infection
	56	Negative	Candida
	56	Negative	Trichomonas infection
	66	LGUN	None
	66, 51	Negative	Bacterial infection
	16, 66, 51	Negative	Bacterial infection
	18, 39, 58	Negative	None

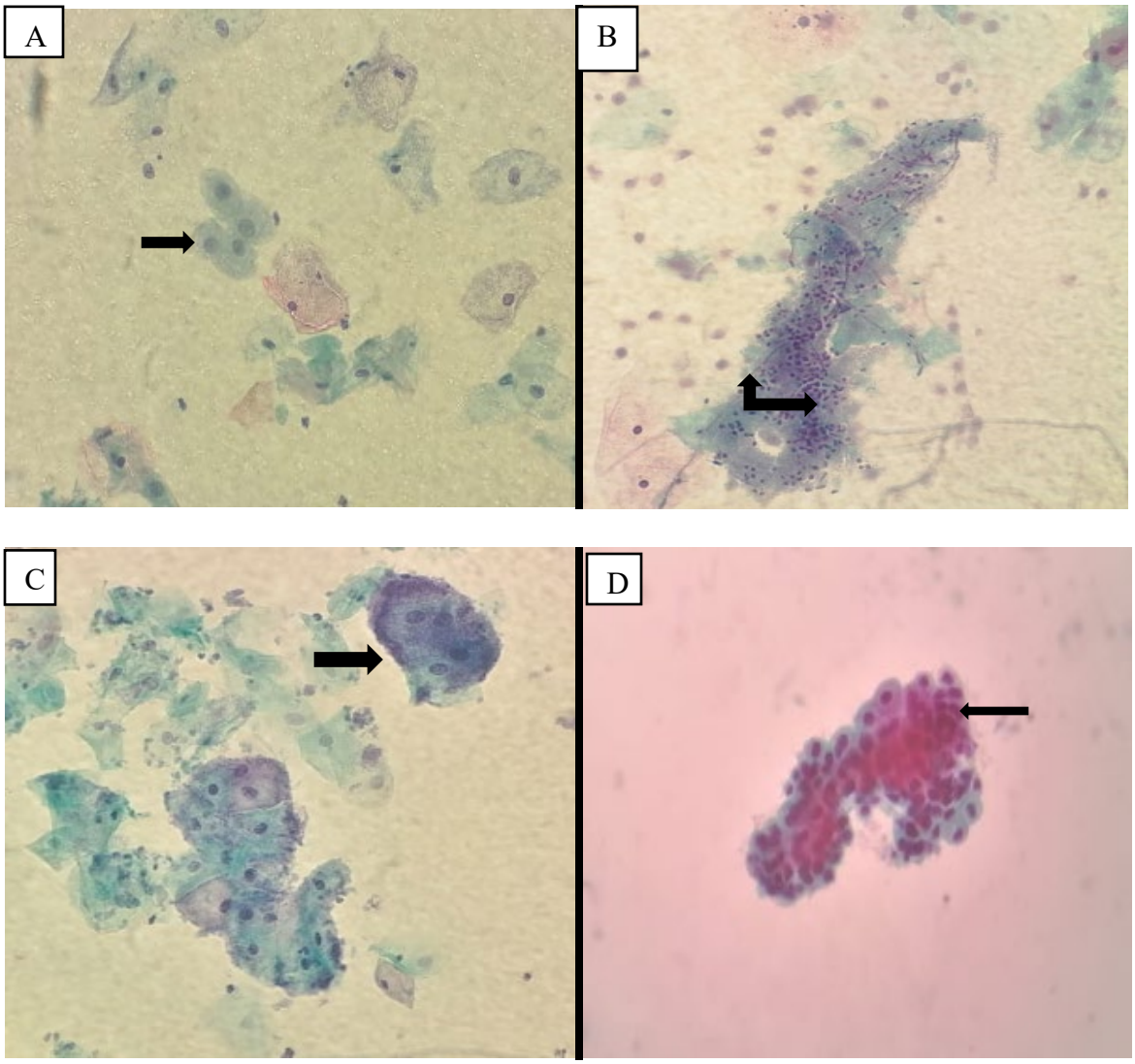

Figure 7: Photomicrographs of urine smears(Papanicolaou stain x40)

