COLLEGE OF HEALTH SCIENCE, SCHOOL OF MEDICINE

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Human papillomavirus in women with pre-cancerous lesion and cervical cancer: the use of urine as an alternative specimen

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List of Abbreviations

CIN	Cervical intraepithelial neoplasia
DNA	Deoxyribonucleic Acid
E gene	Early gene
GLOBOCAN	Global cancer registry
HPV	Human Papillomavirus
HR HPV	High risk HPV
ICC	Invasive cervical cancer
L gene	Late gene
LCR	Long Control Region
LR HPV	Low risk HPV
ORF	Open reading frame
pRb	Retinoblastoma protein
RNA	Ribonucleic Acid
SOP	Standard operating procedure
SPSS	Statistical Package for Social Sciences
STI	Sexually transmitted infection
TASH	Tikur Anbessa Specialized Hospital
VIA	Visual Inspection with Acetic acid
PBS	Phosphate buffer saline

Abstract

Background: In a country where the coverage cervical cancer screening is low optimization of the uptake is critical. The implementation of high precision test is advocated by WHO. To augment the implementation human papillomavirus (HPV) based screening in Ethiopia we compared the performance urine HPV DNA test with cervical swab.

Methods: Paired samples (n=103) of first void urine and cervical swab were collected from patients Gynecology Clinic of Tikur Anbessa Specialized Hospital (TASH). After extraction of DNA using QIAamp® DNA Mini Kit (Qiagen) the HPV infection, coinfection and type-specific HPV distribution was determined using the Anyplex HPV28 DNA genotyping kit (Seegene, Seoul, Korea) and CFX96 IVD (In Vitro Diagnostic) Real-Time PCR System. The kit simultaneously detects, differentiate, and semi-quantify 28 HPV genotypes 19 high risk (Hr)-HPV types; HPVs 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73 and 82 and 9 LR-HPV types; HPVs 6, 11, 40 ,42, 43, 44, 54, 61 and 70. Additionally, blood sample was collected to detect HPV16 L1 anti-capsid antibody using Prevo-check ® (Abvirus Germany GmbH). It is immunologic rapid test that directs against a protein that is produced by HPV 16 infected cells which interferes with cell division. Pap smear was done by a pathologist and histology results was collected from the chart of the patients and a clinical form was used to collect basic information from the patients by the attending midwife nurses.

Result: Of the 103 paired samples, HPV infection prevalence was 83.5% in cervical and 77.7% in urine samples. HPV 16 is the most prevalent in both samples with 56.8% in cervical swab and 54.6% in urine sample followed by HPV 18 (5.8%) in cervical swab and HPV 18 and HPV 39 (6.2%) in urine samples. Multiple infection rate (infection more than one type of HPV) was 22.4% in urine samples and 32.0% in cervical swab. The agreement in the detection of HR-HPV between urine and cervical samples was moderate with a kappa value of 0.57 at 95% CI. Using the cervical HPV results as a reference, the anaylitical sensitivity of urine HPV testing was 88.4% (76/86) and specificity of 76.5% (13/17) and ROC area of 0.82 with (0.7-0.9) 95% CI. The Prevo-check HPV16 L1 antibody test has detected antibody from seven patients have a low clinical sensitivity but specificity of 100%. Of 93 histology result; 69.9% of the participants were diagnosed with SCC. HR-HPV detected in 76.2% and 79.7% from cervical and urine samples.

Conclusion: In a country with low cervical cancer screening uptake collection of urine specimen can be considered as an alternative sample since the sample is easy to obtain, showed good diagnostic performance and may increases uptake of cervical cancer screening in Ethiopia. HPV16 and 18 were the predominant HPV detected from women with CIN2+ and above patients.

Keywords: Human papillomavirus, Urine, Cervical cancer, TASH

1 Introduction

Human papillomaviruses are a group of small, non-enveloped, double stranded DNA, tumorigenic viruses that belong to Papillomaviridae family. Newly emerging HPV types are being added to the list continuously which resulted in vast and diverse family (De Villierss *et al.,* 2004). To date, more than 200 HPV types has been enumerated and grouped in two five major genera; Alpha papillomavirus, Beta papillomavirus, Gamma papillomavirus, Mu papillomavirus and Nu papillomavirus based on the nucleotide sequence of L1 capsid protein (Moody, 2017b) (Bzhalava *et al.,* 2015, Doorbar *et al.,* 2015).

HPV is sexually transmitted viral infection that spreads widely among sexually active population (Mboumba Bouassa *et al.,* 2017) with global incidence of 570,000 cases per year in women and 60,000 cases in men (de Martel *et al.,* 2017). HPV characterized by epithelial tropism (Egawa *et al.,* 2015); that can infect skin and epithelial cells and caused benign infection and sometimes it progresses into malignancy (cervical, anogenital and oropharyngeal cancers) (Morshed *et al.,* 2014, Zheng and Baker, 2006).

HPV are classified into two subgroups; High-risk and Low-risk group based on the degree of oncogenicity capacity (Morshed *et al.,* 2014). Low-risk HPV (Lr- HPV) are mostly responsible for genital warts. This group includes genotypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81, while genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 72, and 82 (probable) are High risk HPV (HR-HPV) with HPV16 and 18 alone contributing for 73% of all cervical cancer cases (de Martel *et al.,* 2017, Ogembo *et al.,* 2015, Ciapponi *et al.,* 2011) (Van Keer *et al.,* 2017, Cheng *et al.,* 2018).

Most HPV infections are transient and asymptomatic or subclinical with slow progression from precancerous lesion to cervical malignancy through cascade of pathological change that can be detected from exfoliated cervical cells (Andrew W. Hahn 2018). The clinical presentation of premalignancy stages ranges from cervical intraepithelial neoplasia (CIN) grade 1, 2, 3 (CIN1, CIN2, CIN3) with increased severity of dysplasia. CIN1/2 lesions often spontaneously regress after being cleared by the immune system following an HPV infection. On the other hand, individuals who advanced to CIN2/3, are at a high risk of developing cervical cancer (Ashrafi and Salman, 2016) (Cheng *et al.,* 2018, Georgescu *et al.,* 2018).

Persistent infection with HR-HPV is required for the development of cervical cancer (Racey *et al.,* 2013, Ashrafi and Salman, 2016, O'Leary *et al.,* 2011b, Tsakogiannis *et al.,* 2017). Early detection of precancerous lesions allows for rapid intervention. Even if, screening of cervical cancer was solely based on cytological examination of exfoliated cervical cells in most countries, it declines significantly cervical cancer incidence and mortality (Racey *et al.,* 2013). However, cytology-based screening is more subjective and relatively insensitive. As a result of this major drawback, there is an urge to replace it with molecular tests like HPV testing or other adjunct markers (Chan *et al.,* 2012, Salazar-Pina *et al.,* 2016).

Screening tests based on HPV DNA testing is more reliable and efficient as compared to other tests like cytology and a negative HPV test provides high reassurance against precancerous lesions for at least 5 years than a woman with negative cytology (Orang'o *et al.,* 2020, Tranberg *et al.,* 2018). Urine HPV DNA testing has been present as alternative screening test (Nilyanimit *et al.,* 2017). In the last decade, the use of urine to identify biomarkers for cervical screening has been closely scrutinized and are likely to yield high-throughput and reproducible results as of cervical swab samples. In addition to that urine sample allows self-collection and pose noninvasive strategy. So, it increases the uptake of the screening of test and helpful to collect samples from hard-to-reach population (Sahasrabuddhe *et al.,* 2014a) (Van Keer *et al.,* 2021).

Various HPV proteins are expressed and released during the virus's life cycle and the progression to malignancy, and they can elicit a host humoral immune response (Combes *et al.,* 2014). Since the L1 gene is the most conserved in the genome and immunogenic (De Villierss *et al.,* 2004), antibody produce against the L1 major capsid protein is thought to be a predictor of accumulated HPV exposure (Salazar-Pina *et al.,* 2016) and help to design serological assay as well as in the development of prophylactic vaccines (Chan *et al.,* 2012, Weiland *et al.,* 2020).

Therefore, we conducted this research to investigate the prevalence of HPV among bio-specimens for Cervical cancer screening and triage of HPV-infected women, including determining the concordance among the tripod screening methods. cervical swab, urine and blood samples were collected at Tikur Anbessa specialized hospital. Papanicolaou stain (PAP smear) was used to determine the cervical cell abnormality. Total DNA was extracted from cervical swab and urine samples using Qiagen DNA Blood Mini Kit and HPV detected and amplified using Anyplex ™ II

HPV28 on real-time PCR. Serum sample separated and stored at -20^{0}c was used to detect antibody against HPV16L1.

1.1 Statement of the problem

Human papillomavirus (HPV) is the main etiological factor of almost all cervical cancer which is the fourth most common cancer in women globally, with an estimated 640,000 new cases and 342,000 deaths in 2020 (GLOBOCAN). Majority (more than two thirds) of the global burden diagnosed in the developing countries, where in 2020, the top 23 countries with the highest rate of cervical cancer found in sub-Saharan Africa, Melanesia, South America, and South-Eastern Asia. Sub-Saharan Africa has the highest regional prevalence and mortality, with rates elevated in Eastern Africa (Sung *et al.*, 2021)

In Sub-Saharan Africa with an estimated 75,000 new cases and 50,000 deaths caused by cervical cancer yearly (Mboumba Bouassa *et al.*, 2017). Ethiopia, being in the region of Sub-Saharan Africa with 31.5 million women aged 15 years and older who are at risk of developing cervical cancer. Current estimate indicates that, cervical cancer ranks as the 2nd leading cause of female cancer in Ethiopia with an estimated 6294 new cases and 4884 deaths in 2018 (Bruni L et al). Despite this, cervical screening program only covers 0.8% female population (Abate, 2015), (Bruni L et al 2019).

In contradiction with this amount of burden of the disease, there is only one diagnostic and cancer treatment center, TASH for nearly 120 million people. In addition to this, patients present lately with advanced stages in which multiple treatment modalities may involve. This creates a long waiting list and financial incapability which results in mortality. Furthermore, women are apprehensive about engaging in cervical screening out of modesty, fear of pain, or cultural factors. Cancer screening that involves pelvic examination must be customized and validated in the context of our society and women's educational status. So, implementing sensitive screening modalities combined with easy sampling methods with minimal pain or discomfort such as self-sampling of urine samples as alternative sample could help to increase the numbers of women who are screened for cervical cancer.

1.2 Significance of the study

Timely screening or HPV detection is more protective against invasive cervical cancer. However, many women do not participate in screening at regular intervals because national cervical cancer screening program only covers less than 1% of the female population in Ethiopia. In addition to this, conventional cytology is being used as the standard screening tests used for the detection of cervical cancer in the country which involve women to have a pelvic examination. This might result in low uptake in the cervical screening program. So, use of urine samples helps to overcome these issues by allowing a screening sample to be taken in the privacy of the woman with its safe, minimal pain and ease of use method of collecting cervicovaginal fluid along with urine specimens without discomfort. In addition to this, detection of HPV in urine sample would also offer a more accessible and acceptable method. Moreover, we did a proof of concept to a new serology assay to detect serum antibodies against HPV16 capsid protein L1. Therefore, evidences from this study will help our society and policy makers by providing pragmatic evidence on acceptable, appropriate and cost-effective method of cervical cancer screening in Ethiopia while it is based in HPV DNA testing.

2 Literature review

2.1 Virology and genome organization of Human Papillomavirus

Human papillomaviruses (HPVs) are small, non-enveloped with icosahedral capsids, double-stranded, with closed circular DNA genomes (Longworth and Laimins, 2004). Their genomes are approximately 8 kilobase pairs (kb) in size (De Villierss *et al.,* 2004), which comprises eight or nine open reading frames (ORFs) (Doorbar *et al.,* 2015) that are located on a single DNA strand (Longworth and Laimins, 2004) that are covalently closed circular, encoding eight genes, from the E1, E2, E4, E5, E6, E7, L1 and L2 open reading frames (ORFs). The genome is wrapped around nucleosomes and coated by a 60 nm diameter T = 7 capsid (Ibeanu, 2014).

ORFs (Open Reading Forms) are classified into early, late regions (Doorbar *et al.,* 2015) depending on the time of viral DNA replication occurrence (Morshed *et al.,* 2014). Despite their small size genome, all papillomaviruses contain well-conserved core genes. The early fragments E1 and E2 which involved in transcription and replication; E4 is structural protein that involves in the thickening and koilocytosis of layers of epidermidis. In addition to that E4 used as biomarker in diagnosis and disease staging since it expressed in gross in cell undergoing viral amplification. E5 protein involves in DNA replication and cellular transformation. It also circumvents the immune system not to able recognize the infected cells. E6 and E7 proteins are crucial in the malignant transformation by impairment of the control of cell cycle regulation and cell maturation. E6 binds to P53 which results in the proteolysis of P53 and degrades all of its functional activity, which enable the virus to replicate enormously. E7 connects to pRb protein and results in its degradation and that leads to loss of control of the cell cycle. Expression of these oncoproteins is controlled by E2, YY2 which is host cellular protein and some inflammatory cytokines (Morshed *et al.,* 2014, Ibeanu, 2011, Georgescu *et al.,* 2018). The two late fragments L1 and L2 compose the viral capsid are the conserved regions, whereas the early oncogene fragments E5, E6 and E7 have great diversity (De Villierss *et al.,* 2004).

In a functional sense, the HPV genome is organized into three regions. The first is a non-coding upstream regulatory region (URR) or long-control region (LCR) with a regulatory function for the transcription of viral genes E6 and E7; representing about 10% of the genome. The second is an early region (E), consisting of six ORFs: E1, E2, E4, E5, E6 and E7; participating in viral replication and oncogenesis and account for 50% of the genome. It lacks an E3 ORF indicating an

initial BPV1 genome sequencing error (Doorbar *et al.*, 2015). The third one is a Late (L) region encoding the structural proteins L1 and L2 representing 40 % of the genome (Fernandes and de Medeiros Fernandes, 2012) (Morshed *et al.*, 2014).

There is no enzymes, lipids and saccharides found within the Papillomavirus structure. The virus is stable at pH 3–7, inactivates at 70^0 C, and is killed at temperatures above 50^0 C after 30 min. It resists solvents, acids, and X-rays. Virus infection generally leads to cell destruction; however, it may also cause cell transformation and tumor development (Morshed *et al.*, 2014).

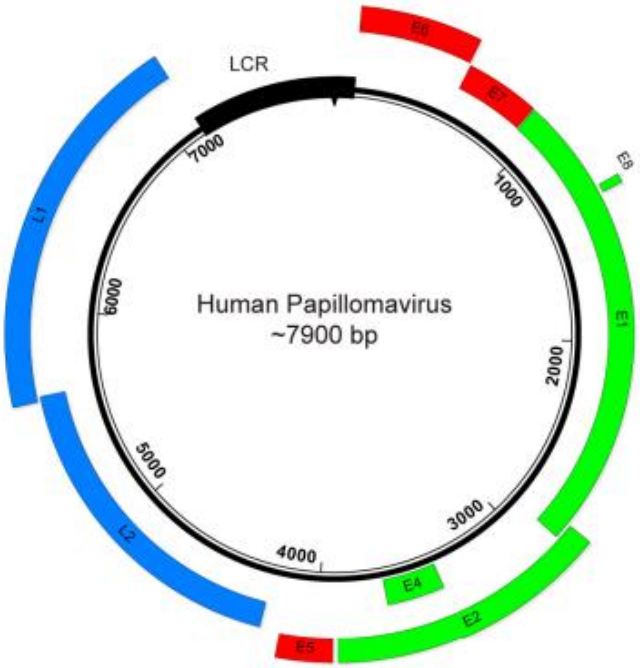


Figure 2-1: Genetic map of HPV (Spurgeon and Lambert, 2017)

2.2 HPV infection

Human papillomaviruses have a predilection for different types of epithelial cells (Fernandes and de Medeiros Fernandes, 2012) (Stanley, 2012). They have a characteristic of epitheliotropic (Morshed *et al.*, 2014). Skin injuries and abrasion, micro-injuries of the epidermis and the mucous membrane are the way for the virus to enter into the body and infect the basal stem cell subsequently cause a lesion (Doorbar *et al.*, 2015). Vertical transmission from mother to child during childbirth is also possible (Morshed *et al.*, 2014).

Cervical transformation zone (TZ) which is located between the squamous epithelium of the ectocervix and the columnar epithelium of endocervix are more susceptible to HPV infection. The basal layer of TZ have fewer overlaying layers of cells and it retains the ability to differentiate which is suitable for the HPV virion production. So, this makes TZ as the site for the initiation of cervical cancer (Fernandes and de Medeiros Fernandes, 2012).

Researches indicate that 90% of genital HPV infections are asymptomatic and cleared within two years. The incubation period varied from 3 weeks to several months for genital warts and several months to years for cervical cell abnormalities. Following the acquisition of HPV, the median period of cervical HPV infection (measured by detection of HPV DNA) is approximately one year. The possible progression of cervical cancer is a complex process with a range of potential outcomes including immune-mediated cervical HPV clearance, progression to the precancerous lesion, precancerous lesion regression, a progression of precancerous lesions to cervical cancer with invasion of local tissue (Andrew W. Hahn 2018).

2.3 Life cycle of HPV

HPVs infect poorly differentiated, primitive epithelial basal lamina to which it is believed that they get access through micro-wound that exposed the basal layer of epithelium (Spurgeon and Lambert, 2017). The receptor for virus entry is vague however it is thought that it binds to heparin sulfate proteoglycan (HSPG) (Longworth and Laimins, 2004; Moody, 2017a; Deligeoroglou *et al.,* 2013) that results in conformational change of the virion that expose the proprotein cleavage of L2 at the amino terminus (Egawa *et al.,* 2015). Up on entry, the initial phase of virus amplification referred 'establishment replication' takes place and amplified viral copy number would be 50-100 viral genome per cell (Moody, 2017a; Pinidis *et al.,* 2016). E1 and E2 viral replication proteins are essential for this initial amplification (Egawa *et al.,* 2015; Doorbar *et al.,* 2012). When infected cell leaves the basal lamina and enters the transit amplifying proliferative compartment of the epithelium, viral episomes or plasmid maintained at constant copy number and viral gene expression is minimal. In this stage of the virus life cycle, the expression of oncoproteins E6 and E7 are under control and very minimal which result in undetectable level (Pinidis *et al.,* 2016). As infected daughter cells reaches terminal differentiation exiting the cell cycle, there is viral gene expression and production of viral DNA to many thousands of copies per cell followed by high expression of early genes E6 and E7 and expression of late genes that results in the release of HPV

progeny from superficial layers of the terminally differentiated stratified epithelium (Spurgeon and Lambert, 2017).

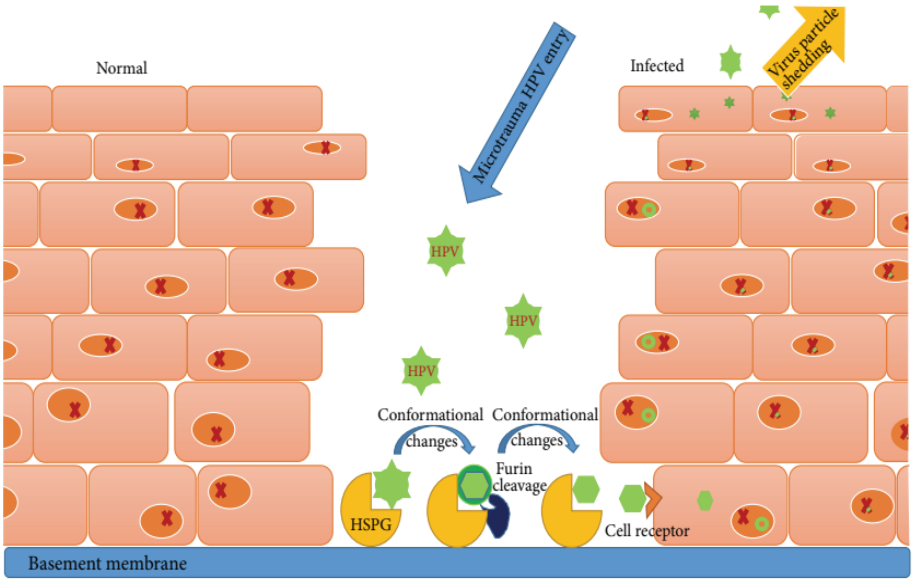


Figure 2-2: Microtrauma allows HPV to enter the cervix's basement membrane. Interaction with HSPG via the L1 protein causes conformational changes on the virus capsid, accompanied by furin cleavage and the unveiling of the cell receptor binding site (Deligeoroglou et al., 2013)

2.4 Immune evasion mechanism

HPV's ingenious method in which viral DNA replication and virus assembly occur in a cell that will be terminally differentiated and die by natural causes is the main immune evasion mechanism of HPV (Pinidis *et al.*, 2016). These are some of the mechanisms that HPV used for immune evasion.