A. Normal urothelial cells with dense cytoplasm, high nucleus to cytoplasmic ratio, round nuclei with regular nuclear membranes, and intermediate cells with abundant cytoplasm.
B. Candida species with pseudohyphae and spores
C. Cocco-bacilli are covering the intermediate cells showing a bacterial infection.
D. Low-Grade Urothelial Neoplasia forming a three-dimensional papillary cluster, increased nuclear to cytoplasmic ratio, nuclear overlapping, hyperchromatic nuclei, and dense cytoplasm.

5.0 DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Discussion

This study aimed to investigate the prevalence of high-risk HPV types in urine samples among pregnant women attending the antenatal clinic in Kenyatta National Hospital. Detecting HPV in pregnancy is more significant or comparable to HPV infection in non-pregnant women [70]. Therefore, using a non-invasive and more widely acceptable method to detect this infection is more beneficial in pregnancy.

The study had one hundred study participants, and most (70%) were in the age category of 26-35 years which is very similar to the finding in the developing world in pregnancy [37]. Generally, this young population had a low parity due to the high levels of education. However, on the other hand, high parity increases the risk of acquiring HPV infection [28]. Fifty-two percent (52%) of the participants had their sexual debut after age 20 and had a low number of sexual partners, lowering the risk of acquiring HPV [71]. Nine percent (9%) of the pregnant women gave an HIV-positive medical history higher than the prevalence of HIV (5.8%) among women in Kenya [72]. HIV increases the risk for acquisition and persistence of HPV due to low immunity [13]. A third of the participants had a history of using oral contraceptive pills. Long-term use of the pills has increased the risk of acquiring HPV [73].

The prevalence of high-risk HPV infection among pregnant women in this study was 22%. A few studies in other countries conducted using urine in HPV testing in pregnancy had varying prevalence rates., 54.0% and 2.7% [14,15]. In Kenya, the high-risk HPV prevalence in urine among non-pregnant women (28%) is high compared to the current study's prevalence [74]. Unfortunately, there are no studies on the HPV infection rate among pregnant women in Kenya. However, these variations in prevalence between the current study and other studies might be due to the differences in HPV testing methods and geographical regions. In addition, hormonal influences and immunological depression during pregnancy may favor the presence and persistence of HPV [32] and, thus, explain a high HPV prevalence.

In pregnancy, HPV infection clears with increasing trimesters. Some studies have shown decreased infection in the third trimester because of the changes in sexual behavior than the biologically induced effect of pregnancy upon HPV infection exposure and persistence [12,23]. According to

Takakuwa et al., 42.9% of pregnant women are newly infected by HPV between the first/second and third trimesters [75]. In this study, most participants tested positive for the HR- HPV in the 3rd trimester at 45.5% (10/22). Multiple HPV infections were identified in the second and third trimesters. Therefore HPV infection can be influenced by hormonal and other effects of pregnancy.

In this study, the HPV Genotypes 14 Real-TM Quant kit was used to analyze the first stream of urine collected from participants on arrival at the antenatal clinic, and thirteen high-risk HPV types were detected. The kit's specificity was 100%, and the potential cross-reactivity was tested against the group control. For the sensitivity of not less than 1000 copies/ml, the kit allows detecting HPV DNA in 100% of the tests. The detection of the HPV DNA was performed on the control standard and its dilutions by negative sample [69]. In this study, thirteen genotypes were detected using the real-time PCR, while Franciscatto et al. detected six genotypes using the microplate colorimetric hybridization assay [14]. The difference in the genotypes was due to the different HPV detection methods and the number of genotypes detected. Therefore, standardizing molecular protocols and techniques is essential for global comparison.