1. The infection and vegetative growth of HPV are extremely dependent on the program of the target cell, the keratinocyte. The differentiation of keratinocytes from basal cell to terminally differentiated superficial squamous takes about 3 weeks, which is the same as the time for the infection to virus release. As a result of this, the progeny viruses wait and released naturally without triggering cellular damage (Stanley, 2012).
2. There is no cytolysis or cytopathic effect as a consequence of virus replication and assembly and as a result of this no inflammation. There seems to be a little to no proinflammatory cytokine release, which is essential for antigen-presenting cells (APCs)

activation and movement into the local environment. Thereby immune surveillance and antigen-presenting cells can't locate and induce an immune response (Chan *et al.,* 2012).

3. HPV infection is local and exclusively an intraepithelial, there is no viremic phase. Therefore, the virus remains in the host for many months, even years, during which the mechanisms of host defense remain ignorant of the pathogen for a long period and only small amounts of viruses are exposed to immune defenses (Stanley, 2012, Deligeoroglou *et al.,* 2013).
4. HPV downregulates the synthesis of interferon. Though the immune system plays an important role in infection clearance and localized cellular immune response, papillomavirus infection does not lead to a robust antibody response (Chan *et al.,* 2012, Stanley, 2012).

2.5 Human Papillomavirus Vaccines

As HPV is identified as an etiologic agent for the occurrence of cervical cancer and its associated disorders (Cheng *et al.,* 2018), different strategies have been developed to prevent or therapeutically treat HPV infections. Currently there are three licensed HPV prophylactic vaccines: **Cervarix**, a bivalent HPV-16/18 (GlaxoSmithKline Biologicals, Rixensart, Belgium); **Gardasil**, a quadrivalent HPV-6/11/16/18 and **Gardasil 9**, a nonvalent HPV-6/11/16/18/31/33/45/52/58 vaccine, both from (Merck, Whitehouse Station, NJ, USA). (Cheng *et al.,* 2018) (Pinto *et al.,* 2018). These preventive vaccines block the entry of HPV into the epithelium of cervix targeting major capsid proteins (L1) by generating HPV-specific neutralizing antibodies through the use of virus-like particles. However, these vaccines were not able to control established infections or lesions because they did not target an antigen that is expressed in HPV related malignancies and their precursors lesions (Lin *et al.,* 2010). There is therefore an urgent need to develop effective therapies for established HPV infections and associated diseases.

Other alternative form of treatment includes therapeutic vaccines which induce cell mediated immune response unlike the prophylactic vaccine that generates neutralizing antibody (Yang *et al.,* 2016, Cheng *et al.,* 2018). Therapeutic vaccine targets tumor specific antigen, HPV E6 and E7 due to several reasons. They are constitutively expressed in malignant cells and critical in cellular transformation. In addition to that, these oncoproteins are only present on tumor cells therefore subvert the problem raised with related to immune intolerance (Hung *et al.,* 2008, Lin *et al.,* 2010).

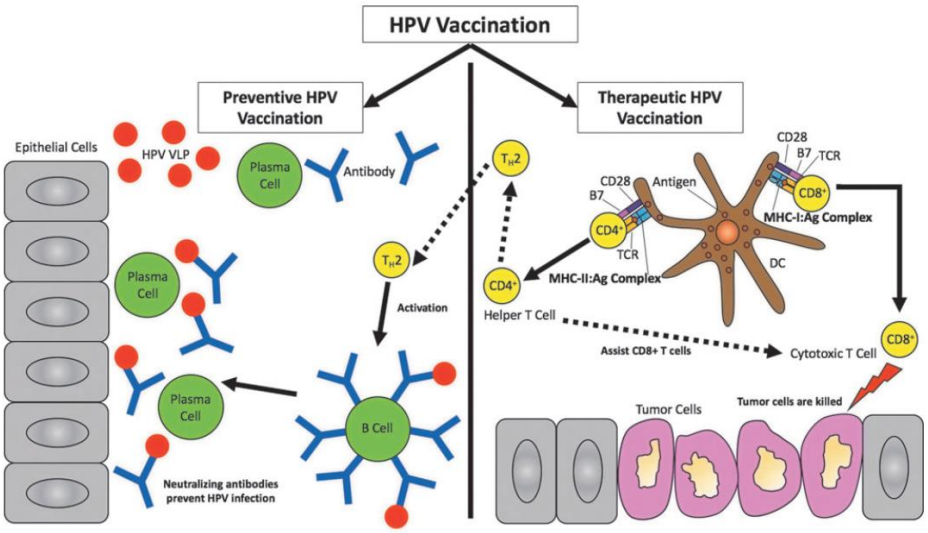


Figure 2-3: HPV vaccination types (Cheng et al., 2018)

2.6 Clinical manifestation

A number of clinical manifestations might appear that depends on the type of HPV infected, the host immune response and the predilection of the virus.

2.7 Laboratory Methods for Detection of Human Papillomavirus Infection

Human papillomavirus replication is completed only in the differentiated epithelial cells. So, this makes it difficult for the isolation of the virus from clinical samples. Papillomavirus growth has been achieved only in an organotypic "raft" culture, a specialized primary human keratinocyte-derived cell culture system, where epithelial cells are grown on semi-solid agar to allow epithelial cell differentiation and thus productive replication of papillomaviruses. Even with this method, only HPV 11 and 31 growths were successful (Chan *et al.*, 2012) hence, in most cases, it's accurate identification relies on molecular biology techniques (Villa, 2009). Nowadays, technology advances enable the development of high throughput HPV assays including DNA-based, mRNA-based, high-risk group-specific and type-specific methods. In general, HPV assay classified into morphological based assay, immunohistochemical staining and nucleic acid tests (Zheng and Heller, 2020).

2.7.1 Morphology based test

Progression of HPV infection to cervical cancer is slow and it's results in a pathological change of epithelium which can be recognized from exfoliated cells (Chan *et al.,* 2012). These pathological changes could be squamous cell irregularity, perinuclear clearance, enlarged nuclei with irregular nuclear contour, and cells are often binucleated with variation in nuclear size and increased cytoplasm density (Coutlée *et al.,* 2005). Detection of precancerous lesions at the early stage enables to intervene promptly. The possible specimens could be conventional pap smear, liquid-based cytology, cervical biopsy or surgical specimens. The major drawback for this test is high interpersonal variation and subjective. In addition to that it has low specificity and sensitivity (Zheng and Heller, 2020).

Most laboratories use a standard set of terms known as 'Bethesda Method' to report cytology result. Samples with no cell defects are graded as "negative for intraepithelial lesion or malignancy" in the Bethesda System. Certain benign results, such as common infections or inflammation, can be noted as negative Pap test. The findings of a Pap examination will also reveal the adequacy of the clinical specimen.

Squamous cell and glandular cell defects are treated differently by the Bethesda System. Squamous cell disorders are categorized into the following groups, which vary in severity from moderate to severe. The most frequent reported abnormality in Pap tests is atypical squamous cells (ASC). This category is split into two categories by the Bethesda System: ASC-US and ASC-H:

- **ASC-US** stands for atypical squamous cells of uncertain significance. The cells don't seem to be fully regular, but the reason for this is unknown. The cells modification either could be results from an HPV infection or as a result of other factors.
- **ASC-H** is an acronym for atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion. Lesions that are ASC-H are more likely to be precancerous than lesions that are ASC-US.
- **LSILs,** are low-grade squamous intraepithelial lesions caused by infection of HPV. LSILs, frequently regress to normal particularly in younger women, as the immune system combat the infection.

- **HSILs** are high-grade squamous intraepithelial lesions that are serious anomalies and, if left unchecked, are more likely to progress to cancer.
- **CIS** (Carcinoma in situ) is a term used to describe abnormal cells that mimic cancer cells that reside on the cervix's surface and have not infiltrated further or metastasize beyond the cervix.
- **SCC** is squamous cell carcinoma. The defective squamous cells have spread further into the cervix, as well as other tissues and organs.

Glandular cell anomalies are results from abnormal change in the glandular tissues of the cervix. These anomalies are classified by the Bethesda scheme into the following categories:

- **AGC** (Atypical glandular cells) are glandular cells which don't appear to be normal
- **AIS** (Endocervical adenocarcinoma in situ) refers to cells that are significantly defective but have not extended beyond the cervix's glandular tissue.
- **Adenocarcinoma** comprises cancer of the endocervical canal, endometrial, as well extrauterine and other cancers. (Wilbur)

2.7.2 Visual inspection using acetic acid

VIA-based screening is straightforward, efficient, and cost-effective, and the results are available right away but it has been reported to show inter-observer variation (Verma *et al.,* 2020). To visualize the cervix, a self-retaining speculum implanted under good lighting and aseptic precautions. After that, the cervix smeared with 5% acetic acid and examined for one minute. Any aceto-white lesion in the transition zone that touched the current Squamo-columnar junction was considered and identified as positive and reported as VIA positive or VIA negative accordingly. (Bhattachan *et al.*, 2019; Shrestha and Joshi, 2020).

2.7.3 Immunohistochemical Staining Tests

P16 immunohistochemistry along with Ki-67 is used as a biomarker in tissue specimens and cervical smears to identify high-risk HPV infections with likelihood of developing into cervical cancer (Zheng and Heller, 2020). $P16^{INK4a}$ is a tumor suppressor and cyclin-dependent kinase (cdk) inhibitor that inhibits E2F-dependent transcription and cell cycle progression by blocking pRb phosphorylation by cdk4 and cdk6. The functional inactivation of pRb by HPV E7 early protein, allows $p16^{INK4a}$ to be overexpressed and accumulated in cells throughout all high-grade lesions

(Verma *et al.,* 2020). Ki-67 is cellular proliferation marker which is non-histone nuclear protein that expressed in all stage of cell cycle. Under normal physiological condition cellular proliferation marker Ki-67 and tumor suppressor p16 are not expressed in same cervical epithelial cell at a time. Therefore, dual staining of P16 and ki-67can be used as a marker to predict HR-HPV cellular transformation and the presence of high-grade cervical lesions by using antibodies against p16 and Ki-67 (Yu *et al.*, 2019).

For many factors, p16INK4a has been successfully used to characterize HPV-related disease; (1) $p16^{INK4a}$ expression is independent of HPV type, (2) $p16^{INK4a}$ expression is directly related to HPV oncogenicity, since continuous expression of E7 is needed to preserve the malignant phenotype, (3) Cycling cells' expression of $p16^{INK4a}$ is a particular marker for HPV-E7 increased expression or other factors that inactivate Rb As a result, $p16^{INK4a}$ serves as a biomarker for the diagnosis of precancer and cervical lesions (Hwang and Shroyer, 2012).

$p16^{Ink4a}$ Immunocytochemistry is done using Rabbit Polyclonal Antibody. The smear soaked in 3% hydrogen peroxide in methanol (hydrogen block) for 30 minutes and then washed with Tris buffer three times. 5% milk block is used for blocking unspecific protein binding and rewashed again using Tris buffer for three times more. The primary antibody which is ($p16^{Ink4a}$) added to the tissue and incubate it overnight at 4^{o}c. Rewashed again with Tris buffer three times. Horse radish peroxidase (HRP) primer added and incubated for 60 minutes. Tris buffer used to rewash again for additional three times. On the slides, Diaminobenzidine (DAB) applied, and the reaction will be observed under a microscope. As soon as crisp golden brown nuclear staining was visible, the slides will be submerged in water. Hematoxylin used to counterstain the slides and then dehydrated in a series of graded alcohol solutions. Following that, the slide passed through xylene, which served as a clearing agent and being ready for examination. Brown stain of cytoplasm taken as positive and signaling P16 expression (Verma *et al.,* 2020).

2.7.4 DNA-based assays

The majority of HPV diagnostics have relied on DNA detection, for which signal and target amplification approaches are available. There are many commercially available tests; (Ikenberg, 2014). Table 2.1 summarizes the overall HPV DNA tests available.

Table 2-1: DNA based HPV assays

HPV Test name	Company name	Regulatory Status	Type of Assay	HPV Types Detected
Cervista HPV HR	**Hologic GEN-PROBE**	FDA approved	Signal Amplification detects 14 high-risk types. (Invader method/ Fluorescence resonance energy transfer FRET)	14 high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
Cervista HPV 16/18	**Hologic GEN-PROBE**	FDA approved	Signal Amplification detects and differentiates HPV 16 and HPV 18. (Invader method/ Fluorescence resonance energy transfer FRET)	HPV 16; HPV 18
APTIMA HPV	**Hologic GEN-PROBE**	FDA-approved for ThinPrep	Transcription-Mediated Amplification (TMA) of mRNA E6/E7; amplicon with Hybridization Protection Assay (HPA)	14 high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)
AMPLICOR HPV test	**Roche**	CE marked (not available in US)	Target amplification. (PCR/ Multiwell Plate hybridization, enzymatic color reaction)	13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68)
LINEAR ARRAY HPV Genotyping Test	**Roche**	NA RUO	L1 consensus PCR with Line-blot assay	37 HPV genotypes. Individual types
Cobas HPV test	**Roche**	FDA approved CE marked	Target amplification. Simultaneous detection of 14 high-risk types + specific genotyping information for types 16 and 18. PCR/ The detection of the amplicon is performed during thermal cycling using oligonucleotide probes labeled with four different fluorescent dyes	Channel 1 - HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 Channel 2 - HPV 16 Channel 3 - HPV 18
Xpert HPV	**Cepheid**	CE marked	E6/E7 target amplification multiplex PCR	14 HR types(16,18,31,33,35,39,51,52,56,58,59,66 and 68)
BD HPV Onclarity Assay	**BD**	CE marked	E6/E7 DNA target amplification, real type PCR	14 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)
Digene HC2 (Hybrid Capture 2) HPV DNA test	**Qiagen**	FDA approved	Full HPV genome detection by HC2 compared to PCR targeting L1	13 HR HPV Types 16,18,31,33,35,39,45,51, 52,56,58,59 and 68
HPV genotyping RH and LX kit	**Qiagen**	L1 consensus PCR with reverse Lineblot detection	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	
careHPV test	**Qiagen**	Application submitted for WHO prequalification	Hybrid Capture 2 full-length RNA probes and signal amplification, chemiluminescence	14 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)
OncoE6 Cervical Test	**Arbor Vita Corporation**	CE marked	Monoclonal antibodies in lateral-flow assay format to HPV 16/18 E6.	16, 18
Real TimeHigh-Risk HPV	**Abott Molecular**	CE marked	PCR	14 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)
INNO-LiPA HPV Genotyping Extra*	**Innogenetics**	RUO	SPF Target amplification. Reverse hybridization line probe assay	6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74, 82
HPV DIRECT FLOW CHIP + E-BRID system	**Master Diagnostic (TaKaRa**	reverse dot blot hybridization	HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82	

	Shuzo Co. Ltd.)			
PapilloCheck HPV Screening	**Greiner Bio-One**	CE marked	PCR, amplicon hybridization on slide for type-specific detection	24 HPV types (6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82
PapilloCheck High-risk	**Greiner Bio-One**	CE marked	PCR, amplicon hybridization on slide for type-specific detection	14 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)
HPV4A ACE Screening kit	**Seegene**	CE marked	PCR, Gel Electrophoresis	HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82
Anyplex II HPV28	**Seegene**	CE marked	multiplex real-time PCR with proprietary detection system	Hr: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82 Lr: 6, 11, 40, 42, 43, 44, 54, 61, 70
Multimetrix Multiplex HPV Genotyping Kit	**MIKROGEN (Germany) also Progen/Multimetrix**	Gp5+/6+ consensus PCR, Luminex	HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82	
CLART HPV 2	**Genomica (Spain)**	?CE marked	mulit-plex PCR with visualization in low density arrays	35 low risk and high risk types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 83, 84, 85, 89)
GenoFlow HPV Array	**DiagCor (Hong Kong)**	PCR, flow-through hybridization (cassette)	16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66/68,73, 82 6, 11, 40/61, 42, 43/44, 54/55, 70, 57/71, 72, 81, 84/26 plus universal HPV	
fHPV Typing	**molGENTIX (Spain)**	CE marked	15 low risk and high-risk types	HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
Trovagene Urine based HPV test	**Trovagene & Strand Life Sciences (India)--kit under development, test currently performed by Trovagene laboratory**	PCR	HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	
13 High-risk HPV Real Time PCR kit	**Hybribio**	multiplex real-time PCR	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	
PreTect HPV-Proofer	**NorChip AS**	Multi-plex NASBA for HPV E6/E7 mRNA	HPV 16/18/31/33/45	
CERVIMAX IHC/WB/FC/IC	**VALDOSPAN GmbH**	Antibodies E7	specialized kits for IHC, WB, FC, IF applications	
NucliSENS easyQ	**Biomerieux**	CE-IVD	NASBA amplification, real-time detection with molecular beacon probe	16, 18, 31, 33, 45
PapType	**Genera Biosystems**	PCR with labeled nucleotides on micro beads	6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	

(https://www3.paho.org/hq/index.php?option=com_content&view=article&id=11925:hpv-tests-for-cervical-cancer-screening&Itemid=41948&limitstart=1&lang=en) Accessed on May16,2021

2.7.4.1 Human Papillomavirus detection from urine sample

HPV testing using urine sample for collecting cervicovaginal samples can be offered as an alternative strategy for women cervical cancer screening programs. It has been approved as tool for screening cervical cancer because it has relatively high sensitivity and specificity as referenced from cervical swab (Van Keer *et al*., 2017). A recent meta-analysis by reported a pooled sensitivity and specificity for detecting any HPV in urine were 87% and 94% respectively, when results from cervical samples were used as a reference (Pathak 2014). Moreover, mucus and debris from exfoliated cells from the genital tract (particularly the cervix) that line the urethra opening and small labia and are washed away with the initial urine flow. It hence follows that the initial flow of urine (first-void) collects most of this debris. However, there is variation in urine collection and testing methods. Use of first void urine, midstream or random has been identified as the source of this heterogeneity. Moreover, ultrafiltration and subsequent DNA extraction, usage of DNA preservative and use of first-void urine has been proved to yield greater number of HPV DNA-positive samples (Vorsters et al., 2014; (Van Keer et al., 2017). So, it is recommended that usage of first-void urine to secure a standardized urine fraction; no extensive washing of genitals before urination prior to collection of other cervicovaginal self-samples not to reduce the amount of human/HPV DNA originating from the (exfoliated) cells from the cervix; storage and extraction by immediately mixing the first-void urine with a conservation medium; preferentially prefilled in the collection vial; processing a sufficient amount of first-void urine to maximize the amount of HPV DNA identified from urine sample (Van Keer et al., 2017).

2.7.5 Human Papillomavirus Ribonucleic Acid Testing

HPV RNA testing is preferred to overcome some limitations that is related with the diagnosis of HPV since oncoproteins E6 and E7 are expressed abundantly in the course of infection. However, RNA analytic for the diagnosis of HPV is becoming difficult because of the general instability of mRNA, expression of these oncoproteins in the non-transforming (non-productive) state of HPV and subsequent transcription in non-neoplastic lesions (Ikenberg, 2014).

2.7.6 Electron microscopy

In productive HPV infections, nonenveloped icosahedral viral particles can be seen, but electron microscopy cannot be used to genotyping them. High-risk HPV types which cause non-productive precancerous are undetectable using this method (Coutlée *et al.*, 2005).

2.8 Risk factors for the acquisition of Human Papillomavirus

Many researchers report stated a number of risk factors that contributed for the acquisition of Human Papillomavirus and development of cervical cancer. Not only infection with HPV contribute for the progression of cervical cancer but also other risk factors such as number of sexual partners, early sexual debut, number of pregnancies, immune related diseases, other sexually transmitted infection, human immunodeficiency virus infection, smoking of cigarette, oral contraceptive use might lead to the route of carcinogenesis (Schneider, 1993; Ibeanu, 2011; Chelimo *et al.*, 2013).

2.9 Global and national burden of Cervical cancer

Cervical cancer is the fourth most common cancer in women, accounting for 6.6 percent of all female cancers in 2018, with an approximate of 570,000 new cases. Nearly 90% mortality from cervical cancer occurred in low- and middle-income countries (WHO: cervical cancer). Ethiopia is the second most populated country in Sub-Saharan Africa, with more than 54 million females (https://countrymeters.info/en/Ethiopia Accessed November 14, 2018). Cervical cancer ranks as the 2nd leading cause of female cancer in Ethiopia (www.hpvcentre.net on 27 July 2017) with an estimation of 20.9 million women at risk of contracting cervical in year 2010, with an estimated 4,648 new cases and 3,235 deaths each year (Guideline for Cervical Cancer Prevention and Control in Ethiopia).

Majority of cancer diagnosed lately in Sub-Saharan Africa; patient delay is the prominent factor due to lack of awareness about cervical cancer symptoms, cultural attitude towards seeking medical treatment, health literacy and very limited prevention care. Late-stage cancer associated with poor survival rates. For many women in low-income countries including Ethiopia, treatment modalities like radiotherapy, surgery might be limited, inaccessible and patients must travel many kilometers far away from their homeland.

A systematic review was conducted by Awoke *et al* in 2018 from a total of 859 women to determine the molecular type distribution of human papillomaviruses (HPVs) in Ethiopia. The top five identified genotypes were HPV 16 (45.3%), HPV 52 (9.4%), HPV 18 (8.2%), HPV 58 (6.9%) and HPV 45 (5.2%). Moreover, table 2.2 summarizes HPV prevalence from previous studies in Ethiopia.

Table 2-2: Comparison of HPV prevalence from previous studies in Ethiopia

Sample size	HPV genotyping	Study design	HPV prevalence			Top five genotypes	References
			overall	HR-HPV	LR-HPV		
83	RIATOL qPCR HPV genotyping	Hospital based study	22.7% (15/66)	18.2% (12/66	6.1% (4/66)	51, 31, 58, 68, 6, 67	Haile *et al* 2019
893	Multiplexed genotyping (MPG)	Population based study	23.2%	20.5	10.3	16, 35, 52, 31, 45	Teka *et al* 2021
233	Nested PCR	Hospital based study	69.1%.	83.2%	15.2%	16,35,45,31,56	Wolday *et al* 2018
170	Line probe assay (Inno-LiPA).	Study from paraffin embedded cervical biopsy	93%	93%	13%	16, 52,58,18	Abate *et al* 2013
180	Modified general primer-PCR and Luminex assay.	Study from women employed by Ethiopian airlines in AA	20.6%	12.6%	2.18%	----	Mekuria et *al*, 2020
537	GP5+/6 + − and SPF10-primer based system	Hospital based study		16.0%	3.9%	16, 52, 56, 31	Leyh-Bannurah *et al* 2013

3 Objectives

3.1 General objective

To determine HPV genotype in women with precancerous and invasive cervical lesions (CIN2+) and compare concordance to detect HPV from two bio-specimens (cervical swab and first void urine).

3.2 Specific objectives

1. To determine the circulating HPV genotypes in women with different grades of lesion in TASH gynecology clinic.
2. To identify the most common HPV genotypes detected in urine and cervical samples in women with different grades of lesion
3. To evaluate the performance of human papillomavirus (HPV) DNA testing in urine samples compared to that of cervical sample
4. To determine the detection of HPV L1 Anti-capsid antibody from different grades of dysplasia
5. To compare the performance of HPV testing in two biospecimens against cytology and histology

4 Methodology

4.1 Study area

The study was carried out at Tikur Anbessa Specialized Hospital (TASH), Gynecology out-patient department (Gyn-OPD). TASH is a large referral teaching hospital, under the administration of Addis Ababa University, located in Addis Ababa, Ethiopia. TASH is also the center of excellence for the training of undergraduate and postgraduate students in different disciplines. This hospital is the only central referral hospital and cancer diagnostic and treatment center in the country that provides services for patients from all over the country. Women comprises about 73% of the total patients attending TASH, and cervical cancer is the commonest disease comprising over one-third of all female patients treated (Hailu and Mariam, 2013). The Gynecology Department also provides evaluation, examination, surgical treatment, and screening of new and referral cases of cervical cancer, among other services.

4.2 Study design and period

A hospital-based cross-sectional study was conducted from October 2019 to February 2020.

4.3 Population

4.3.1 Source population

All-new women patients who visited Gynecology OPD during the study period at Tikur Anbessa Specialized Hospital (TASH)

4.3.2 Study population

All women aged 18 years old and above who had cervical complaint at gynecological clinics in TASH.

4.4 Sample size and sampling procedure

4.4.1 Sample size

All new eligible women patients who were attending the cervical cancer screening program from October 2019 to February 2020 were included.

4.4.2 Sampling technique

A consecutive convenience sampling technique was used.

4.5 Inclusion criteria and Exclusion criteria

4.5.1 Inclusion criteria

1. Sexually active women aged 18 years old and above

4.5.2 Exclusion criteria

1. Pregnant women
2. Women who had history of hysterectomy, chemotherapy and radiotherapy
3. Women who had cognitive or physical impairment that prevent them to give informed consent/participation

4.6 Study variables

4.6.1 Dependent variables

- HPV infection
- HPV genotype
- Grade of pre-cancerous lesion

4.6.2 Independent variables

- Age
- schooling
- smoking
- number of sex partners
- condom use
- marital status
- family income
- age at first sexual intercourse
- age at first pregnancy
- a long duration of oral contraceptive use
- other sexually transmitted infections such as HIV, Syphilis

4.7 Sample collection

4.7.1 Socio-demographic data

All eligible women attending the cervical cancer screening program in TASH were informed about the research objective and asked for their permission. After obtaining the consent, basic data collected by attending midwife nurses using a paper-based questionnaire that is adopted from similar studies. The questionnaire request basic demographic (age, schooling, marital status, family income) and social characteristics (tobacco use, alcohol, and illicit drug use), age at first sexual intercourse, number of sex partners (lifetime and in the past year), and clinical data (previous pregnancy, age at first pregnancy, number of children), prior cervical cancer screening, and, contraceptive practice, frequency of condom use, history of sexually transmitted disease to obtain information regarding their sexual habits and other risk factors related to acquiring HPV infection.

4.7.2 Collection of blood sample

5 ml venous blood was collected from each woman and serum was separated for anti-HPV16 L1 antibodies using Abvirus® HPV serological test.

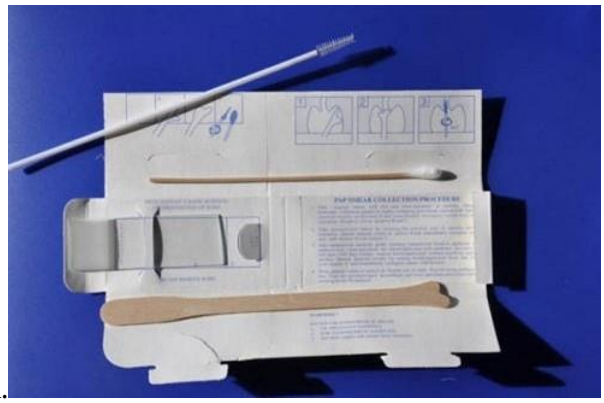
4.7.3 Collecting cervical swab and urine samples

Cervical swabs and urine were collected from each woman on the same day. A urine sample was collected prior to cervical samples as recommended by Munoz et al in order not to disrupt the natural history of HPV infection (Munoz *et al.*, 2013). The first sample, which is First void urine specimen (FVU) was self-collected. The cervical swabs were collected using Evalyn and pap smear brushes by a trained nurse or gynecologist.

4.7.4 Urine sample collection

The study participants asked and given adequate orientation not to urinate at least one hour before collecting the urine sample (www.novosanis.com). They were also asked not to wipe the labia before urinating. Before collecting the cervical samples using Evalyn Brush and Pap swab for HPV genotyping and Papanicolaou test respectively, 20 mL of first void urine was collected using standard urine collection vial (Colli Pee™, Novosanis, Belgium) that is designed to collect the first 14 ml of first-void urine immediately into 7 ml of a urine-conservation medium to avoid DNA degradation and the subsequent urine will outflow to the toilet (Franceschi *et al.*, 2016). The urine sample kept in an ice-cold container immediately after the collection. The urine kept in an ice-cold

container then transferred to the Department of Microbiology, Immunology and Parasitology molecular laboratory, Addis Ababa University, where the extraction of DNA and HPV testing/genotyping performed.

A.  B. 

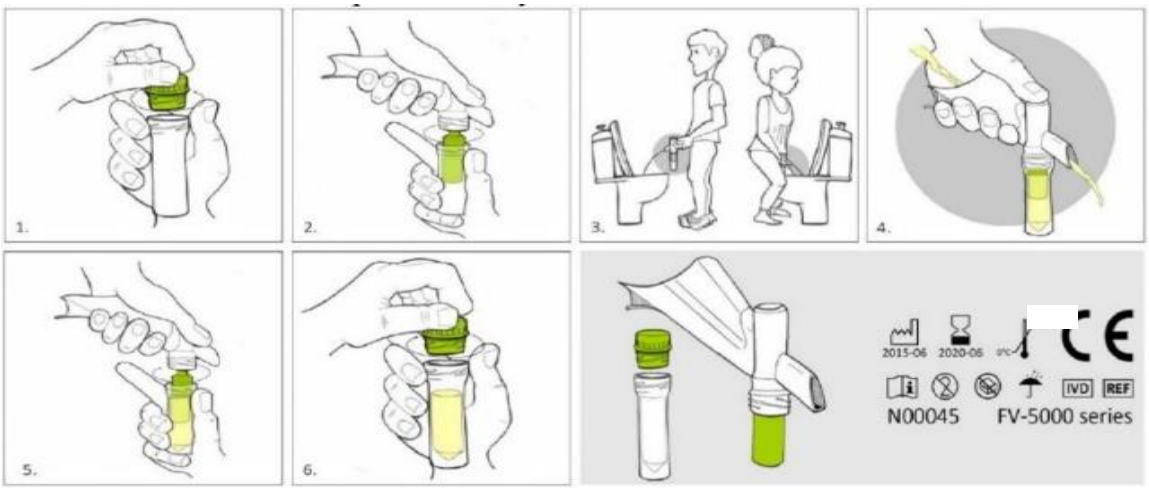
C. 

Figure 4-1: Pap smear Kit ®(A), Evalyn cervical brush (B) and Colli-pee (C) used for collecting cervical swabs and urine respectively.

4.8 Cervical swabs processing

After the urine collection, during a pelvic examination, a Evalyn Brush (Rovers Medical Devices, The Netherlands) and disposable pap smear kit were used for the collection of exfoliated cervical cells.

4.8.1 Evalyn brush

The Evalyn brushes maintained at room temperature until being processed. The Evalyn brushes maintained at room temperature then removed using 2ml Eppendorf tube. Each Eppendorf tube

with the brush inside was filled with1ml of PBS and vortexed vigorously for 1min. The tubes kept at room temperature overnight. The next day, the tubes with brushes vortexed vigorously for 1min and centrifuged for 5min at 2500 rpm. Using the 1000µl pipette tips the brush was removed from the tube carefully and by tipping carefully they being placed in a new 2ml Eppendorf tube and kept at -20^{0}c, if it is needed for future use. The Eppendorf tubes with PBS and cells were used for extracting DNA using QIAamp® DNA Mini Kit (Qiagen).

4.8.2 Pap smear brush

Conventional pap smear was prepared using Ayre spatula and endocervix brush from (Disposable pap smear kit, Human) that ensures hassle free collection as it comes as a collective kit where you can collect fix the samples on to the slide and a transport box which is very convenient to send samples individually. The Pap Smear Kit consist of sterile cytology brush, wooden spatula and cotton swab with glass slide to make the smear on. Then, the smears were preserved in 95% ethanol and kept at room temperature until being processed. The cytology findings were reported following the Bethesda classification (Nayar and Wilbur, 2015).

4.9 Urine sample processing

Upon arrival at the molecular laboratory, the urine sample was stored at -80 °C until being processed. Subsequently, the urine sample was thawed and 10 ml of urine sample was aliquoted in falcon tube and centrifuged at 4,000g for 10 minutes in an Amicon Ultra-4 50 K filter device (Merck Millipore, Belgium) to concentrate all DNA, including cell-free DNA fragments. Centrifugation was repeated for 10 min if the remaining volume on the filter was more than 1 ml (Franceschi *et al.*, 2016). After filtration, 1 ml of phosphate buffer saline (PBS) was added to the filtrate and subsequently transferred to 2 ml Eppendorf tube. The transferred sample then centrifuged at 14000g for 10 minutes and the supernatant discarded until 200 µl ul of lysate being left. DNA extraction then carried out using the Qiagen DNA Extraction kit (QIAGEN, Germany).

4.10 HPV genotyping

The HPV presence and genotype were determined using Anyplex™ II HPV28 detection kit (Seegene, Seoul, Korea). Anyplex™ II HPV28 is a new PCR assay designed for HPV genotyping. It can detect 28 HPV types including 19 high-risk (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70). It

is a multiplex realtime PCR using tagging oligonucleotide cleavage and extension (TOCE) techn ology for simultaneous detection and genotyping of high risk (HR) and low risk HPV types.

The genotyping was performed as recommended by the manufacturer (Seegene, Seoul, South Korea) with 5 µl DNA in each of the two 20-µl reaction mixtures with primer set A or B. It uses HPV-specific dual priming oligonucleotides (DPO) for multiplex (real-time) PCR.

The assay thus distinguishes semi quantitatively 28 HPV genotypes in only 2 reactions by taking advantage of the 5 dyes that can be resolved on the CFX96 real-time PCR instrument (Bio-Rad). The kit provided with positive and negative control. Data recording and interpretation were automated with the Seegene viewer software according to the manufacturer's instructions.

In PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimens. An internal control (IC) is incorporated in to the product as an endogenous whole process control in order to monitor nucleic acid isolation, and to check for possible PCR inhibition. The IC is co-amplified with the target nucleic acids within the clinical specimens.

Figure 4-2: Bio-Rad CFX96 ™ Real time PCR

4.11 HPV 16 L1 Rapid test

The Prevo-check® is a rapid in vitro diagnostic test for the qualitative detection of antibodies against HPV16 L1 in whole blood and serum. The test is diagnostic aid for determining the

HPV16L1 antibody status in the context of the early detection of HPV16 induced carcinomas of head and neck and anogenital region. 25µl of serum mixed with the HPV reagent and allow the mixture to incubate for 10 minutes and then apply 4 drops of the mixture from the tube into the sample opening (S) of the test cassette and observe the result after 10 minutes.

Figure 4-3: Prevo-check® rapid test

4.12 Histology result

Histology was one of the patient standard care method employed for the patients who suspected of cervical cancer in TASH, Ethiopia. Patient hospital number (I care number) registered when collecting basic socio-demographic data was used to collect histology results. The principal investigator collects the histology results from each of study participants medical records using their I-care number.

4.13 Laboratory workflow

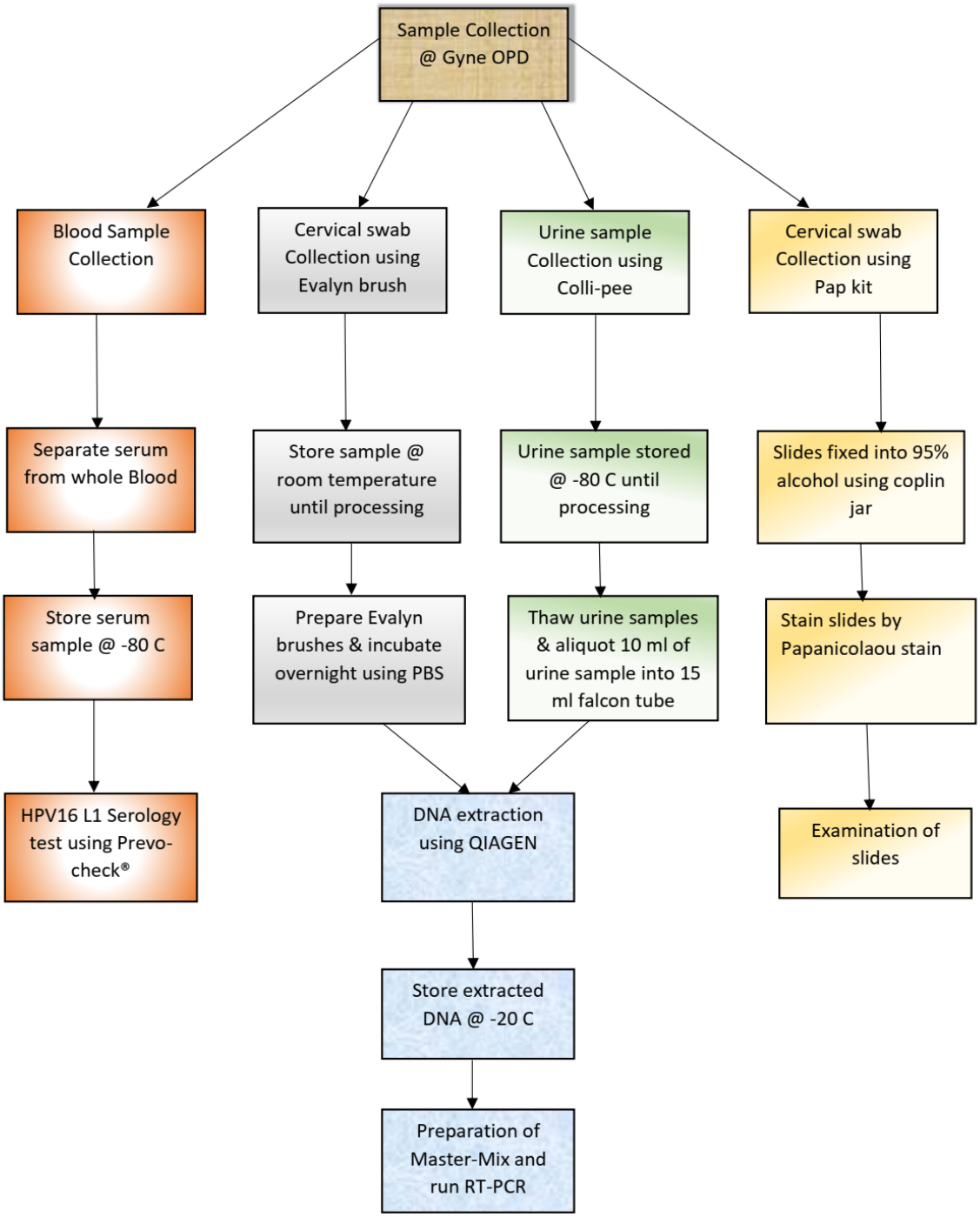


Figure 4-4: Laboratory work flow chart

4.14 Statistical data analyses

Data were cleaned and entered into Excel then exported into SPSS - statistical software program for Windows, version 25.0 for analysis. Frequency tables, graphs and charts were used to display socio-demographic characteristics, overall and type-specific HPV prevalence using the proportion of patients who tested HPV DNA positive for a given HPV DNA type. The agreement rate, kappa coefficient with 95% confidence interval, was calculated to estimate the concordance between the results from urine and cervical samples. P-value was used to calculate differences between paired proportions. The accuracy of urine HPV DNA testing, as measured by sensitivity and specificity, was evaluated using the results obtained from cervical samples as reference. Concordance between test was assessed using the Kappa statistic (Cohen's Kappa) and defined as for "Poor" ($\kappa \leq 0.20$), "Fair" ($0.21 \leq \kappa \leq 0.40$), "Moderate" ($0.41 \leq \kappa \leq 0.60$), "Good" ($0.61 \leq \kappa \leq 0.80$), or "Very good" ($\kappa \geq 0.81$) (Altman, 1990). Categorical variables were compared with categorical variables (such as age groups vs. histological type) using a chi-square test. Alpha level of significance was set at 0.05.

4.15 Ethical consideration

Ethical approval was obtained from the Research and Ethics Review Committee (DRERC) of the Department of Medical Microbiology, Immunology and Parasitology, College of health science Addis Ababa University (Reference no DERC/18/19/01-B). Official permission letter was obtained from TASH. After informed consent was obtained from each study participant adequate explanation about the objective of the study, the benefits and risks were given in a language they understood and inclusion was solely on a voluntary base. This study caused a minimal risk to the study participants as the samples being collected were blood, urine and cervical swabs. Sample collection was performed after adequate information given and demonstration of the collection procedure.

All the data obtained from the research was kept confidential by keeping them locked in the office of the principal investigator.

4.16 Operational definitions

High-risk HPV refers to those samples that were identified to harbor DNA of high-risk HPV genotypes in single or multiple HPV infection with other high-risk HPV types

Low-risk HPV refers to those samples that were identified to harbor DNA of low-risk HPV genotypes in single or multiple HPV infection with other low-risk HPV types

Multiple HPV infection refers to isolation of more than one HPV types from a single sample

Self-employed refers to those women who reported that they run private business-like shop, grocery, restaurant or related businesses.

House wife refers to a woman who raise their children by staying at home

I-care number refers to the patient's identification number that is unique and specific for that particular patient.

5 Result

From a total of 105 women enrolled in this study, two study participants were excluded because one of the cervical samples was not collected form one of the women and detection of HPV DNA from one of urine samples was invalid resulting in 103 paired cervical and urine samples. One sera of patient were not collected which resulted in total of 102 blood sample were tested for HPV16 L1 antibody test.

5.1 Socio-Demographic data

The sociodemographic of the participants is described in Table 5.1. The age range of the participants was from 26 to 80 (mean age: 50.4 years ± 11.9 SD years). The majority of the participants were aged 50 and above (52.4%) followed by 40.8% of women were from the age group of 35-49.

Among the study participants, illiterate (without formal education) rank highest proportion (59.2%; N=61, 49.1%-68.8%, 95%CI), followed by those who attend primary school (16.5%; N=17 9.9%-25.1%, 95% CI) and high school (12.6%; N=13, 6.9%-20.6%, 95% CI).

Regarding the marital status of our participants, most of them were married, 57.3% (N=59, 47.2%-67.0%. 95% CI), followed by widow 15.5% (N=16; 9.1%-24.0%, 95% CI) and divorced 13.6% (N=14; 7.6%-21.8%, 95%CI). About 58.8% (N=60; 48.6%-68.5%; 95%CI) of the participants had started sexual activity before the age of 18. The majority of the participants were pregnant more than four times 82.5% (N=85) and they were low income in status with monthly income less than birr 1500 (52.4%).

Table 5-1: Sociodemographic characteristics of study participants.