Genotypes 18 and 56 were the most prevalent in this study. However, genotypes 16, 66, and 70 are the most prevalent in urine among non-pregnant women in Kenya [74]. The findings in this current study are dissimilar from those of Mantzana et al., which showed HPV 16 type is the most prevalent genotype in pregnancy [15]. Franciscatto et al. found that the highest prevalent genotype in pregnant women was 31, followed by 16 and 33 [14]. The prevalence of HPV genotypes can vary among populations in different regions [76]. Hence, more research should be conducted on pregnancy.

A single HPV infection was identified in 13 (59.1%) of the cases; 6 (27.3%) had two HPV types and more than two HPV types in 3 (13.6%) cases. The majority of the participants report being in a monogamous relationship, which could be why many had a single infection. However, the sexual behavior or the number of HPV types in a partner may increase the risk of acquiring multiple infections [77].

Of the one hundred urine samples processed for cytology, 6% had insufficient cells for cytological interpretation. Hence 94% of the samples were analyzed and reported as follows – 93% were NHGUC, and only one had abnormal cytology of LGUN according to the Paris system of reporting urinary cytology [78]. Currently, no studies are available describing the cytological lesions in urine

smears among pregnant women. Among the high-risk HPV- positive participants, a majority(86%) of the urine samples were NHGUC, and one(5%) smear had a lesion(LGUN). HPV-associated neoplasia in the urothelial lesions is generally low [79]. The low number of abnormal cellular changes in this relatively young population can be attributed to the natural history of the virus [80]. However, the virus's progression to cervical cancer is more in women older than 40 years because of the long duration of the virus in this group leading to cervical cancer [81]. Thus, Human Papilloma Virus infection is cleared in most cases due to natural cell-mediated immunity [29].

5.2 Conclusion

The prevalence of high-risk HPV infection among this cohort of pregnant women is relatively high in urine samples. However, the prevalence of HPV- associated urothelial neoplasia was very low, with only one case reported.

5.3 Recommendations

Larger studies with paired samples be conducted to validate urine as an alternative screening sample for genitourinary HPV-associated neoplasia, especially during pregnancy when a cervico-vaginal sample is undesirable.

5.4 Limitations

Urine usually has degenerated cells which may have contributed to the low number of cellular abnormalities identified in the study. Therefore, the results cannot be generalized to entire populations to comment on the population prevalence of HPV-associated cytological changes in the urine.

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7.0 APPENDICES

Appendix 1: Study Questionnaire

Patient Identification: Date: ……

Study Number: …….

1) **Age**: [ ]

2) **Marital status**:

Single: [ ] Married: [ ]

3) **Occupation**:

Unemployed: [ ]

Employed: [ ]

Self-employed: [ ]

4) Contacts:

5) **Education level**:

Primary: [ ]

Secondary: [ ]

University/College: [ ]

Never attended: [ ]

6) Age of sexual debut (First time of sexual intercourse): [ ]

7) How many sexual partners have you had in the last year? [ ]

Names of Nurse…… Sign……. Date…….

Principal investigator…… Sign……. Date……

Appendix 2: Cytology Request and Result form

Patient Identification: Study Number: …….

Age: …….

1.0 <u>Specimen</u>

- Sample collection date: ……
- Sample collection time: ……
- Quantity: ……...
- Color: …….

2.0 <u>Cytology report</u>

- Specimen type: Liquid Based Preparation
- Specimen adequacy: …….
- Microscopic description:

  ……

  …….
- Diagnosis: ……...