Variables		**Total (%)**
Age Group N=103	20-34 Years	7 (6.8%)
	35-49 Years	42(40.8%)
	>=50 Years	54(52.4%)
Residence N=103	Urban	57(55.3%)
	Rural	46(44.7%)

Educational status **N=103**	Illiterate	61(59.2%)
	Read only	2(1.9%)
	Read and write	4(3.9%)
	Primary school	17(16.5%)
	High school	13(12.6%)
	Diploma	1(1%)
	College or University	5(4.9%)
Occupation **N=103**	unemployed	1(1%)
	Self employed	6(5.8%)
	Relative support	1(1%)
	House wife	53(51.5%)
	Merchant	3(2.9%)
	Private employee	2(1.9%)
	Government employee	7(6.8%)
	Farmer	30(29.1%)
Age at first intercourse **N= 102**	< 18 Years	60(58.8%)
	>= 18 Years	42(41.2%)
Marital status **N=103**	Single	3(2.9%)
	Married	59(57.3%)
	Divorced	14(13.6%)
	Widow	16(15.5%)
	Multi-partner	11(10.7%)
Family panning used **N=103**	None	51(49.5%)
	Hormonal	43(41.7%)
	IUCD	9(8.7%)
Number of Pregnancy **N=103**	None	7(6.8%)
	1	1(1%)
	2	4 (3.9%)
	3	6(5.8%)
	>= 4	85(82.5%)

Monthly income N=95	<1500 Birr	54(52.4%)
	1500-2999 Birr	17(16.5%)
	3000-4999 Birr	12(11.7%)
	>= 5000 Birr	12 (11.7%)

5.2 Association of sociodemographic variables with HPV infection

The highest proportion of HPV infection was detected in the age group 35-49 years with prevalence of 90.5% (N=38) from cervical samples and 81% (N=34) from urine samples. Table 5.2 summarizes the HPV infection association with sociodemographic characteristics. Age group, marital status, educational status, occupation, number of pregnancies are significantly associated with HPV infection with p value of 0.007, 0.004, 0.02, 0.00, 0.002 respectively in cervical swab.

Table 5-2HPV infection prevalence and association across with the sociodemographic characteristics

Characteristics		**HPV Infection**				**Total %**
		Cervical Swab		**Urine Samples**		
		N (%)	p	N (%)	p	
Age Group N=103	**20-34 Years**	3(42.9%)	0.007	4(57.1%)	0.375	7 (6.8%)
	35-49 Years	38(90.5%)		34(81%)		42(40.8%)
	>=50 Years	45(83.3%)		42(77.8%)		54(52.4%)
Residence N=103	**Urban**	44(77.2%)	0.055	40(87.0%)	0.042	57(55.3%)
	Rural	42(91.3%)		40(70.2%)		46(44.7%)
Marital status N=103	**Single**	1(33.3%	0.004	2(66.7%)	0.312	3(2.9%)
	Married	54(91.5%)		49(83.1%)		59(57.3%)
	Divorced	8(57.1%)		8(57.1%)		14(13.6%)
	Widow	14(87.5%)		12(75%)		16(15.5%)
	Multi-partner	9(81.8%)		9(81.8%)		11(10.7%)
Educational status N=103	**Illiterate**	56(91.8%)	0.020	52(85.2%)	0.183	61(59.2%)
	Read only	1 (50%)		1(50%)		2(1.9%)
	Read and write	4(100%)		4(100%)		4(3.9%)
	Primary school	12(70.6%)		11(64.7%)		17(16.5%)
	High school	10(76.9%)		8(61.5%)		13(12.6%)
	Diploma	0(%)		1(100%)		1(1%)

	College or University	3(60%)		3(60%)		5(4.9%)
Occupation N=103	**Unemployed**	1(100%)	0.000	1(1.0%)	0.012	1(1.0%)
	Self employed	4(66.7%)		5(83.3%)		6(5.8%)
	Relative support	0(0.0%)		0(0.0%)		1(1.0%)
	House wife	47(88.7%)		41(77.4%)		53(51.5%)
	Merchant	0(0.0%)		0(0.0%)		3(2.9%)
	Private employee	2(100%)		1(50.0%)		2(1.9%)
	Government employee	4(57.1%)		5(71.4%)		7(6.8%)
	Farmer	28(93.3%)		27(90.0%)		30(29.1%)
Age at first intercourse N= 102	**< 18 Years**	53(88.3%)	0.182	48(80.0%)	0.645	60(58.8%)
	>= 18 Years	33(78.6%)		32(76.2%)		42(41.2%)
Family planning used N=103	**None**	43(84.3%)	0.299	40(78.4%)	0.627	51(49.5%)
	Hormonal	34(79.1%)		32(74.4%)		43(41.7%)
	IUCD	9(100%)		8(88.9%)		9(8.7%)
Number of Pregnancy N=13	**None**	3(42.9%)	0.002	4(57.1%)	0.703	7(6.8%)
	1	0 (0%)		1(100%)		1(1%)
	2	3 (75%)		3(75%)		4 (3.9%)
	3	4 (66.7%		5(83.3%)		6(5.8%)
	>= 4	76(89.4%)		67(78.8%)		85(82.5%)
STI History N=102	**No**	67(83.8%)	0.83	61(763%)	0.58	80(78%)
	Yes	18(81.8%)		18(81.8%)		22(21.6%)
Monthly income(birr) N=95	**<1500**	47(87%)	0.64	46(85.2%)	0.139	54(56.8%)
	1500-2999					17(17.9%)
	3000-4999	10(83.3%)		7(58.3%)		12(12.6%)
	≥5000	9(75%))		8(66.7%)		12(12.6%)

5.3 Prevalence of HPV

The prevalence of HPV was determined from both cervical and urine samples. Shown below on Table 5.3, the overall HPV infection prevalence rate was 83.5% (N= 86; 76-91, 95% CI) in cervical samples, while the prevalence in urine samples was 77.7% (N=80; 69-86 %, 95% CI).

Table 5-3: Overall prevalence of HPV among biospecimens and HPV types identified with their corresponding specimen type

Sample Types	**HPV infection n (%)**		**HPV types**	
	Negative	Positive	LR-HPV	HR-HPV
Urine sample	23(22.3%)	80(77.7%)	17(16.5%	74(71.8%)
Cervical swab	17(16.5%)	86(83.5%)	13(12.6%)	84(81.6%)

From 19 HPV genotypes targeted by Anyplex ^{TM} II HPV28 PCR System, 17 high risk genotypes (HPV 16,18,31,33,35,39,45,51,52,53,56,58,59,66,68,73,82) from cervical samples and 16 high risk genotypes (HPV 16,18,26,31,33,35,39,45,52,53,56,58,59,66,73,82) from urine samples were detected in our study participants. HPV26 genotype was detected only from urine sample with prevalence rate of 1% (n=1) while HPV51 and HPV68 genotypes were detected only from cervical swab samples with prevalence rate of 1.7%(n=2) and 0.8%(n=1) respectively.

Among the tested women, 16.5% (n=17, 9.9%-25.1%, 95%CI) and 12.6% (n=13, 6.9%-20.6%, 95%CI) had low risk HPV infection in urine and cervical swab respectively. Low risk HPV genotypes were detected more in urine samples than cervical swabs. In contradiction with this, High risk HPVs were detected more frequently in cervical swabs with prevalence rate of 81.6%; (n=84, 72.7%-88.5%, 95%CI) in cervical sample than urine sample 71.8% (n=74, 62.1%-80.3%, 95%CI) infection rate as shown in Table 5.3.

5.4 Prevalence of multiplicity of HPV infection

Single and multiple infection rate (infection by more than one type of HPV) was determined in paired samples as described below in (Table 5.4). Single infection prevalence was 50.5% (N=52; 40.5% -60.5; 95%CI) in cervical sample and 52.4% (N=54; 42.4%- 62.4%; 95%CI) in urine

sample. The rate of multiple infection rate was 31.5% (N=32) in cervical samples and 22.3% (N=23) in urine sample.

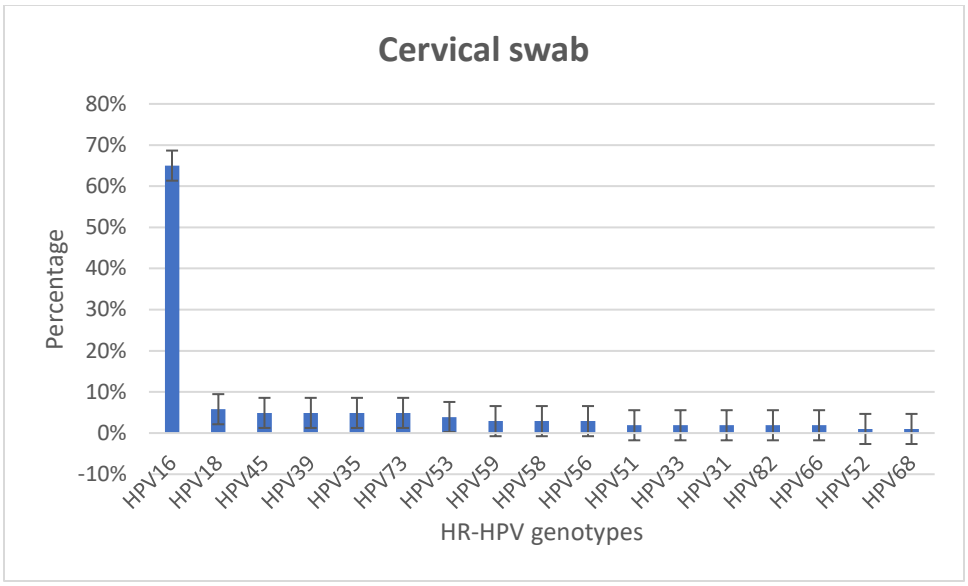
Table 5-4: Number of HPV Types among biospecimens

Number of HPV Types	Sample Types	
	Cervical swab n (%)	**Urine Sample n (%)**
None/negative	17(16.5%)	23(22.3%)
1	54(52.4%)	57(55.3%)
2	22(21.4%)	15(14.6%)
3	9(8.7%)	4(3.9%)
4	1(1.9%)	4(3.9%)
Total	103(100%)	

5.5 Type-specific distribution of HPV genotypes

From the 19 HR-HPV and nine LR-HPV types that can be detected by using Anyplex ™ II HPV28 PCR System, HPV 16 was the most prevalent type in paired samples: 51.5 % (N=53; 42-61%, 95% CI) and 65% (N=67; 56-74%, 95% CI) in urine and cervical samples respectively which was followed by HPV 18 which had 5.8% prevalence (N=6; 1 to 10% 95%CI) in both cervical and urine samples. In addition to HPV 18, HPV 39 was also the second greatest prevalent type that was identified in urine sample. HPV 35, HPV 39, HPV 45 and HPV 73 were equally the third greatest prevalent HPV types identified in cervical sample with 4.9%; (N=5, 1.6-11, 95% CI) while HPV 45 contributed in the urine sample with the same proportion (Figure 5.1).

A.



B.

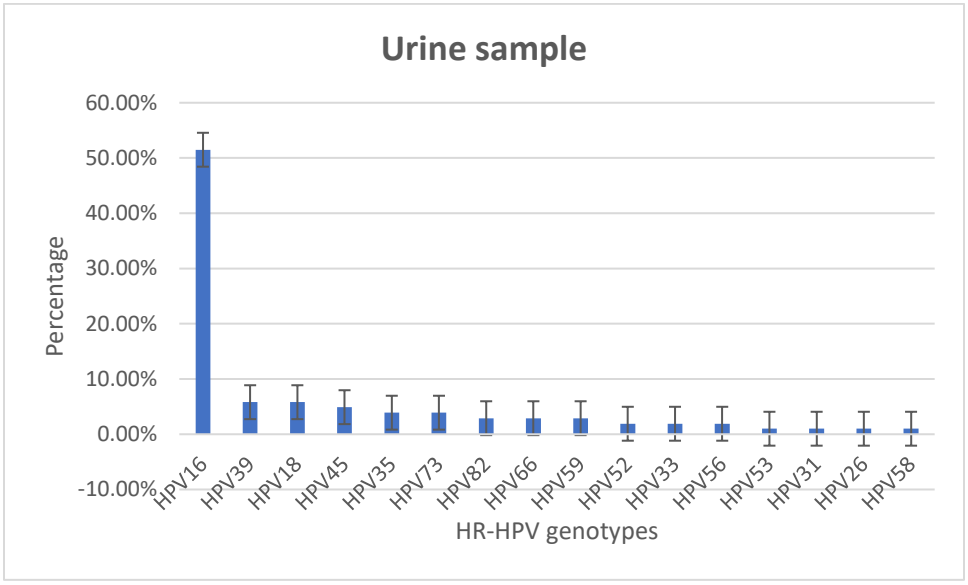
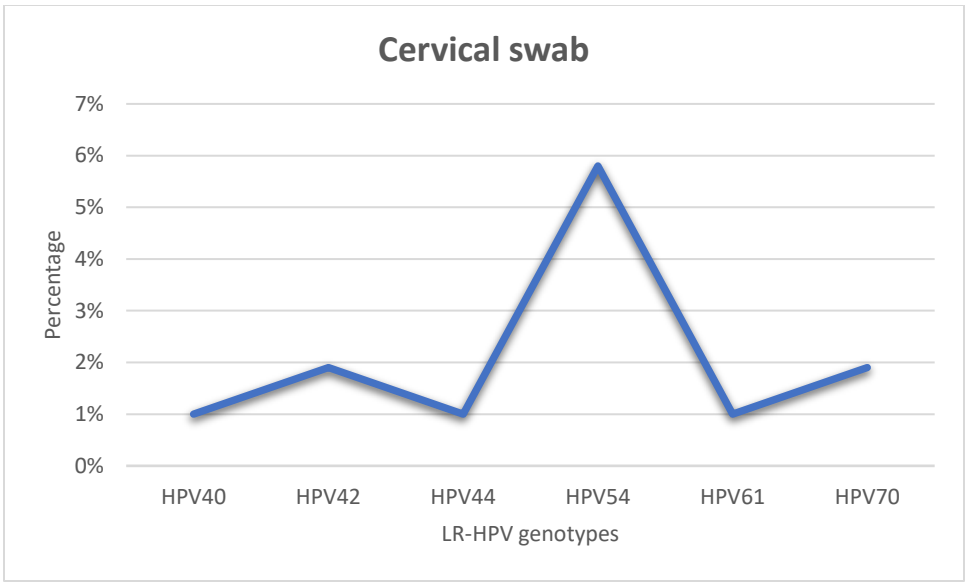


Figure 5-1: Prevalence and genotype distribution of HR-HPV types from cervical (A) and urine (B) samples.

From eight LR-HPV genotypes detected in our study, HPV 54 is the most prevalent genotype from both cervical and urine samples with prevalence rate of 5.8% (N=5; 2.2%-12.2%, 95%CI) and 6.8% (N=7; 2.8%-13.5%, 95%CI) respectively. However, other HPV types distributed differently in between the sample sources (Figure 5.2).

A.



B.

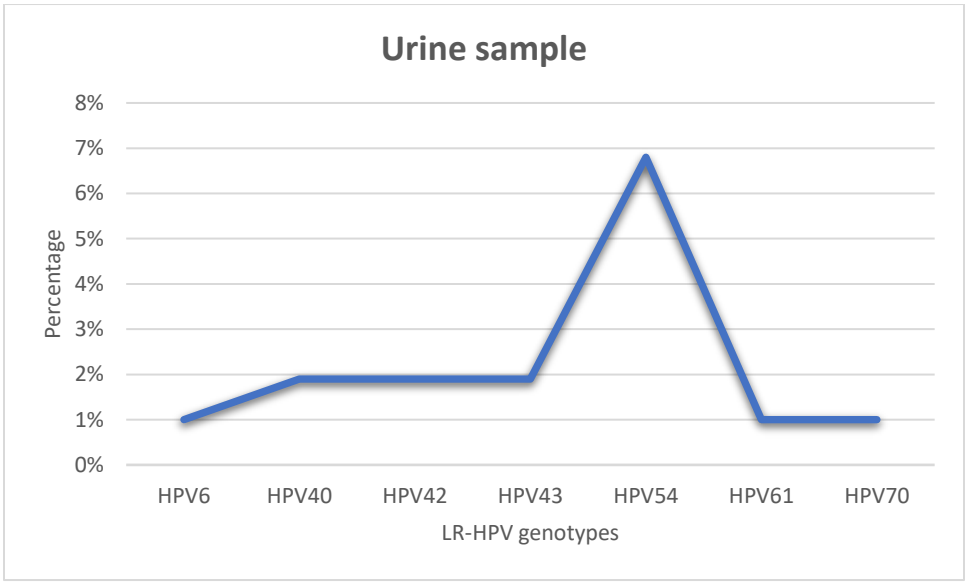


Figure 5-2: prevalence and genotype distribution of LR-HPV types from cervical (A)and urine samples(B).

5.6 Prevalence of prophylactic vaccine preventable HPV genotypes

Among the 9-valent vaccine included HPV types, in our study, HPV 16 is the most commonly identified type with prevalent rate of 65% (55-74.2%: 95%CI) and 51.5% (41.1-61.4%: 95%CI) from cervical swab and urine samples respectively. HPV 18 is the second prevalent with rate of 5.8% (2.2-12.2%: 95%CI) followed by HPV45 with 4.9% (1.6-11.0%: 95%CI) from both paired samples. Moreover, HPV 11 from the two LR-HPV types incorporated in the Gardasil-4 and Gardasil-9 was not detected from both cervical swab and urine samples (Table 5-5).

Table 5-5: The proportion of nine valent vaccine included HPV types among paired specimens

HPV type included in vaccines	Urine sample (%)	Cervical swab (%)
HPV 6	1.3 %	0.0%
HPV 11	0.0%	0.0%
HPV 16	66.3 %	77.9%
HPV 18	7.5 %	7.0%
HPV 31	1.3 %	2.3%
HPV 33	2.5%	2.3%
HPV 45	6.3 %	5.8%
HPV 52	2.5 %	1.2%
HPV 58	1.3 %	3.5%

Prevalence of HPV types incorporated in the Cervarix and Gardasil-4 vaccines was the same among the cervical sample with detection rate of 83.7%. However, HPV types detected in the nine valent vaccine was higher than the two vaccines in the both in the cervical and urine samples with prevalence rate of 90.7% and 77.9% respectively as shown below on Figure 5.3.

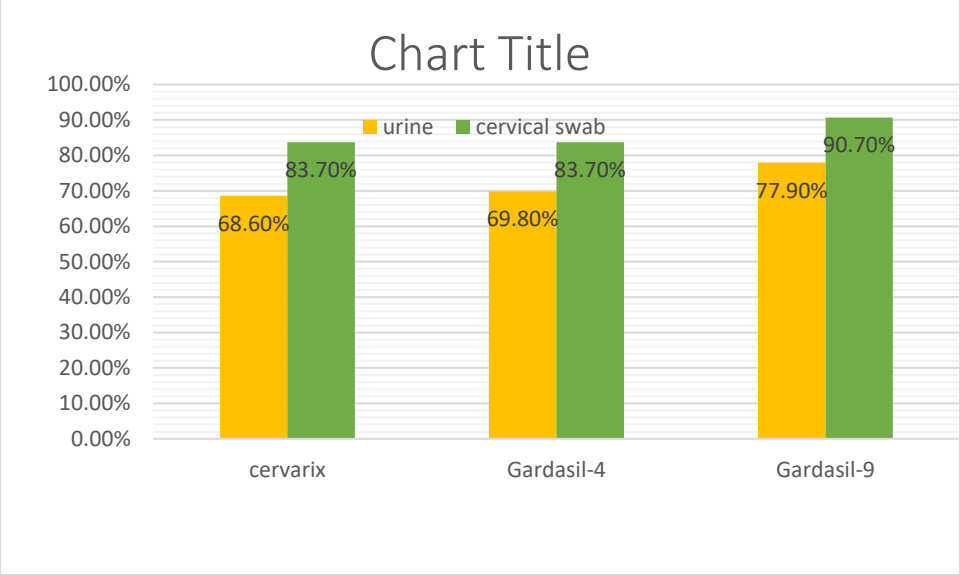


Figure 5-3: Prevalence of HPV types included in prophylactic vaccine

5.7 Diagnostic performance of urine HPV DNA testing and agreement between paired samples

Paired collected samples were analyzed and an overall agreement between cervical and urine samples for the molecular detection of HPV infection was found to be 86.4% with Cohen kappa of 0.58.

Urine sample's diagnostic performance calculated using the cervical sample as a gold standard revealed 88.4% sensitivity, 76.5% specificity, 56.5% negative predictive value (NPV), 95% positive predictive value (PPV) (Table 5.6).

A total of 14(13.6%) study participants result were discordant. In ten cases of HPV positive in cervical swab were found to be HPV negative in urine samples whereas, four cases of HPV positive in urine sample were HPV negative in cervical swabs.

Table 5-6: Diagnostic performance and concordance for detection of HPV (any type) between cervical swabs and urine samples

Urine sample	**Cervical sample**		**Total**	**Agreement % (95%CI)**	**κ-value**	**sensitivity**	**specificity**
	HPV negative (Any type) n (%)	**HPV positive (Any type) n (%)**					
HPV negative (Any type)	13(12.6%)	10(9.7%)	23(22.3%)	86.4% (79.3-92.7)	0.58	88.4%	76.5%
HPV positive (Any type)	4(3.9%)	76(73.8%)	80(77.7%)				
Total	17(16.5%)	86(83.5%)	103				

- Any HPV Type is any of the HPV types detected via Anyplex II detection system
- k value is interpreted as for "Poor" ($\kappa \leq 0.20$), "Fair" ($0.21 \leq \kappa \leq 0.40$), "Moderate" ($0.41 \leq \kappa \leq 0.60$), "Good" ($0.61 \leq \kappa \leq 0.80$), or "Very good" ($\kappa \geq 0.81$) (Altman, 1990)

Table 5.7 summarizes type specific agreement and concordance calculated from paired samples (cervical and urine swab) for 14 HR-HPV genotypes. HPV 45 detected with k value of 1, 100%

reliability. Urine HPV DNA testing sensitivity for detection of HPV 39 and 45 was high i.e (100%). The overall type specific agreement of HPV detection was higher (>80%) though Cohen kappa value was small, that ranges from moderate to very good. The specificity for the detection of 14 HR-HPV was more than 90%. However, HPV 51 and HPV68 was not identified form urine sample though two and one cases were identified from cervical swabs respectively.

Table 5-7: Diagnostic performance and concordance for detection of type specific 14 HR-HPV genotypes

HPV genotypes	Agreement N (%)	Kappa (95% CI)	Sensitivity	Specificity
HR-HPV	87(84.5%)	0.571	84.5%	84.2%
LR-HPV	89(86.4%)	0.455	61.5%	90.0%
HPV 16	85(82.5%)	0.647	76.4%	94.4%
HPV 18	99(96.1%)	0.646	66.7%	97.9%
HPV 31	100(100%)	-.013	0.0%	99.0%
HPV 33	103(100%)	1.0	100%	100%
HPV 35	100(97.1%)	0.652	60.0%	99.0%
HPV 39	102(99.0%)	0.904	100.0%	99.0%
HPV 45	103(100.0%)	1.0	100.0%	100.0%
HPV 51	---	---	---	---
HPV 52	102(99.0%)	0.662	100%	99%
HPV 56	102(99.0%)	0.795	66.7%	100%
HPV 58	101(98.1%)	0.493	33.3%	100%
HPV 59	101(98.1%)	0.657	66.7%	99.0%
HPV 66	102(99.0%)	0.795	100%	99.0%
HPV 68	---	---	---	---

Next, we analyze the ROC curve for urine sample as refenced from cervical swab and AUC was 0.820 (0.7 to 0.95, 95%CI) (Figure 5.4).

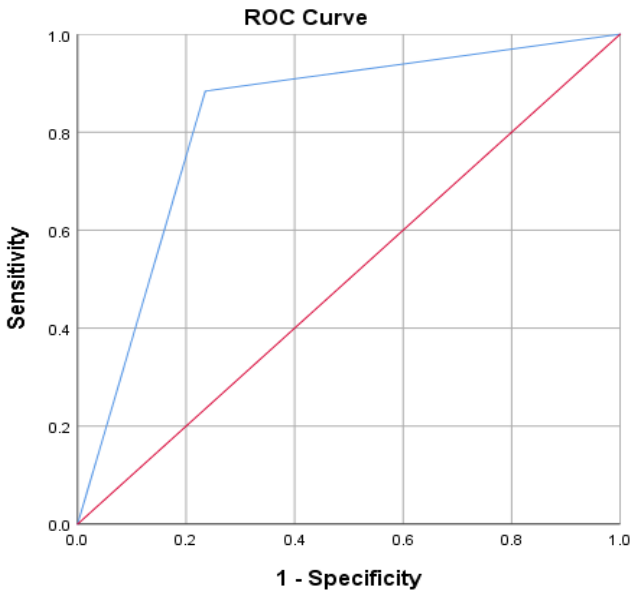
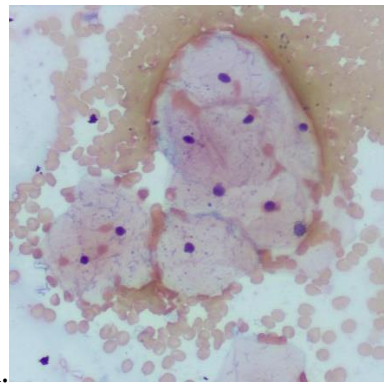
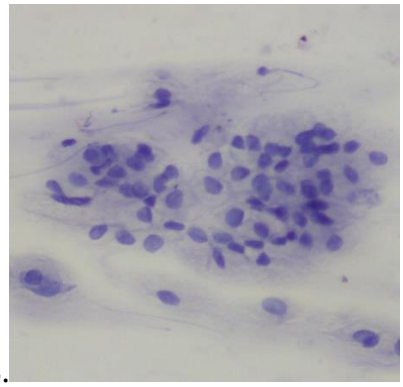
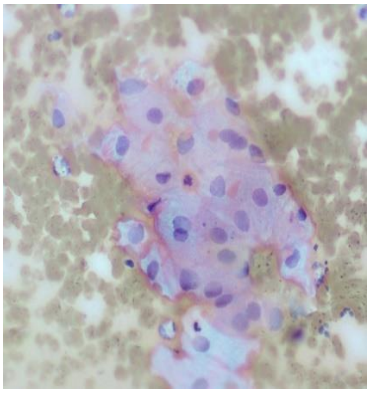


Figure 5-4: ROC analysis of urine samples

5.8 Pap smear test and HPV detection

From a total of 103 study participants, only 50 of them were tested for Papanicolaou test. From the 50 Pap tested women 56.0 % (N=28) of overall abnormal cytology findings according to the Bethesda classification (Nayar and Wilbur, 2015). Of which, 10% (N = 5), 2 % (N= 1), 2% (N= 1), 10 % (N =5), 28% (N=14) and 4% (N=2) cases were classified as atypical squamous cells of undetermined significance (AS-CUS), atypical squamous cells – cannot exclude a high-grade squamous intraepithelial lesion (ASCH), low-grade squamous intraepithelial lesions (LGSIL), high-grade squamous intraepithelial lesions (HG-SIL), Squamous Cell Carcinoma and Adenocarcinoma respectively. However, 30% (N=15) of the pap test were unsatisfactory for evaluation as shown in Table 5.8 and Figure 5.5.

A.  B.  C. 

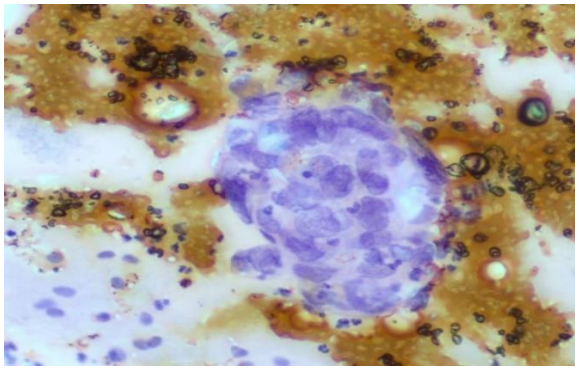
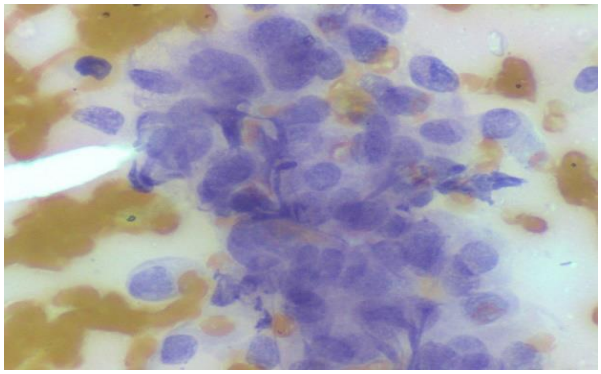
D.  E. 

Figure 5-5: Normal cells (A); Abnormal cytology results, patient with ASCUS (B), patient with HGSIL (C), patient with adenocarcinoma (D), patient with SCC(E).

Table 5-8: Overall HPV (any type) and HPV 16/18 detection in paired sample sources according to the cytological results.

Pap result	**HPV detection N (%)**			
	Any HPV type		HPV 16 and 18	
	Urine sample	Cervical swab	Urine sample	Cervical swab
Normal (N=7)	3(42.9%)	2(28.6%)	0(0%)	0(0%)
ASCUS (N=5)	2(40%)	4(80%)	1(50%)	3(60%)
ASCH (N=1)	0(0%)	0(0%)	0(0%)	0(0%)
LGSIL (N=1)	0(0%)	0(0%)	0(0%)	0(0%)
HGSIL (N=5)	4 (80%)	5(100%)	3(60%)	4(80%)
SCC (N=14)	13(92.9%)	13(92.9%)	8(57.1%)	9(64.2%)
Adenocarcinoma (N=2)	2(100%)	2(100%)	1(50%)	1(50%)
Inadequate (N=15)	12(80%)	13(86.7%)	11(73.3%)	12(80%)
Total (N=50)	36(72%)	39 (78%)	24(48%)	29(58%)

The association of Pap smear result grouped as normal/abnormal finding to the detection of HPV in paired samples showed that from the total of 28 women who had abnormal cytological finding seven and four cases were HPV negative from urine and cervical swab samples respectively. As shown in Table 5.10 the pap smear test and HPV DNA test has shown significant association when cervical swab is used (p=0.001) but not when urine is used (p=0.115).

Table 5-9: Association of Pap smear test with HPV infection in paired samples

PAP result	HPV infection N (%)						Total
	Urine sample		P value	Cervical swab		P value	
	Negative	Positive		Negative	Positive		
Normal	4(57.1%)	3(42.9%)	0.115	5(71.4%)	2(28.6%)	0.001	7
Abnormal	7(25.0%)	21(75.0%)		4(14.3%)	24(85.7%)		28
Inadequate	3(20%)	12(80%)		2(13.3%)	13(86.7%)		15
Total	14(28%)	36(82%)		11(22%)	34(88%)		50

5.9 Histology and HPV detection

Among the 103 study participants, histology results were available for 93 women (9 were missing and 1 was inconclusive) from the chart view. Accordingly, 65 (69.9%) squamous cell carcinoma, nine (9.7%) CIN I, six (6.6%) adenocarcinoma and two CIN II and adenosquamous carcinoma (2.2% each) in decreasing order of frequency (Figure 5.6).

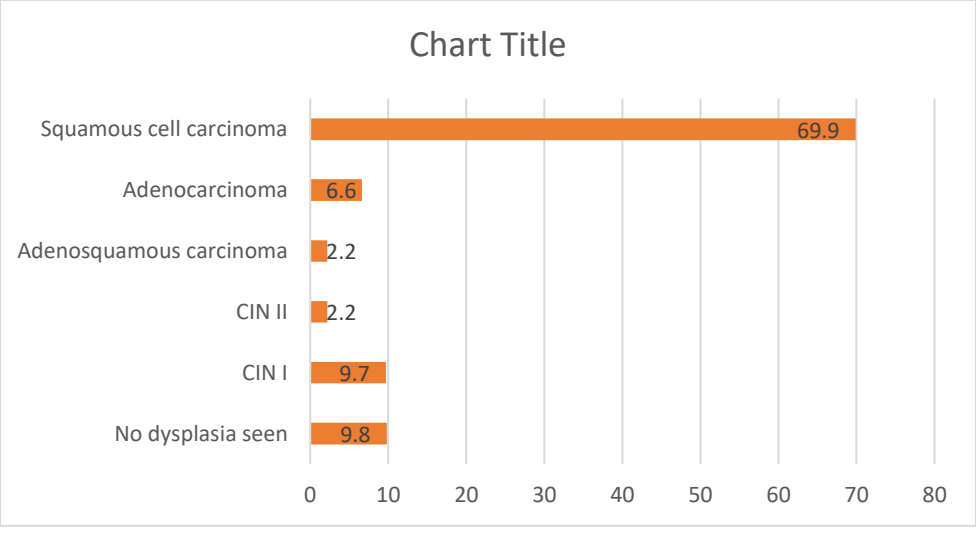


Figure 5-6: Frequency distribution of Biopsy results

Of the total 86 HPV positive women in cervical sample, 64(74.4%) of them were diagnosed with squamous cell carcinoma (SCC) by histology. Similarly, 61(76.3%) of urine sample DNA testing were found to be SCC. Furthermore, 64(76.2%) and 59(79.7%) of SCC were found to be HR-HPV in cervical swab and urine samples respectively. The proportion of detection of HR-HPV was a bit higher for the women diagnosed with SCC in urine sample than cervical sample with proportion of 79.7% and 76.20% respectively. Moreover, carcinogenic HPV types were detected in four (4.80%) and three (4.1%) of histologically reported as no dysplasia from cervical and urine samples respectively (Table 5.11).

5.9.1 Type specific HPV genotypes across the disease category

From the total of LR-HPV genotypes detected in paired samples, only 38.5% and 47.1% from cervical and urine sample respectively were contributing for ≤ CIN II precancerous lesions. HPV 16 was the most detected in SCC with prevalence rate of 45(84.9%) and 52(77.6%) in urine and cervical sample respectively. Furthermore, Women diagnosed as squamous cell carcinoma and adenocarcinoma in cytology test reported 12/14(85.7%) and 2/2(100%) similarly with histology respectively. In contradiction with this, 40% of cytology graded as ASCUS reported as SCC in histology (Table 5.11).

5.9.2 Diagnostic performance of urine HPV DNA testing using histology as a reference

Urine HPV DNA testing was 44.4% and 50% sensitive for the detection of CIN I and CIN II with specificity of 14.3% and 17.6% respectively. For the cervical swab samples, the sensitivity for CIN I was 33.3% and 100% for CIN II. The specificity was of 7.1% for CIN I and 13.2% for CIN II. For the detection of invasive cervical cancer, the sensitivity of paired samples was more than 90% with specificity greater than 50% (Table 5.10).

Table 5-10: Diagnostic performance of paired samples by using histology as a reference

Biopsy result	Sample type	Overall agreement	Cohen k-value	Sensitivity	Specificity
No dysplasia	Urine	17.2%	-0.095	44.4%	14.3%
	Cervical swab	14%	-0.076	55.6%	9.5%
CIN I	Urine	17.2%	-0.095	44.4%	14.3%
	Cervical swab	9.7%	-0.13	33.3%	7.1%

CIN II	Urine	18.3%	-0.017	50%	17.6%
	Cervical swab	15.1%	0.006	100%	13.2%
ICC	Urine	83.9%	0.495	91.3%	55%
	Cervical swab	87.1%	0.553	97.3%	50%

Table 5-11: HPV infection rate in association with biopsy results

		Biopsy Result								
		No biopsy	**No dysplasia**	**CIN I**	**CIN II**	**Adenosquamous carcinoma**	**Adenocarcinoma**	**Squamous cell carcinoma**	**Inconclusive**	**Total**
HPV positive (Any type)	**Cervical**	4(4.7%)	5(5.8%)	3(3.5%)	2(2.3%)	1(1.2%)	6(7.00%)	64(74.4%)	1(1.2%)	86
	Urine	4(5.0%)	4(5.0%)	4(5.0%)	1(1.3%)	1(1.3%)	5(6.30%)	61(76.3%)	0(0.0%)	80
Pap result	**ASCUS**	1(20%)	1(20%)	1(20%)	0(0.0%)	0(0.00%)	0(0.00%)	2(40.0%)	0(0.0%)	5
	ASCH	0(0.0%)	0(0.0%)	1(100%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	1
	LGSIL	0(0.0%)	1(100%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	1
	HGSIL	0(0.0%)	1(20.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	3(60.0%)	1(20.0%)	5
	SCC	1(7.10%)	0(0.0%)	1(7.10%)	0(0.0%)	0(0.0%)	0(0.0%)	12(85.70%)	0(0.0%)	14
	Adenocarcinoma	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	2(100.0%)	0(0.0%)	0(0.0%)	2
	Inadequate	2(13.30%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	2(13.30%)	11(73.30%)	0(0.0%)	15
	Negative	3(50.00%)	2(33.30%)	1(16.70%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	6
High risk HPV	**Cervical**	3(3.6%)	4(4.80%)	3(3.60%)	2(2.4%)	1(1.20%)	6(7.10%)	64(76.20%)	1(1.2%)	84
	Urine	3(4.10%)	3(4.1%)	2(2.7%)	1(1.4%)	1(1.4%)	5(6.8%)	59(79.7%)	0(0.0%)	74
Low risk HPV	**Cervical**	1(7.7%)	2(15.4%)	1(7.7%)	1(7.7%)	0(0.0%)	0(0.0%)	8(61.5%)	0(0.0%)	13
	Urine	2(11.8%)	2(11.8%)	3(17.6%)	1(5.9%)	0(0.0%)	0(0.0%)	9(52.9%)	0(0.0%)	17
HPV 16	**Urine**	1(1.9%)	0(0.0%)	2(3.8%)	1(1.9%)	1(1.9%)	3(5.7%)	45(84.9%)	0(0.0%)	53
	Cervical	2(3.0%)	3(4.5%)	3(4.5%)	2(3.0%)	0(0.0%)	4(6.0%)	52(77.6%)	1(1.5%)	67
HPV 18	**Urine**	1(16.7%)	1(16.7%)	0(0.0%)	0(0.0%)	1(16.7%)	0(0.0%)	3(50.0%)	0(0.0%)	6
	Cervical	0(0.0%)	1(16.7%)	0(0.0%)	0(0.0%)	1(16.7%)	0(0.0%)	4(66.7%)	0(0.0%)	6

5.10 Prevalence of HPV 16 L1 Antibody

Of 102 serum samples of study participants were tested using rapid HPV 16L1 antibody test. The overall detection rate of HPV 16 L1 is 6.8% (2.8-13.5%: 95%CI). HPV 16 L1 detected in 10.4 % (7/67) and 13.2% (7/53) from HPV 16 DNA positive from cervical and urine sample respectively. The sensitivity of Prevo-check® was low but has 100% specificity for both samples when HPV DNA testing being used as a reference.

Of 65 women with SCC, seven were Prevo-check positives. However, data for CIN I and CIN II was inconclusive due to low number of HPV 16 positive cases.

Table 5-12: Association of prevo-check positivity with different degree of dysplasia

	No dysplasia		**CIN1**		**CIN2**		**Adeno carcinoma**		**Squam. cell carcinoma**	
	N	Prev+	N	Prev+	N	Prevo +	N	Prevo +	N	Prevo+
HPV16 only	2	0%	1	0%	1	0%	4	0%	37	5 (14%)
HPV16 + others	1	0%	1	0%	1	0%	0	0%	15	2 (13%)
No HPV16	6	0%	7	0%	0	0%	4	0%	13	0 (0%)
Total	9	0%	9	0%	2	0%	8	0%	65	7 (10.8%)

6 Discussion

For long decades diagnosis of cervical cancer solely based on the analysis of epithelial cells for any morphological changes that is caused by HPV infection. This universal stain for the screening of cervical cytology is Papanicolaou stain which has been used in many laboratories (Roy Biswas *et al.,* 2008). The programmatic implementation of these test together with vaccination against HPV in developed countries has contributed significantly to the decline of cervical cancer cases (Tsakogiannis *et al.,* 2017). The implementation cytology based cervical cancer in developing countries is difficult for a number of reasons (Zur Hausen, 2002; Catarino *et al.,* 2015). Besides, even countries who have been implementing these in the last five decades are revising their strategy for screening (Teixeira *et al.,* 2020). The world health organization has also recommended the use of high precision test for screening cervical cancer (Munoz *et al.,* 2013a). Among these high precision tests, HPV DNA testing using self-sampling is advocated for higher sensitivity and above all access to difficult to reach women (Pathak *et al.,* 2014).

To the best of our knowledge, genotyping of HPV on urine sample is the first study conducted in Ethiopia. In this study, HPV infection rate from urine samples was 78% (69.0-86.0 %; 95% CI). This is in total agreement with studies from UK (79.0%) (Cuzick *et al.,* 2017) and Chile (71.1%) (Buchegger *et al.,* 2018), slightly agree with Bhutan (62.9%) (Tshomo *et al.,* 2017) and Colombia from HIV positive women (63.2%, 63.9%) respectively (Munoz *et al.,* 2013a; Munoz *et al.,* 2013b). However, studies from Thailand (32.3%) (Nilyanimit *et al.,* 2017) and Japan (42.9%) (Hagihara *et al.,* 2016) reported a lesser prevalence rate.

Overall, HPV infection rate was 83.5% (76.0-91.0%; 95%CI) in cervical swab. This finding agrees with a pilot study by Mihret et al 2014 and systematic review by Deribe et al 2019 that reported 85% & 67.8%-93% HPV prevalence respectively. Furthermore, similar study in Ethiopia, 69.1% (Wolday *et al.,* 2018) and African countries such as in Angola and Nigeria revealed a slightly lesser HPV infection rate of 71.9 % & 69.8%% respectively (de Almeida Damião *et al.,* 2016; Kabir *et al.,* 2019). This disparity may be explained by the fact that most of our study participants were diagnosed either with severe cervical cancer lesion and/or cervical cancer and the difference in the HPV DNA test used targeted HPVs, (i.e., 14HR-HPV vs 19HR-HPV) might have a contribution for the difference.