- **<u>Category:</u>**

☐ Unsatisfactory/ Non-diagnostic

☐ Negative for High-Grade Urothelial Carcinoma(NHGUC)

☐ Atypical Urothelial Cells(AUC)

☐ Suspicious for High-Grade Urothelial Carcinoma(SHGUC)

☐ High-Grade Urothelial Carcinoma(HGUC)

☐ Low-Grade Urothelial Neoplasia

☐ Positive for Malignancy

3. <u>Recommendation:</u>

- **Normal screening/ Routine follow up:** _____
- **Treatment:** _____
- **Other specify:** _____

Principal investigator _____ Date _____

Pathologist _____ Date _____

Appendix 3: Hr-HPV DNA Testing Request and Result Form

Patient Identification: Study Number: _____

Age: _____

TEST RESULT

- Positive for Hr-HPV: _____
- Negative for Hr-HPV: _____

HPV-DNA genotype

_____

Principle Investigator _____ Date _____

Molecular scientist _____ Date _____

Appendix 4: HPV Referral Form

Patient Identification: Patient's name: _____

Age: _____

Hospital number: _____

TEST RESULT

- Positive for Hr-HPV: _____
- Hr-HPV genotype: _____

Recommendation

Colposcopy and Biopsy

Principal Investigator _____ Date _____

Molecular scientist _____ Date _____

Appendix 5: Papanicolaou Staining Procedure

1. Fix in 95% Ethanol for a minimum of 15 minutes
2. Hydrate in decreasing concentrations of Ethanol of 80%,70%, and 50%, ten dips in each.
3. Rinse in tap water
4. Stain in Harris Hematoxylin for 4 minutes
5. Rinse in tap water
6. Differentiate in 0.05% acid alcohol, ten dips
7. Rinse in tap water
8. Blue in Scott's tap water ten dips
9. 95% Ethanol ten dips
10. OG-6 stain for 1.5 minutes
11. 95% Ethanol ten dips
12. EA-50, or Modified EA-50, or EA-65 stain for 3 minutes
13. 95% Ethanol ten dips, two changes
14. Absolute Ethanol ten dips, two changes
15. Clear in 3 changes of xylene, ten dips each
16. Mount in D.P.X
17. Examine by 10X and 40X objectives

The Papanicolaou Stain Principle

The Papanicolaou test is a screening test used for visualization of pre-cancerous and cancerous processes in cells exfoliated from epithelial surfaces of the body. The stain is polychromatic containing multiple dyes that display many variations of cellular morphology, showing a degree of cellular maturity and metabolic activity.

The Pap stain contains five steps to be followed, namely; fixation, nuclear staining with hematoxylin, cytoplasmic staining with Orange G 6 and Eosin Azure, dehydration, clearing with xylene and mounting with DPX. The hematoxylin stain is a primary stain which stains the nucleus purple. Orange G-6 and Eosin Azure stain the cytoplasm of matured and keratinized squamous cells orange. Eosin Azure also stains the cytoplasm of immature squamous cells blue.

In the Papanicolaou procedure, smears are fixed in ethanol for a minimum of 15 minutes, hydrated in running tap water, and stained in hematoxylin. The excess stain is removed by using a differentiating solution, acid alcohol, and then blued under running tap water or use Scott's tap water. The cytoplasm is then stained using counter stains Orange G-6 and Eosin Azure, followed by dehydration in absolute alcohol for two changes for removal of excess water. The clearing is vital because it replaces alcohol with xylene, which is miscible with mounting media. The smears are then coverslipped using the mounting media DPX.

The smear will be examined under the microscope to look for abnormalities. This test aims to detect pre-cancerous and cancerous changes in the epithelial cells that are caused by sexually transmitted human papillomaviruses. The test is essential in the detection of infections and abnormalities.

Microscopy and Result Interpretation (Paris system)

Microscopy encompased the description of the cells in Liquid Based Cytology concerning the standard structure and functions of the normal cell. The reporting of results was according to the Paris System for Reporting Urinary Cytology.

They include:

- Negative for High-Grade Urothelial Carcinoma(NHGUC)
- Atypical Urothelial Cells(AUC)
- Suspicious for High-Grade Urothelial Carcinoma(SHGUC)
- High-Grade Urothelial Carcinoma(HGUC)
- Low-Grade Urothelial Neoplasia(LGUN)