Persistent infection with HR-HPV is needed for the malignant transformation of precancerous lesion to cervical cancer (O'leary *et al.*, 2011a; Taku *et al.*, 2020). In this study, the overall HR-HPV prevalence was 81.6% from cervical swab and 71.8% from urine samples. This finding in total agreement with the study conducted by Wolday et al and Deribe et al from Ethiopia with prevalence of 83.2% and 80.4-100% respectively (Wolday *et al.*, 2018 & Deribe *et al.*, 2019). On previous studies back in 2013, the prevalence of HR-HPV was 93.0% from paraffin embedded cervical biopsy samples (Abate *et al.*, 2013). This report was a bit higher than our finding. This higher reported could be resulted from the contamination during cutting paraffin embedded biopsy samples.

In this study, the top five most prevalent HR-HPV found from the total of 19 HR-HPV genotypes identified were HPV 16 (65%), HPV 18(5.8%), HPV (35,39,45,73) each 4.9%, HPV 53 (3.9%), HPV (56,58,59) each 2.9% in decreasing order from cervical swab whereas HPV 16(51.5%), HPV (18,39) each 5.8%, HPV 45(4.9%), HPVs (35&73) each 3.9%, HPVs (59,66,82) each 2.9% were identified from urine sample. This finding is worldwide phenomena particularly for the very first three genotypes identified matches with (top three) Nigerian (Kabir *et al.*, 2019) and (top two) Egypt studies (Youssef *et al.*, 2016). Oncogenic HPV 16 was the most prevalent identified type which is in total consensus with studies of Abate, Leyh-Bannurah Wolday and Teka, and from Ethiopia (Abate et al., 2013; Leyh-Bannurah *et al*., 2014; Wolday *et al*., 2018; Teka *et al*., 2021). However, HPV 18 which is the second most prevalent type that was detected from our study was the 4^{th} and 5^{th} in studies by Abate et al and Wolday et al respectively (Abate *et al.*, 2013; Wolday *et al.*, 2018). In addition to this, it was not on the list of the top five most prevalent genotype identified from other studies in Ethiopia. This variation in HR-HPV prevalence may be attributed to difference in population and severity of cervical lesions which resulted in variation of HPV types that circulate in the population. Thus, the relative contribution of these HR-HPV for HPV attributed malignancy varied across different geographical locations (de Martel *et al.*, 2017).

We determined the diagnostic performance of urine-based HPV DNA testing and concordance on individual HPV genotypes identified in 103 paired cervical and urine samples. We found an overall concordance of 86.4% HPV DNA testing between urine and paired cervical swab samples among women in Ethiopia. It has a relatively moderate reliability rate with kappa value of 0.57 but it has comparable clinical performance with study conducted in Bhutan that reported more than 70%

concordance (Tshomo *et al.,* 2017) and UK with 82.6% (Cuzick *et al.,* 2017). However, with the same protocol as ours from Japan, reported a higher agreement of 98.4% with kappa of 0.792 (Hagihara *et al.,* 2016) which could indicate a potential use for the clinical application of this sample source. Moreover, the higher sensitivity could be resulted from use of pellet fraction of urine samples for the detection of HPV. Nevertheless, additional studies must be carried out in the general population for determining clinical applicability, storage conditions, suitable extraction method, the most appropriate urine fraction to be used in the molecular analysis, and other factors that could affect the diagnostic performance of this sample source.

Moreover, the agreement of HR-HPV detection rate from urine and cervical swab sample was 84 % which is in total agreement with a study from Thailand that reported 86.2% (Khunamornpong *et al.,* 2016) and Denmark 88.7% with Cohen's kappa of 0.66 (Tranberg *et al.,* 2020). On the other hand, results obtained from Sahasrabuddhe *et al.,* (2014b) and Buchegger *et al.,* (2018) was slightly lower than our finding with concordance of 79.2% and 73.7% with Cohen's kappa of 0.55 and 0.42 respectively. This slight difference could be explained by the fact that we used different protocol for genotyping and detection of HPV. Moreover, we tried to determine genotype level concordance across paired samples. Except HPV 16 with 82.5% (k=0.647), all remaining 13 HR-HPV had more than 95% concordance. This shows urine sample could be an alternative sample for detection of HR-HPV infections in precancerous and cervical cancer patients.

Analytical sensitivity and specificity of urine sample shows 88.4% and 76.5% respectively by using cervical sample as a reference. Similarly, a meta-analysis by Pathak *et al* 2014 showed urine pooled sensitivity of 87% and specificity of 94%. Another study conducted by the same genotype detection kit as our study reported a lesser sensitivity of 68.40% and higher specificity of 99.90% (Hagihara *et al.,* 2016). Other studies form Thailand, Bhutan, Colombia revealed a sensitivity and specificity of (56.2%, 70.6%), (80%, 61%) and (68.80%, 50%) respectively (Nilyanimit *et al.,* 2017; Tshome *et al.,* 2017; Munoz *et al.,* 2013b). Our result revealed urine sample ability to diagnose patients with and without HPV infection based on the cervical swab test and determine AUC of 0.8 which was excellent according to Mandrekar (2010). A lesser AUC of was reported by Munoz *et al.,* (2013a).

In our study, positive predictive (PPV) and negative predictive value (NPV) of urine sample was 95% and 56.50% respectively. There is similar finding from Thailand with 80.00% and 88.20% (Khunamornpong *et al.,* 2016). In contrary lower PPV and NPV reported by Nilyanimit *et al.,* (2017) (53.8% and 72.7%) and Munoz *et al.,* (2013a) (76.70% and 40%). This high PPV in our report indicates that women who have a positive test result actually have a probability of 95% harboring HPV infection. Thus, urine sampling could be used for clinical application as an alternative sample source.

HR-HPV analytical sensitivity and specificity of urine sample was 84.5% and 84.2% respectively. A study from Japan, Thailand, USA, and Denmark showed sensitivity and specificity of (63.90% & 96.50%) (74.7% & 99.9%), (68.60% & 93.20%), (96.20% & 40%), respectively (Sahasrabuddhe *et al.,* 2014b; Hagihara *et al.,* 2016; Khunamornpong *et al.,* 2016; Tranberg *et al.,* 2020). In addition to this, specificity of urine sample for detection of 14 HR-HPV was more than 94%. So, this result tells us urine sample could correctly rule out person who do not have HPV infection and avoids unnecessary follow up of the women in return it will decrease anxiety, medical costs of the patients. This high sensitivity and specificity resulted by strictly following standardized sample handling starting from collecting first void urine with UCM (urine conservation medium) to avoid degradation of DNA and processing and storage at optimum temperature to obtain maximum DNA yield as recommended by Vorster and his colleagues (Vorsters *et al.,* 2014).

Prophylactic vaccines have been designed to prevent malignancy and genital wart causing HPV infection. The three licensed and commercially available prophylactic vaccines are Cervarix, Gardasil-4 and 9-valent Gardasil that protect HPV 16&18, HPV6,11,16&18 and all HPV types included in Cervarix and Gardasil-4 in addition five HR-HPV types (HPV31,33,45,52 and 58) respectively (Kim and Kim, 2017). Our result shows HPV 16 is the most prevalent type from vaccine targeted genotypes with infection rate of 77.9% and 66.3% from cervical swab and urine sample respectively. In addition, HPV 16 and 18 accounts for the overall prevalence of 83.7% in cervical swab and 68.6% in urine sample. Generally, the three vaccines: Cervarix, Gardasil-4 and Gardasil-9 have a protection of 83.7%, 83.7% and 90.7% which was somehow greater for the first two vaccines than Nigerian study with protection of 59.4%, 65.3% and 89.1% respectively (Kabir *et al.,* 2019). A similar study by Teka et al showed the lesser protection of 65.6% and 79.0% by Cervarix and nine-valent vaccine respectively (Teka *et al.,* 2021). However, our report agreed with

global survey which projected efficacy of Gardasil-9 in Africa was around 91.7% (Zhai and Tumban, 2016)

In this study, other HR-HPV genotypes prevalence (HPV26, 35, 39, 41, 51, 53, 56, 59, 66, 68, 73 and 82) that are not included in nine-valent vaccine was determined. From the total 86 HPV DNA positive participants, 18 (21.0%) of them were not covered by nine valent vaccine. This is lower than the study from Zimbabwe that reported 47% (Thistle *et al.*, 2020). Considering this, identifying HPV genotypes that circulates in the population and improved HPV vaccine that provides full immunity against all high-risk HPV infections is still needed.

The overall prevalence of HPV among women who had negative pap result was 28.6% (2/7) in cervical swab. However, it is higher in urine sample with 42.9% (3/7). This is slightly lower than the study in Kenya (31.3%) (Omire *et al.*, 2020), and Morocco (34.3%) (Souho *et al.*, 2016). From our study, we can tell that woman who had pap negative results were HPV infected. Taking this into account, women who undergone pap tests should be carefully managed and HPV DNA testing should be given side by side to decrease their risk of developing cervical dysplasia in the future.

The association between cytological finding being classified as normal and abnormal and presence of HPV showed that 14.3% (4/28) and 25% (7/28) of women who had abnormal pap results are HPV negative in cervical and urine samples respectively. A slightly similar result of 20.4% and 19.6% in cervical and urine sample respectively from HIV positive women was reported by Munoz *et al.*, (2013a). This could be resulted from a number of cases like vaginal infection with other sexually transmitted infection including chlamydia, gonorrhea or, in very rare cases, herpes. Yeast infections can also cause changes in cervical cells. In rare instances, even inflammation perhaps from having sex recently can lead to an abnormal Pap test result. Moreover, this discordance could result from false positive result of cytology.

HR-HPV infection rate was proportionally increased with the degree of dysplasia in the cervix. It ranges from 4.8% in no dysplasia to 76.2% in women who had SCC. This prevalence was higher in urine with proportion of 79.7% in SCC women. This result is similar with a study conducted by Buchegger *et al.*, (2018). This could be explained by the fact that the detection of HPV DNA increased proportionally as exfoliated cervical cells increased due to cervical lesions advancement.

The clinical sensitivity of urine sample for the detection HR-HPV in women who had CIN II (N=2) was 50% with the specificity of 23.1%. However, the corresponding sensitivity of urine was as high as 89% with 70% specificity SCC cases. A study by Sahasrabuddhe *et al.,* 2014a reported sensitivity of 80.8% and a clinical specificity of 53.3%. Another study by Bernal *et al.*, 2014 reported a clinical sensitivity and specificity for detection of HR-HPV in CIN 2 were 95% and 52.4% respectively. Similarly, another study reported a clinical sensitivity and specificity of 52.40% and 71.7% respectively (Buchegger *et al.,* 2018). Our finding is lower than other studies might be cause of small number of CIN II study participants which would not be representative to conclude.

Infection with genital HPV often results in antibody-mediated immune responses, the majority of which are targeted the viral capsid proteins (Rocha-Zavaleta *et al.,* 2004). The viral capsid proteins L1 and L2 are the most conserved structural proteins which comprise 40% of the genome (Morshed *et al.,* 2014). They are target for designing prophylactic vaccines as well (Buck *et al.,* 2013). HPV capsid protein based immunological assays have been shown to detect type specific antibodies presumably due to the presence of conformational epitope that are specific to the given HPV types. Surprisingly, HPV16 capsid present not only specific but also a common antigenic epitope with some of Hr & Lr HPV types (Rocha-Zavaleta *et al.,* 2004; Scherpenisse *et al.,* 2013). Thus, cross reactivity of HPV16L1 antibody have been found against sequence of L1 of other HPV types. In addition to this, infection after HPV results in production of antibody in 50-70% of individuals (Scherpenisse *et al.,* 2013). However, our finding was far from this. Prevo-Check® result showed only 6.8% positivity from 86 HPV16 DNA positive participants. This is not clear to us and raise a question why antibody was not detected? So, additional studies are necessary to answer this question.

7 Strength and Limitation

This study represents the first of its kind to determine the HPV genotype distribution and diagnostic performance of urine sample as compared to paired sample of cervical swab in Ethiopia from different degree of dysplasia. In addition to usage of urine as an alternative source of sample, we collected cervical and blood sample to perform cytology and serological tests respectively. So, we are able to determine molecular epidemiology of HPV types among cervical and pre-cancerous patients and stratification of the HPV types according to pathological finding.

The major pitfall of this study could be its small sample size. In addition to this, the study was conducted in only one specialized hospital in the country and the women were having cervical complaint. Thus, this limits the representativeness of the study participants for the general population. So, large scale studies that comprise population and hospital based should be planned for different regions of the country.

8 Conclusions

- ✓ The top five most prevalent genotypes identified were HPV 16 (65%), HPV 18(5.8%), HPV (35,39,45,73) each 4.9%, HPV 53 (3.9%), HPV (56,58,59) each 2.9% from cervical swab. Meanwhile, HPV 16(51.5%), HPV (18,39) each 5.8%, HPV 45(4.9%), HPV (35&73) each 3.9%, HPV (59,66,82) each 2.9% were detected from urine sample from the total number of cases.
- ✓ The overall prevalence of HPV across paired samples of cervical swab and urine sample was 83.5% and 77.7% and HR-HPV prevalence was 81.6% and 71.8% among our study participants respectively. High prevalence of infection might be due to most of our study participants were cervical cancer patients.
- ✓ Prevalence of vaccine included HPV types were high.
- ✓ Our study revealed good diagnostic performance of urine sample for the detection of cervical HPV infection. So, urine sample could be used as an alternative sample in women who do not wish to have pelvic examination since it has overall agreement more than 85% and sensitivity and specificity of 88.4% and 76.5% with PPV and NPV of 95% and 56.5% respectively. In addition, the calculated AUC was 0.82.
- ✓ HPV DNA detected from women who had normal pap result.
- ✓ The Prevo-check serology test kit for detection of antibody against HPV16L1 has low sensitivity. Additional studies are necessary.
- ✓ Sociodemographic variables such as age group, marital status, education, occupation and number of pregnancies has significant association with HPV detection form cervical sample with p value 0.007, 0.004, 0.02, 0.00 and 0.002. Whereas, age at first intercourse, family planning used, history of STI and monthly income has not significance association with p value greater than 0.05.

9 Recommendations

- ✓ Urine HPV testing should be done on large scale that include women from different degree of dysplasia.
- ✓ Commencement of Gardasil-9 vaccine should be planned for vaccinating school girls in national vaccine program in Ethiopia.

10 References

Abate E, Aseffa A, EL-tayeb M, EL-hassan I, Yamuah L, Mihret W, Bekele L, Ashenafi S. EL-dawi N. & Belayneh, M. 2013. Genotyping of human papillomavirus in paraffin embedded cervical tissue samples from women in Ethiopia and the Sudan. *Journal of medical virology,* 85**,** 282-287.

Abate S. 2015. Trends of cervical cancer in Ethiopia. *Gynecol Obstet (Sunnyvale),* 5**,** 2161-0932.

Ali K E, Mohammed I A, Difabachew M N, Demeke D S, Haile T, Ten hove RJ, KUMSSA T H, Woldu Z L, Haile E L & Tullu K D. 2019. Burden and genotype distribution of high-risk Human Papillomavirus infection and cervical cytology abnormalities at selected obstetrics and gynecology clinics of Addis Ababa, Ethiopia. *BMC cancer,* 19**,** 1-9.

Altman D. G. 1990. *Practical statistics for medical research*, CRC press.

Andrew W. Hahn D. H. S. 2018. human-papillomavirus-infection.

Ashrafi G. H. & Salman N. A. 2016. Pathogenesis of human papillomavirus: immunological responses to HPV infection.

Bernal S, Palomares J C, Artura A, Parra M, Cabezas J L, Robles A & Mazuelos E M 2014. Comparison of urine and cervical samples for detecting human papillomavirus (HPV) with the Cobas 4800 HPV test. Journal of Clinical Virology, 61, 548-552.

Bhattachan K, Dangal G, Karki A, Pradhan H. K, Shrestha R, Parajuli S, Poudel R, Bajracharya N, & Tiwari K. 2019. Evaluation of Abnormal Cervix with Visual Inspection under Acetic Acid and Colposcopy. *Journal of Nepal Health Research Council,* 17**,** 76-79.

Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J, Bosch FX, de Sanjosé S. ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in Ethiopia. Summary Report 17 June 2019. [Accessed on April 26, 2021]

Buchegger K, Viscarra T, Andana A, Ili C, López J, Zanella L, Carmona-lópez M. I, Fernández, J. J, Espinel I. C. & Sánchez R. 2018. Detection and genotyping of human papillomavirus virus (HPV): a comparative analysis of clinical performance in cervical and urine samples in Chilean women. *International journal of clinical and experimental pathology,* 11**,** 5413.

Buck CB, Day PM. & Trus B L. 2013. The papillomavirus major capsid protein L1. Virology, 445, 169-174.

Bzhalava D, Eklund C. & Dillner J. 2015. International standardization and classification of human papillomavirus types. *Virology*, 476**,** 341-344.

Catarino R, Petignat P, Dongui G & Vassilakos P. 2015. Cervical cancer screening in developing countries at a crossroad: Emerging technologies and policy choices. World journal of clinical oncology, 6, 281.

Chan P. K, Picconi M. A, Cheung T. H, Giovannelli L. & Park J. S. 2012. Laboratory and clinical aspects of human papillomavirus testing. *Crit Rev Clin Lab Sci,* 49**,** 117-36.

Chelimo C, Wouldes T. A, Cameron L. D. & Elwood J. M. 2013. Risk factors for and prevention of human papillomaviruses (HPV), genital warts and cervical cancer. *Journal of Infection,* 66**,** 207-217.

Cheng M. A, Farmer E, Huang C, Lin, J, Hung C. F. & Wu T. C. 2018. Therapeutic DNA vaccines for human papillomavirus and associated diseases. *Human gene therapy,* 29**,** 971-996.

Ciapponi A, Bardach A, Glujovsky D, Gibbons L. & Picconi M. A. 2011. Type-specific HPV prevalence in cervical cancer and high-grade lesions in Latin America and the Caribbean: systematic review and meta-analysis. *PLoS One,* 6**,** e25493.

Combes J. D, Pawlita M, Waterboer T, Hammouda D, Rajkumar T, Vanhems P, Snijders P, Herrero R, Franceschi S. & Clifford G. 2014. Antibodies against high-risk human papillomavirus proteins as markers for invasive cervical cancer. *International journal of cancer,* 135**,** 2453-2461.

Coutlée F, Rouleau D, Ferenczy A. & Franco E. 2005. The laboratory diagnosis of genital human papillomavirus infections. *Canadian Journal of Infectious Diseases and Medical Microbiology,* 16.

Cuzick J, Cadman L, Ahmad A. S, Ho L, Terry G, Kleeman M, Lyons D, Austin J, Stoler M. H. & Vibat C. R. T. 2017. Performance and diagnostic accuracy of a urine-based human papillomavirus assay in a referral population. *Cancer Epidemiology and Prevention Biomarkers,* 26**,** 1053-1059.

De almeida Damião P, Oliveira-silva M, Moreira M. Â, Poliakova N, De lima M. E. R., ChiovoJ. & Nicol A. F. 2016. Human Papillomavirus types distribution among women with cervical preneoplastic, lesions and cancer in Luanda, Angola. *The Pan African Medical Journal,* 24.

De martel C, Plummer M, Vignat J. & Franceschi S. 2017. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *International journal of cancer,* 141**,** 664-670.

De villierss E. M, Fauquet C, Broker T. R, Bernard H. U. & Zur hausen H. 2004. Classification of papillomaviruses. *Virology,* 324**,** 17-27.

Deligeoroglou E, Giannouli A, Athanasopoulos N, Karountzos V, Vatopoulou A, Dimopoulos K. & Creatsas G. 2013. HPV infection: immunological aspects and their utility in future therapy. *Infectious diseases in obstetrics and gynecology,* 2013.

Doorbar J, Egawa N, Griffin H, Kranjec C. & Murakami I. 2015. Human papillomavirus molecular biology and disease association. *Rev Med Virol,* 25 Suppl 1**,** 2-23.

Doorbar J, Quint W, Banks L, Bravo I. G, Stoler M, Broker T. R. & Stanley M. A. 2012. The biology and life-cycle of human papillomaviruses. *Vaccine,* 30 Suppl 5**,** F55-70.

Egawa N, Egawa K, Griffin H. & Doorbar J. 2015. Human papillomaviruses; epithelial tropisms, and the development of neoplasia. *Viruses,* 7**,** 3863-3890.

Fernandes J. V. & De medeiros fernandes T. A. A. 2012. Human papillomavirus: biology and pathogenesis. *Human Papillomavirus and Related Diseases-From Bench to Bedside-A Clinical Perspective.* InTech.

Franceschi S, Chantal umulisa M, Tshomo U, Gheit T, Baussano I, Tenet V, Tshokey T, Gatera M, Ngabo F, Van damme P, Snijders P. J. F, Tommasino M, Vorsters A. & Clifford G. M. 2016. Urine testing to monitor the impact of HPV vaccination in Bhutan and Rwanda. *International Journal of Cancer,* 139**,** 518-526.

Georgescu S. R, Mitran C. I, Mitran M. I, Caruntu C, Sarbu M. I, Matei, C, Nicolae I, Tocut S. M. & Popa M. I. 2018. New insights in the pathogenesis of HPV infection and the associated carcinogenic processes: the role of chronic inflammation and oxidative stress. *Journal of immunology research,* 2018.

Hagihara M, Yamagishi Y, Izumi K, Miyazaki N, Suzuki T, Kato H, Nishiyama N, Koizumi Y, Suematsu H. & Mikamo H. 2016. Comparison of initial stream urine samples and cervical samples for detection of human papillomavirus. *Journal of Infection and Chemotherapy,* 22**,** 559-562.

Haile E. L, Cindy S, Ina B, Belay G, Geertruyden jean-pierre V, Sharon R, Lisbeth L. R. & Paul B. J. 2019. HPV testing on vaginal/cervical nurse-assisted self-samples versus clinician-taken specimens and the HPV prevalence, in Adama Town, Ethiopia. *Medicine*, 98.

Hailu A. & Mariam D. H. 2013. Patient side cost and its predictors for cervical cancer in Ethiopia: a cross sectional hospital-based study. *BMC cancer,* 13**,** 69.

Hui Y, Hansen K, Murthy J, Chau D, Sung C J & Quddus M R. 2016. Relevance of the Pap test: a report of HPV-DNA test-negative high-grade squamous intraepithelial lesions of the female lower genital tract. Acta cytologica, 60, 445-450.

Hung C. -F, Ma B, Monie A, Tsen S.-W. & Wu, T. 2008. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert opinion on biological therapy,* 8**,** 421-439.

Hwang S. J. & Shroyer K. R. 2012. Biomarkers of cervical dysplasia and carcinoma. *Journal of oncology,* 2012.

Ibeanu O. A. 2011. Molecular pathogenesis of cervical cancer. *Cancer biology & therapy,* 11**,** 295-306.

Ibeanu O. A. 2014. Molecular pathogenesis of cervical cancer. *Cancer Biology & Therapy*, 11**,** 295-306.

Ikenberg H. 2014. Laboratory diagnosis of human papillomavirus infection. *Human Papillomavirus,* 45**,** 166-174.

Kabir A, Bukar M, Nggada H. A, Rann H. B, Gidado A. & Musa A. B. 2019. Prevalence of human papillomavirus genotypes in cervical cancer in Maiduguri, Nigeria. *The Pan African Medical Journal,* 33.

Khunamornpong S, Settakorn J, Sukpan K, Lekawanvijit S, Katruang N. & Siriaunkgul S. 2016. Comparison of human papillomavirus detection in urine and cervical samples using high-risk HPV DNA testing in northern thailand. *Obstetrics and gynecology international,* 2016.

Kim H. J. & Kim H. J. 2017. Current status and future prospects for human papillomavirus vaccines. *Archives of pharmacal research,* 40**,** 1050-1063.

Leyh-bannurah S.-R., Prugger C., De koning M. N., Goette H. & Lellé R. J. 2014. Cervical human papillomavirus prevalence and genotype distribution among hybrid capture 2 positive women 15 to 64 years of age in the Gurage zone, rural Ethiopia. *Infectious agents and cancer,* 9**,** 33.

Lin K, Roosinovich E, Ma B Hung CF & Wu TC. 2010. Therapeutic hpv DNA vaccines. *Immunologic research,* 47**,** 86-112.

Longworth MS & Laimins LA. 2004. Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev,* 68**,** 362-72.

Mandrekar JN. Receiver operating characteristic curve in diagnostic test assessment. Journal of Thoracic Oncology. 2010 Sep 1;5(9):1315-6.

Mboumba bouassa R. S, Prazuck T, Lethu T, Jenabian M. A, Meye J. F. & Belec L. 2017. Cervical cancer in sub-Saharan Africa: a preventable noncommunicable disease. *Expert Rev Anti Infect Ther,* 15**,** 613-627.

Mekuria S, Jerkeman M, Forslund O, Fikru S. & Borgfeldt C. 2020. Detection of HPV mRNA in Self-collected Vaginal Samples Among Urban Ethiopian Women. *Anticancer research,* 40**,** 1513-1517.

Mihret W, Yusuf L, Abebe M, Yamuah LK, Bekele L, Abate E, et al. A pilot study on detection and genotyping of human papilloma virus isolated from clinically diagnosed Ethiopian women having cervical intraepithelial neoplasia. Ethiop Med J. 2014;1:49–52.

Moody C. 2017a. Mechanisms by which HPV induces a replication competent environment in differentiating keratinocytes. *Viruses,* 9**,** 261.

Moody C. A. 2017b. Mechanisms by which HPV Induces a Replication Competent Environment in Differentiating Keratinocytes.

Morshed K, Polz-gruszka D, Szymański M. & Polz-dacewicz M. 2014. Human Papillomavirus (HPV)–structure, epidemiology and pathogenesis. *Otolaryngologia Polska,* 68**,** 213-219.

Munoz M, Camargo M, Soto-de leon S. C, Sanchez R, Parra D, Pineda A. C, Sussmann O, Perez-prados A, Patarroyo M. E. & Patarroyo M. A. 2013a. Human papillomavirus detection from human immunodeficiency virus-infected Colombian women's paired urine and cervical samples. *PLoS One,* 8**,** e56509.

Munoz M, Camargo M, Soto-de leon S. C, Sanchez R, Pineda-Peña AC, Perez-Prados A, Patarroyo ME & Patarroyo, MA. 2013b. Classical molecular tests using urine samples as a potential screening tool for human papillomavirus detection in human immunodeficiency virus-infected women. *Journal of clinical microbiology***,** JCM. 01302-13.

Nayar R & Wilbur DC. 2015. *The Bethesda system for reporting cervical cytology: definitions, criteria, and explanatory notes*, Springer.

Nilyanimit P, Chansaenro JJ, Karalak A, Laowahutanont P, Junyangdikul P & Poovorawan Y. 2017. Comparison of human papillomavirus (HPV) detection in urine and cervical swab samples using the HPV GenoArray Diagnostic assay. *PeerJ,* 5**,** e3910.

Ogembo RK, Gona PN, Seymour AJ, Park HS, Bain PA, Maranda L & Ogembo JG. 2015. Prevalence of human papillomavirus genotypes among African women with normal cervical cytology and neoplasia: a systematic review and meta-analysis. *PLoS One,* 10**,** e0122488.

O'leary MC, Sinka K, Robertson C, Cuschieri K, Lyman R., Lacey M, Potts A, Cubie HA & Donaghy M. 2011b. HPV type-specific prevalence using a urine assay in unvaccinated male and female 11- to 18-year olds in Scotland. *British Journal of Cancer,* 104**,** 1221-1226.

O'leary M, Sinka K, Robertson C, CuschierI K, Lyman R, Lacey M, Potts A, Cubie H & Donaghy M. 2011a. HPV type-specific prevalence using a urine assay in unvaccinated male and female 11-to 18-year olds in Scotland. *British journal of cancer,* 104**,** 1221.

Omire A, Budambula NL, Kirumbi L, Langat H, Kerosi D, Ochieng W & Lwembe R. 2020. Cervical Dysplasia, Infection, and Phylogeny of Human Papillomavirus in HIV-Infected and HIV-Uninfected Women at a Reproductive Health Clinic in Nairobi, Kenya. *BioMed Research International,* 2020.

Orang'o EO, Were E, Rode O, Muthoka K, Byczkowski M, Sartor H, Broeck DV, Schmidt D, Reuschenbach M & Von Knebel Doeberitz M. 2020. Novel concepts in cervical cancer screening: a comparison of VIA, HPV DNA test and p16 INK4a/Ki-67 dual stain cytology in Western Kenya. *Infectious Agents and Cancer,* 15**,** 1-10.

Pathak N, Dodds J, Zamora J & Khan K. 2014. Accuracy of urinary human papillomavirus testing for presence of cervical HPV: systematic review and meta-analysis. *Bmj,* 349**,** g5264.

Pinidis P, Tsikouras P, Iatrakis G, Zervoudis S, Koukouli Z, Bothou A, Galazios G & Vladareanu S. 2016. Human papilloma virus' life cycle and carcinogenesis. *Maedica,* 11**,** 48.

Pinto LA, Dillner J, Beddows S & Unger ER. 2018. Immunogenicity of HPV prophylactic vaccines: serology assays and their use in HPV vaccine evaluation and development. *Vaccine,* 36**,** 4792-4799.

Racey CS, Withrow DR & Gesink D. 2013. Self-collected HPV testing improves participation in cervical cancer screening: a systematic review and meta-analysis. *Can J Public Health,* 104**,** 159-166.

Rocha-Zavaleta L, Ambrosio JP, de Lourdes Mora-Garcia M, Cruz-Talonia F, Hernandez-Montes J, Weiss-Steider B, Ortiz-Navarrete V, Monroy-Garcia A. Detection of antibodies against a human papillomavirus (HPV) type 16 peptide that differentiate high-risk from low-risk HPV-associated low-grade squamous intraepithelial lesions. Journal of general virology. 2004 Sep 1;85(9):2643-50.

Roybiswas R, Paral CC, Dey R & Biswas SC. 2008. Rapid economic, acetic acid, Papanicolaou stain (REAP)-Is it suitable alternative to standard PAP stain. *Al Ameen J Med Sci,* 1**,** 99-103.

Sahasrabuddhe V V, Gravitt PE, Dunn ST, Brown D, Allen RA, Eby YJ, Smith K, Zuna RE., Zhang RR & Gold MA. 2014a. Comparison of human papillomavirus detections in urine, vulvar, and cervical samples from women attending a colposcopy clinic. *Journal of clinical microbiology,* 52**,** 187-192.

Sahasrabuddhe VV, Gravitt PE, Dunn ST, Robbins D, Brown D, Allen R A, Eby YJ, Smith KM., Zuna RE & Zhang RR. 2014b. Evaluation of clinical performance of a novel urine-based HPV detection assay among women attending a colposcopy clinic. *Journal of Clinical Virology,* 60**,** 414-417.

Salazar-Pina DA, Pedroza-Saavedra A, Cruz-Valdez A, Ortiz-Panozo E, Maldonado-Gama M, Chihu-Amparan L, Rodriguez-Ocampo AN, Orozco-Fararoni E, Esquivel-Guadarrama F & Gutierrez-Xicotencatl L. 2016. Validation of Serological Antibody Profiles Against Human Papillomavirus Type 16 Antigens as Markers for Early Detection of Cervical Cancer. *Medicine (Baltimore),* 95**,** e2769.

Schneider A. 1993. Pathogenesis of genital HPV infection. *Sexually Transmitted Infections,* 69**,** 165-173.

Scherpenisse M, Schepp R M, Mollers M, Meijer CJ, Berbers GA & Van der klis FR. 2013. Characteristics of HPV-specific antibody responses induced by infection and vaccination: cross-reactivity, neutralizing activity, avidity and IgG subclasses. PloS one, 8, e74797

Shrestha B & Joshi R. 2020. Evaluation of Visual Inspection of Cervix with Acetic Acid and Liquid Based in Cervical Cancer Screening with Cervical Biopsy. *Journal of Nepal Health Research Council,* 18**,** 426-430.

Souho T, EL Fatemi H, Karim S, EL Rhazi K, Bouchikhi C, Banani A, Melhouf MA, Benlemlih M & Bennani B. 2016. Distribution of carcinogenic human papillomavirus genotypes and association to cervical lesions among women in Fez (Morocco). *PLoS One,* 11**,** e0146246.

Spurgeon ME & Lambert PF. 2017. Human papillomavirus and the stroma: bidirectional crosstalk during the virus life cycle and carcinogenesis. *Viruses,* 9**,** 219.

Stanley MA. 2012. Epithelial Cell Responses to Infection with Human Papillomavirus. *Clinical Microbiology Reviews,* 25**,** 215-222.

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A & Bray F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*.

Taku O, Businge C B, Mdaka ML, Phohlo K, Basera W, Garcia-Jardon M, Meiring TL, Gyllensten U, Williamson AL & Mbulawa ZZ. 2020. Human papillomavirus prevalence and risk factors among HIV-negative and HIV-positive women residing in rural eastern cape, South Africa. *International Journal of Infectious Diseases,* 95**,** 176-182.

Teixeira JC, Vale D B, Bragança JF, Campos C S, Discacciati M G & Zeferino L C. 2020. Cervical cancer screening program based on primary DNA-HPV testing in a Brazilian city: a cost-effectiveness study protocol. BMC public health, 20, 1-8.

Teka B, Gizaw M, Ruddies F, Addissie A, Chanyalew Z, Skof A S., Thies S., Mihret A, Kantelhardt E J. & Kaufmann A M. 2021. Population-based human papillomavirus infection and genotype distribution among women in rural areas of South Central Ethiopia. *International Journal of Cancer,* 148**,** 723-730.

Thistle P, Parpia R, Pain D, Lee H, Manasa J. & Schnipper L E. 2020. Prevalence and subtype distribution of high-risk human papillomavirus among women presenting for cervical cancer screening at Karanda Mission Hospital. *JCO Global Oncology,* 6**,** 1276-1281.

Tranberg M, Jensen J S, Bech BH. & Andersen B. 2020. Urine collection in cervical cancer screening–analytical comparison of two HPV DNA assays. *BMC infectious diseases,* 20**,** 1-10.

Tranberg M, Jensen J S, Bech B H, Blaakær J, Svanholm H & Andersen B. 2018. Good concordance of HPV detection between cervico-vaginal self-samples and general practitioner-collected samples using the Cobas 4800 HPV DNA test. *BMC infectious diseases,* 18**,** 348.

Tsakogiannis D, Gartzonika C, Levidiotou-Stefanou S & Markoulatos P. 2017. Molecular approaches for HPV genotyping and HPV-DNA physical status. *Expert reviews in molecular medicine,* 19.

Tshomo U, Franceschi S, Tshokey T, Tobgay T, Baussano I, Tenet V, Snijders P J, Gheit T, Tommasino M & Vorsters A. 2017. Evaluation of the performance of Human Papillomavirus testing in paired urine and clinician-collected cervical samples among women aged over 30 years in Bhutan. *Virology journal,* 14**,** 1-6.

Van keer S, Pattyn J, Tjalma W A, Van ostade X, Ieven M, Van damme P & Vorsters A. 2017. First-void urine: A potential biomarker source for triage of high-risk human papillomavirus infected women. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 216**,** 1-11.

Van keer s, Van splunter A P, Pattyn J, DE Smet A, Herzog S A, Van ostade X, Tjalma W A, Ieven M, Van damme P & Steenbergen R D. 2021. Triage of human papillomavirus infected women by methylation analysis in first-void urine. *Scientific Reports,* 11**,** 1-10.

Verma L, Shamsunder S, Malik S & Arora R. 2020. To evaluate the role of p16ink4a immunocytochemistry for detection of cin2+ in women detected screen positive by visual inspection using acetic acid. *Journal of Cytology,* 37**,** 82.

Villa LL. 2009. Laboratory Methods for Detection of Human Papillomavirus Infection. *Human Papillomavirus.* Springer.

Vorsters A, Van Den Bergh J, Micalessi I, Biesmans S, Bogers J, Hens A, DE Coster I, Ieven M & Van Damme P. 2014. Optimization of HPV DNA detection in urine by improving collection, storage, and extraction. *European journal of clinical microbiology & infectious diseases,* 33**,** 2005-2014.

Weiland T, Eckert A, Tomazic P V, Wolf A, Pondorfer P, Vasicek S, Graupp M, Holzmeister C, Moser U & Andrianakis A. 2020. DRH1–a novel blood-based HPV tumour marker. *EBioMedicine,* 56**,** 102804.

Wolday D, Derese M, Gebressellassie S, Tsegaye B, Ergete W, Gebrehiwot Y, Caplan O, Wolf DG. & maayan S. 2018. HPV genotype distribution among women with normal and abnormal cervical cytology presenting in a tertiary gynecology referral Clinic in Ethiopia. *Infectious agents and cancer,* 13**,** 1-8.

Yang A, Farmer E, Wu TC. & Hung CF. 2016. Perspectives for therapeutic HPV vaccine development. *Journal of biomedical science,* 23**,** 75.

Youssef MA, Abdelsalam L, Harfoush RA, Talaat IM, Elkattan E, Mohey A, Abdella RM, Farhan MS, Foad HA. & Elsayed AM. 2016. Prevalence of human papilloma virus (HPV) and its genotypes in cervical specimens of Egyptian women by linear array HPV genotyping test. *Infectious agents and cancer,* 11**,** 1-10.

Yu L, Fei L, Liu X, Pi X, Wang L. & Chen S. 2019. Application of p16/Ki-67 dual-staining cytology in cervical cancers. *Journal of Cancer,* 10**,** 2654.

Zhai L. & Tumban E. 2016. Gardasil-9: A global survey of projected efficacy. Antiviral research, 130, 101-109.

Zheng R. & Heller DS. 2020. High-Risk Human Papillomavirus Identification in Precancerous Cervical Intraepithelial Lesions. *Journal of lower genital tract disease,* 24**,** 197-201.

Zheng ZM. & Baker CC. 2006. Papillomavirus genome structure, expression, and post-transcriptional regulation. *Frontiers in bioscience: a journal and virtual library,* 11**,** 2286.

Zur hausen H. 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nature reviews cancer, 2, 342-350.

11 Annexes

11.1 Information sheet for study participants

11.1.1 Participant information sheet (English version)

Title: HPV in women with severe cervical cancer lesion and cancer: the use of urine as an adjunct specimen

Investigator: Ededia Firdawoke (Bsc, MSc fellow at Addis Ababa University College of Health Science).

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Advisors:

- Dr. Tamirat Abebe (Ass. Professor),
- Mr. Birhanu Teka (Phd fellow)

This participants information sheet is for cervical cancer screening of women aged 18 and above in Addis Ababa, Ethiopia and who I am inviting to participate in a research project entitled "Cervical cancer diagnosis and Human Papilloma Virus based screening: Value of urine as an adjunct specimen". I am Ededia Firdawoke, an MSc fellow at Addis Ababa University College of Health Science. I am conducting a research on cervical cancer, which is very prevalent in this country, as part of the requirement for the partial fulfillment of the master's degree in Medical Microbiology at Addis Ababa University. I am going to give you information and invite you to be part of this research. Before you decide to be part of the research you can take time and talk to someone else you like if you want to do about the research. This form may contain words that you do not understand, please ask me to stop as we go through the information and I will take time to explain; or if you wish to ask questions later, you may contact me by the above contact information.

Purpose of the study: Human papillomavirus (HPV) is the main cause of cervical cancer. Cervical cancer is the second most prevalent cancer in this country and it affects women when they are essential for the community, so timely screening is more protective against invasive cervical cancer. So, this research is intended to determine the diagnostic value of urine sample along with the cervical sample to determine the prevalence of cervical cancer.

Your participation in this research is entirely voluntary and it is up to you to decide whether or not to take part. You have the right not to complete this questionnaire in full and can skip a specific question you do not want to answer.

Study Procedure: This study include questionnaire, attached hereunder, will involve you by allowing me to collect urine, self-sampled cervical and blood sample from you as well as telling me what you experience in life in general. You are being invited to take part because I feel that your experience can contribute much to the prospected research.
If you agree to participate in the study, you will be asked to provide a self - collected cervical swab, 20 ml urine and 5 ml blood sample and also asked some questions. The time to complete the survey will vary; however, it is anticipated that no more than thirty minutes will be necessary.

Risks: I don't believe there are any risks from participating in this research.

Confidentiality: All information which is collected about you during the course of the research will be kept strictly confidential. Your name and any other information that can directly identify you will be represented using codes. You are required to answer the questions based on your personal experience on the questionnaire. You may decline to answer any or all questions and you may terminate your involvement without the need to explain at any time you choose.

Benefits: There may or may not direct benefit to you during the research, you will be beneficiary from the information to be generated from this research in such a way that obtained information will be used to design the most accessible and approachable screening methods to diagnose cervical cancer.

Participant consent form

I have read the foregoing information, or it has been read to me. I have a clear understanding of the objectives and conditions of the study. I have had the opportunity to ask questions about it and any questions I have been asked have been answered to my satisfaction. I am providing permission for samples to be taken from me are self-collected cervical swab, 20 ml urine and 5 ml blood. I understand that I will be given a copy of this consent form. I understand my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

Participants' signature_____ Date_____

Investigators' signature_____ Date_____

11.1.2 ለጥናቱ ተሳታፊዎች የሚሆን መረጃ (Amharic version)

ርዕስ፡- የማህፀን ጫፍ ካንሰር ምርምር እና የሽንት ናሙና ጥቅም

ተመራማሪ፡ ኢዲዲያ ፍርድአወቅ (የማስተርስ ዲግሪ ተማሪ፣ አዲስ አበባ ዩኒቨርስቲ)

- ስልክ ቁጥር፡0920307519
- ኢሜል፡ jedediahfirdawoke@gmail.com

አማካሪዎች፡

- ዶ/ር ታምራት አበበ (ረዳት ፕሮፌሰር)
- አቶ ብርሀኑ ተካ (የፒቼዲ ተማሪ)

ይህ ለጥናቱ ተሳታፊዎች የሚሆን መረጃ የተዘጋጀው እድሜያቸው አስራስምንት(18) እና ከዚያ በላይ ለሆናቸው ሴቶች ሲሆን ጥናቱ የሚያጠናው የሽንት ናሙናን ከማህፀን ጫፍ ከሚወሰድ ፈሳሽ ጋር በማድረግ የማህፀን ጫፍ ካንሰርን ለመመርመር መጥቀም መቻሉን ለማየት ነው። እኔ ኢዲዲያ ፍርድአወቅ እባላለሁ በአዲስ አበባ ዩኒቨርስቲ የማስተርስ ዲግሪ ተማሪ ነኝ። በማህፀን ጫፍ ካንሰር ላይ ምርምር እያደረኩ ሲሆን ይህ ጥናት ለማስተርስ ዲግሪ መመረቂያ ፅሁፍ የሚሆን ነው። በቅድሚያ ስለጥናቱ የሚሆን መረጃ ከሰጠኺችሁ በኺላ በጥናቱ ላይ ተሳታፊ እንድትሆኑ ጋብዟችኺለሁ። በጥናቱ ላይከመሳተፋችሁ በፊት ከሌላ ሰው ጋር ስለ ጥናቱ ተመካክራችሁ መምጣት ትችላላችሁ።በዚህ መረጃ መጠይቅ ወረቀት ላይ ያልገባችሁ ነገር ካለ ጥያቄ መጠየቅ ትችላላችሁ። ስለ ጥናቱ መነጋገር ከፈለጋችሁም ከላይ ባለው ስልክ መነጋገርም አንችላለን። የጥናቱ ጥቅም፡ ሂዩማን ፓፒሎማ ቫይረስ የማህፀን ጫፍ ካንሰር አሚጨ ተዋህሲያን ነው። የማህፀን ጫፍ ካንሰር ደግሞ በኢትዮጲያ ውስጥ በሁለተኛ ደረጃ ተስፋፍቶ የሚገኝ የካንሰር አይነት ነው። ሰለዚህም በጊዜ የማህፀን ጫፍ ካንሰር ምርመራ ማድረግ ለዚህ በሽታ ተጋላጪነትን ይቀንሳል። በመሆኑም በዚህ ጥናት የሽንት ናሙናን ከማህፀን ጫፍ ከሚወሰድ ፈሳሽ ጋር በማድረግ የማህፀን ጫፍ ካንሰርን ስርጭት ለማየት ማገልገል መቻሉን ይሆናል። የጥናቱ ቅድመ ተከተል ፡ በዚህ ጥናት ለመሳተፍ ፍቃደኛ ከሆኑ ከእርስዎ በእራስዎት እጅ የሚሰበሰብ የማህፀን ፈሳሽ ፤ 20 ሚሊ ሊትር ሽንት እና 5 ሚሊ ሊትር ደም እወስዳለሁ።

ከዚህ ባለፈ የፅሁፍ መጠይቅም አብሮ ተካትቷል። ይህን ለማድረግ ቢበዛ 30 ደቂቃ ቢፈጅ ነው። **ጉዳት፡** ከላይ የተጠቀሱትን ናሙና በመስጠትዎ የሚደርስቦት ጉዳት አይኖርም።

ሚስጥራዊነት፡- የእርስዎን ሚስጥር ለመጠበቅም ሁሉም የሚሰጡት መረጃ በሚስጥር የተጠበቀ ነው።በፅሁፍ መጠይቅ ላይ የሚጠየቁትን ጥያቄ የሚመልሱት ከራስዎ ሂወት ልምድ አንፃር ይሆናል። በጥናቱ ላይ ተሳትፎዎን በፈለጉበት ሰዓት ማቋረጥም ይችላሉ።ይህን በማድረግዎ የሚደርስብዎት ነገር የለም።

ጥቅም፡- በዚህ ጥናት ለእርስዎ በቀጥታ የሚከፈል ወይም የሚያገኙት ጥቅም ባይኖርውም ውጤትዎን ግን ደውለን እናሳውቆታለን። ከዚህ ባለፈ ከዚህ ጥናት ከሚገኘው ውጤት/መረጃ/ ሁላችንም ጠተቃሚ የሚያደርግ እና ለማህፀን ጫፍ ካንሰር መመርመሪያ ጥሩ፤ ቀላሉ የመመርመሪያ ዘዴ የትኛው እንደሆነ ማወቅ ያስችለናል።

ለተሳታፊዎች ፍቃድ መጠየቂያ

የተሰጠውን መረጃ አንብቤዋለሁ ወይም ተነቦልኛል። ስለ ጥናቱ አላማ በደምብ ተረድቻለሁ። ጥያቄ ለመጠየቅም ችያለሁ። የጠየኩትን ጥያቄም በደምብ ተመልሶልኛል። ለጥናቱ የሚያገለግለውን በእራስዬ እጅ የሚሰበሰብ የማህፀን ፈሳሽ ፤ 20 ሚሊ ሊትር ሽንት እና 5 ሚሊ ሊትር ደም ለመስጠት ተስማምቻለሁ። በዚህ ጥናት ላይ መሳተፍ በፍቃደኝነት እንደሆነ እና በፈለኩበት ጊዜ ማቋዋረጥ እንደምችል ተረድቻለሁ። በዚህ ጥናት ለመሳተፍ ፍቃደኛ ነኝ።

የተሳታፊ ፊርማ_____ ቀን_____

የጥናቱ ተመራማሪ ፊርማ_____ ቀን_____

11.2 Questionnaire

Title: HPV in women with severe cervical cancer lesion and cancer: the use of urine as an adjunct specimen

Patient name _____ Date of interview _____

Patient card number _____ Study ID _____

Patient phone number _____

Demographic data

1. Residence
   ☐ Urban ☐ Rural
2. Age in years _____
3. Educational Status
   ☐ Illiterate
   ☐ Read and write
   ☐ Primary school
   ☐ High school
   ☐ Certificate
   ☐ Diploma certificate
   ☐ College or university and above
4. What is your occupation/source of income?
   ☐ House wife
   ☐ Self employed
   ☐ Private employee
   ☐ Merchant
   ☐ Farmer
   ☐ Unemployed
   ☐ Other specify _____
5. What is your average income? _____
6. Marital status
   ☐ Single
   ☐ Married
   ☐ Divorced
   ☐ Widow
   ☐ Other, specify _____

Reproductive history

1. Age at first sexual intercourse_____

2. Have you ever been pregnant?

Yes ☐ No ☐

3. Age at first pregnancy_____

4. How many children do you have? _____

5. Have you ever used family planning method?

Yes ☐ No ☐

6. If yes, which of family planning method you were using?

Pills, Duration _____
IUCD _____
Injection _____
Condom _____
Others, specify _____

7. Have you ever had sexually transmitted infections? Syphilis? HIV?

Yes ☐ No ☐

Cervical cytology request form

1. Patient name_____
2. Participant ID _____
3. Age _____
4. Residence address _____
5. Hospital number _____
6. Clinical Hx _____

☐Date of last menses

☐Pregnant

☐Postpartum

☐Premenopausal

☐Postmenopausal

7. Previous pap _____
8. Contraceptive

☐Oral

☐IUCD

☐Other

☐None

9. Clinical diagnosis _____
10. Present complaint _____
11. Collection date _____
12. Physician name _____

11.3 SOP

11.3.1 Cervical sample collection

Title: Cervical sample collection and processing
Document type: standard protocol of operation (SOP)
Written by: Brhanu Teka & Ededia Firdawoke
Date :2/7/2019

DAY 1

1. Clean the work surfaces
   Use alcohol to clean the working bench and place a new green working paper.

2. Prepare the materials needed
   Make sure you have enough 2ml Eppendorf tubes and gloves.

3. Take out the Evalyn brushes

4. Label the 2ml Eppendorf tubes according to numbers of Evalyn brushes

5. Using the 2ml Eppendorf tube remove the Evalyn brush
   Place the brush inside the tube, close the cap of the tube and pull

6. **Change gloves between each brush!!!**
   Very important step to prevent cross-contamination

7. Fill each tube with 1ml of PBS
   Change tips between samples

8. Vortex the tubes with brushes vigorously for 1min

9. Keep the tubes at room temperature overnight (or if longer at 2-8C)

DAY 2

10. The next day: Prepare new 2ml and 1.6ml Eppendorf tubes for each brush
    Label according to brush numbering

11. Vortex the tubes with brushes vigorously for 1min

12. Centrifuge for 5min at 2500rpm

13. By using the 1000µl pipette tips (non-filter) remove the brush from the tube careful not to spill around
    Place the tip of the 1000µl pipette tip inside the top of the brush, and by tipping carefully place in a new 2ml Eppendorf tube you already prepared

14. Save the tubes with brushes at -20C

15. After removing the brush, under the hood **thoroughly mix the left-over PBS (re-suspend the pallet)** and aliquot 100ul into a 1.6ml Eppendorf tube you already prepared.

Note: Make sure the Hood has been sterilized with UV light before use. If not turn UV on for 10min

16. The leftover of PBS is to be stored at -20C

17. The tubes with 100ul are now ready to be extracted.

18. Continue with SOP "DNA Extraction" or store at 2-8C for shorter period. For longer storage of aliquots keep them at -20C.

11.3.2 Urine sample collection

Title: Urine sample collection and processing
Document type: standard protocol of operation (SOP)
Written by: Ededia Firdawoke
Date :2/7/2019

Procedure

1. Unscrew the tube cap
2. Screw the collector tube on the colli-pee® device
3. Collect urine: The device will automatically collect the correct volume.

   There is no need to interrupt the urine stream.
4. Disconnect the collector tube from the colli-pee®
5. Close the tube tightly. You will feel and hear a "click" upon correct closure.
6. Dispose of the Colli-Pee®

7. Wash your hand
8. Mix the specimen with UCM (urine conservation medium)
9. Samples can be kept at room temperature for 72 hours. If not, put it in refrigerator until further processing.
10. Take samples from the freezer
    - Leave to thaw
11. Transfer 10 ml of urine to 15ml falcon tube and put the remaining 10ml into refrigerator
12. Centrifuge for 5 minutes @ 5000 rpm
13. Discard the supernatant
14. Transfer the pellet into 2ml microcentrifuge tube
15. Centrifuge @14000rpm for 5 minutes
16. Discard the supernatant
17. Suspend the pellet in 180µ l PBS
18. Add 20 µl of proteinase K
19. Add 200 µl of lysis buffer
20. Extract using the Qiagen DNA kit

11.3.3 Nucleic acid extraction

Title: Extraction of Nucleic Acid using Qiagen
Document type: standard protocol of operation (SOP)
Written by: Ededia Firdaoke
Date :10/03/2020

Principle

QIAamp DNA Mini and QIAamp DNA Blood Mini Kits are designed for rapid purification of an average of 6 µg of total DNA (e.g., genomic, viral, mitochondrial) from 200 µl of whole human blood, and up to 50 µg of DNA from 200 µl of buffy coat, 5 x 106 lymphocytes, or cultured cells that have a normal set of chromosomes. The procedure is suitable for use with whole blood treated with citrate, heparin, or EDTA; * buffy coat; lymphocytes; plasma; serum; and body fluids.

Note: Samples may be either fresh or frozen

Reagents and material needed

- suitable lab coat
- Disposable gloves,
- protective goggles
- Ethanol (96–100%) *
- 1.5 ml microcentrifuge tubes
- Pipet tips with aerosol barrier
- Microcentrifuge (with rotor for 2 ml tubes)
- Vortexer
- Water bath or heating block at 56°C
- Phosphate-buffered saline (PBS) may be required for some samples

Preparation of reagents

A) **QIAGEN Protease stock solution** (store at 2–8°C)

- When using the QIAamp DNA Blood Mini Kit (50), pipet 1.2 ml protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label.
- When using the QIAamp DNA Blood Mini Kit (250), pipet 5.5 ml protease solvent into the vial containing lyophilized QIAGEN Protease, as indicated on the label.

Note: Dissolved QIAGEN Protease is stable for up to 12 months when stored at 2–8°C.

B) **Buffer AL**† (store at room temperature, 15–25°C)

- Mix Buffer AL thoroughly by shaking before use.
- Buffer AL is stable for 1 year when stored at room temperature.

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

C) **Buffer AW1**† (store at room temperature, 15–25°C)

- Buffer AW1 is supplied as a concentrate.
- Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.
- Buffer AW1 is stable for 1 year when stored closed at room temperature.

D) **Buffer AW2*** (store at room temperature, 15–25°C)

- Buffer AW2 is supplied as a concentrate.
- Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.
- Buffer AW2 is stable for 1 year when stored closed at room temperature.

Note: * Contains sodium azide as a preservative; † Contains chaotropic salt.

Amounts (volume) of starting material

- Use the amounts of starting material indicated below

Sample	Amount
Blood, plasma, serum	200 µl
Buffy coat	200 µl
Tissue	25 mg*
Cells (diploid)	5 x 10^6 cells

Note:

- Small samples should be adjusted to 200 µl with PBS before loading. For samples larger than 200 µl, the amount of lysis buffer and other reagents added to the sample before loading must be increased proportionally.
- Application of the lysed sample to the QIAamp Mini spin column will require more than one loading step if the
- initial sample volume is increased. The amounts of Buffer AW1 and Buffer AW2 use in the wash steps do not need to be increased.

Things to do before starting

■ Equilibrate samples to room temperature (15–25°C).

■ Heat a water bath or heating block to 56°C for use in step 4.

■ Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.

■ Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions.

■ If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C

Procedure

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2. Add 200 µl cervical sample to the microcentrifuge tube.

Note: If the sample volume is less than 200 µl, add the appropriate volume of PBS.

Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C for 10 min.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s.

7. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

Note: If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

8. Carefully apply the mixture from step 7 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim.

9. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*

Note: Close each spin column to avoid aerosol formation during centrifugation.

10. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim.

11. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.

12. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate. *

13. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

14. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate.

15. Centrifuge at full speed for 1 min.

Note: This step helps to eliminate the chance of possible Buffer AW2 carryover.

16. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water.

17. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

11.3.4 Master mix preparation

Title: Anyplex^{TM} II HPV28 Master mix preparation
Document type: standard protocol of operation (SOP)
Written by: Ededia Firdawoke
Date :10/03/2021

Principles

Anyplex TM II HPV28 Detection represents Seegene's proprietary technologies and is based on a TOCETM technology which makes it possible to detect multi-pathogens in a single fluorescence channel on real-time PCR instruments.

In current melting curve analysis, temperature differences are often observed among DNAs that have high sequence variation, resulting in issues the field of clinical diagnostic where accurate and reproducible test results are critical. However, TOCETM technology is designated not to be affected by sequence variations; therefore, it guaranteeing consistent Tm values.

AnyplexTM II HPV28 Detection can perform multiplex examination by either End point-CMTA (End point-Catcher Melting Temperature Analysis) or cyclic-CMTA (cyclic-Catcher Melting Temperature Analysis) method. cyclic-CMTA method which represents a new class of molecular tests can discriminate major pathogen in the co-infected samples.

The AnyplexTM II HPV28 Detection is a multiplex real-time PCR assay that permits the simultaneous amplification, detection and differentiation of target nucleic acids of 19 high-risk HPV types (16, 18, 26, 31,33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70) as well as Internal Control (IC)

Reagents

Anyplex™ II HPV28 Detection			
Symbols	**Contents**	**Volume**	**Description**
PRIMER	4X HPV28 A TOM	500 µL	TOCE Oligo Mix (TOM): - Amplification and detection reagents
PRIMER	4X HPV28 B TOM	500 µL	TOCE Oligo Mix (TOM): - Amplification and detection reagents
PREMIX	EM1	500 µL X 2	- DNA polymerase - Uracil-DNA glycosylase (UDG) - Buffer containing dNTPs
CONTROL +	HPV28 PC1	100 µL	Positive Control (PC) : - Mixture of pathogen clones
CONTROL +	HPV28 PC2	100 µL	Positive Control (PC) : - Mixture of pathogen clones
CONTROL +	HPV28 PC3	100 µL	Positive Control (PC) : - Mixture of pathogen clones
WATER	RNase-free Water	1,000 µL X 2	Ultrapure quality, PCR-grade

Storage and Handling

- All components of the Anyplex™ II HPV28 Detection should be stored at ≤-20℃.

- All components are stable under recommended storage conditions until the expiry date stated on the label.
- The performance of kit components is not affected for up to 5 freezing and thawing.
- If the reagents are to be used only intermittently, they should be frozen in aliquots.

Materials required but not provided

- Disposable powder free gloves (latex or nitrile)
- Pipettes (adjustable) and Sterile pipette tips
- 1.5 mL microcentrifuge tube
- Nucleic acid extraction kit (see Nucleic Acid Extraction)
- CFX96TM Real-time PCR system (Bio-Rad)
- Vortex mixer
- Optical Flat 8-Cap Strips (Cat. No. TCS0803, Bio-Rad)
- Ice Maker
- Desktop centrifuge
- 96-Well Skirted PCR Plate, white well (Cat. No. HSP-9655, Bio-Rad)
- PX1 PCR plate sealer (auto-sealer, Cat. No. 181-4000, Bio-Rad)
- Clean bench

Preparation for Real-time PCR

The correct tubes and caps must be used (see MATERIALS REQUIRED BUT NOT PROVIDED).

Note: Aerosol resistant filter tips and tight gloves must be used when preparing specimens. Use an extreme care to ensure no cross-contamination.

Note: Completely thaw the reagents on ice.

Note: Briefly centrifuge the reagent tubes to remove drops from the inner cap.

A. Prepare PCR Master mix**.**

- **Prepare two 1.5mL microcentrifuge tube and labelled as 'A' and 'B'**

5 µL	4X HPV28 A TOM or B TOM
5 µL	EM1
5 µL	RNase-free Water
15 µL	Total volume of PCR Mastermix

Note: Calculate the necessary amount of each reagent needed based on the number of reactions (samples + controls).

B. Mix by inverting 5 times or quick vortex, and briefly centrifuge.

C. Aliquot 15 µL of the PCR Master mix into PCR tubes.

D. Add 5 µL of each sample's nucleic acids into the tube containing PCR Master mix

15 µL	PCR Mastermix
5 µL	sample's nucleic acid
20 µL	Total volume of reaction

Note: Use a new sterile pipette tip for each sample.

Note: For Negative Control (NC), use 5 µL of RNase-free Water instead of sample's nucleic acid.

Note: For Positive Control (PC), use 5 µL of each HPV28 PC1, PC2 and PC3.

Note: Please be careful not to cross-contaminate the PCR Master- mix and samples with the Positive Control.

Note: Do not label the cap of the reaction tubes as fluorescence is detected through the cap.

11.3.5 Pap smear

Title: Papanicolaou (PAP) Stain
Document type: Standard protocol of operation (SOP)
Written by: Ededia Firdawoke
Date :2/7/2019

Procedure

1. Place slide in 95% alcohol for 5 minutes.
2. Place slide in 70% alcohol for 5 minutes.
3. Place slide in 50% alcohol for 5 minutes
4. Place slide in distilled water for 2 minutes
5. Apply adequate Modified Mayer's Hematoxylin to completely to cover cell smear and incubate for 5 minutes.
6. Rinse slide 1 time in distilled water to remove excess stain.
7. Rinse slide in tap water for 2 minutes.
8. Rinse slide in 2 changes of distilled water.
9. Dip slides several times in 95% alcohol and blot excess off
10. Apply adequate OG6 Stain Solution to completely cover cell smear to excess and incubate for 2 minutes.
11. Rinse slide gently using absolute alcohol.
12. Apply adequate EA50 Stain Solution to completely cover cell smear to excess and incubate for 3 minutes.
13. Rinse slide gently using absolute alcohol.
14. Quickly dehydrate slide in 3 changes of absolute alcohol.
15. Rinse slides into xylene
16. Mount in synthetic resin/DPX.

11.3.6 HPV 16 L1 Anticapsid antibody test

Title: HPV 16 L1 Anticapsid antibody test
Document type: Standard protocol of operation (SOP)
Written by: Ededia Firdawoke
Date :2/7/2020

Procedure

1. Take a tube with HPV reagent and to it add 1 drop (25ug) of serum.
2. Mix the sample with the HPV reagent by repeatedly pipetting up and down.
3. Seal the tube. Repeated shaking will ensure that any residual fluid from the HPV reagent remaining in the tube lid is included in the mixing process
4. Lightly cap the base of the sealed tube once against a solid surface in order to return any reagents stuck in the lid
5. Place the tube into a holder and allow the mixture to incubate for 10 minutes
6. Dispose the first pipette
7. Remove the test cassette from the foil pouch and place the cassette onto a flat surface. Use it as soon as possible, within one hour at the most
8. Take the second pipette and insert 4 drops of the mixture from the tube into the sample opening (S) of the test cassette. While doing so, avoid trapping any air in the sample opening (S) and do not put any fluid into the reaction field.
9. Start timer
10. Assess the test results after 10 minutes. If more than 15 minutes have elapsed, you must no longer assess the results. This is in order to avoid erroneous results!

11.4 Declaration

I hereby declare that this MSc dissertation is my original work. I have written and submitted to AAU-CHS Department of microbiology, Immunology and Parasitology never to any other institution in any form for evaluation. All the information here is dully acknowledged and have never used any other source except those cited ones.

Name: Ededia Firdawoke

Signature: _____

Date _____

Title: HPV in women with pre-cancerous lesion and cervical cancer: the use of urine as an alternative specimen

This thesis has been submitted with our approval as advisors.

Tamrat Abebe (PhD, Assistant professor) _____

Brhanu Teka (Msc, PhD candidate) _____