Title: Point of care, cervical cancer diagnostic test strip that detects HPVs 16 and 18 and their oncoproteins E6 and E7 in first void urine.

FOA: PAR-17-441

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DECLARATION

I, Nalwoga Christine, declare that this proposal is original and has never been submitted to any university or institution for any award.

I therefore submit it for award of Bachelor’s degree in Biomedical Engineering at Makerere University.

PRINCIPAL INVESTIGATOR

Date: 18/12/2018

NALWOGA CHRISTINE

This research proposal has been submitted with the approval of my supervisor.

Signature: 

Date: 04 DEC 2018

MR. JULIUS MUGAGA
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Abstract

Cervical cancer is the second most common cause of cancer-related deaths among women worldwide, with more than 80% of the cases in the developing world. Cervical cancer screening and immediate treatment action reduces its overall prevalence. Various methods have been authorized for early detection of cervical cancer with the most common ones being the pap smear test and cytology test. Although these methods are accurate and, in most cases, diagnose cervical cancer, they necessitate invasive sample acquisition from the cervix thus cause extreme discomfort, are slow to produce results, require skilled personnel and appropriate tools, equipment and infrastructure to produce accurate results making them expensive; these characteristics make cervical cancer screening unpopular for women in low and middle-income countries.

First void urine of 100 women between the ages of 21 and 45 years having a positive cervical cancer diagnosis in Kampala, Uganda will be collected and tested for presence of cervical cancer biomarkers; high risk human papillomaviruses (hrHPVs) 16 and 18 as well as their E6 and E7 oncoproteins, using polymerized chain reaction restriction-fragment length polymorphism (PCR-RELP) for the HPVs and Pretect® HPV-Proofer assay for the oncoproteins; presence of these factors in cervical lesions confirms cervical intraepithelial neoplasia (CIN) II, III and invasive cervical cancer. Ability to detect these biomarkers in urine confirms it as an alternative sample to diagnose cervical cancer. A point of care (POC) lateral flow assay (LFA) strip designed with nanoparticle technology, capable of identifying the aforementioned parameters in urine will be designed; appropriate nanoparticles and LFA formats will be chosen. We expect hrHPVs 16 and 18 and their E6 and E7 oncoproteins to be detected in urine and a POC strip capable of effectively detecting them, successfully designed. An effective POC strip capable of effective cervical cancer diagnosis will introduce a faster, non-invasive, affordable, convenient and more socially acceptable technology for use in LMICs, reducing cervical cancer prevalence. Upon receiving funding, we will design and implement this technology first in Uganda and then the rest of East Africa, therein increasing enrollment of patients for cervical cancer diagnosis, reduce time taken to achieve results, increase early treatment hence reduce cervical cancer prevalence.

Key words: cervical cancer, human papillomavirus (HPV), high risk HPVs (hrHPVs), first void urine, oncoproteins, cervical intraepithelial neoplasia (CIN)
Project narrative

The proposed project is relevant to the health sector because a point of care non-invasive system for diagnosing cervical cancer is an alternative faster and more convenient method compared to the current invasive, slow methods. More women in LMICs will be encouraged to get tested, reducing the prevalence of cervical cancer in developing countries. Thus, the proposed research is relevant to the part of the NCI’s mission to fund breakthroughs in development of innovative techniques, agents, methodologies, models, or their applications.
SPECIFIC AIMS
Cervical cancer is the second most common cause of cancer-related deaths among women worldwide[1], with more than 80% of the cases in the developing world[2]–[5]. This cancer emerges from cervical intraepithelial neoplasia (CIN), induced by infection with high risk types of human papillomavirus (HPV) [2], [6]. Human papillomavirus DNA (HPV-DNA) is detected in 10%–90% of cervical cancer specimen[7]. HPVs 16, 18 and 45 are most prevalent and may present tumors early[5], [8] with HPV-16 and 18 termed as class 1 carcinogens[9]; HPV16 accounts for 50% to 60% of the cervical cancer cases in most countries, followed by HPV18 (10%–12%) and HPVs 31 and 45 (4%–5% each)[7]. However, these same HPV-DNAs were found in 46% of the population with normal cervical smears[7], hence further studies are needed to specify the conditions and circumstances in which these HPV types cause cancer[8]. Furthermore, most CIN lesions regress with time, with only a few persisting to invasive carcinoma[6]. HPV-DNA screening, cytotology screening and pap smear test are used to detect precancerous lesions and treat them before they progress to invasive cancer[4], [10]–[12], recommended at the onset of sexual activity and at ages 21 and above. Pap smear test is the first line of screening approved and recommended for women aged 21-34 years[5], [13] despite having low sensitivity and a high false negative rate[1]. HPV-DNA screening is most recommended for verifying presence or absence of carcinogenic lesions for women above the age of 35 years[5] and only done for ages below that for cases of an abnormal pap test[7]; HPV-DNA screening alone leads to over-diagnosis for women between the ages of 25-34 years[7], [14]. For most developing countries however, these currently approved methods of screening are un popular, less frequent and hence ineffective for diagnosis of precancerous lesions because <5% of the population is screened for cervical cancer compared to the 45-50% of women in the developed world[3]. This is because of a lack of policy guidelines, adequate skill set, shortage of staff and test materials in the health facilities[3], [15] in addition to the people’s unwillingness to test due to the invasive nature of testing [16], [17] and exposure un common in such regions due to the conservative nature of the people. There is need for a faster, more affordable and socially acceptable method for screening of cervical cancer in low and middle-income countries (LMICs).

Innovation: To develop a non-invasive, affordable, and socially acceptable point of care (POC) testing diagnostic tool to diagnose presence or absence of cervical cancer by positively identifying high risk types of HPV and viral oncoproteins E6 and E7, present in the first void urine sample taken from women in LMICs. We hypothesize that this technology will reduce diagnostic time by 5 folds, making diagnosis timely, increase enrollment numbers for cervical cancer testing by 75% hence treatment. Approach: A nanoparticle-based test paper strip utilizing the lateral flow assay (LFA) technique will be used to test for the presence of HPV 18 and 16 oncoproteins E6 and E7. These proteins play an important role in HPV-dependent malignant transformation[1], [12]. They impair control of cell cycle regulation and maturation hence responsible for uncontrolled cell proliferation that leads to cases of high-grade and low-grade CIN lesions[18]–[20]. Their presence in urine/cervical smears confirns presence of CIN[17] increasing the specificity and sensitivity of the tests in screening for high risk CIN lesions[12], [15]. POC systems have become the most famous diagnostic tools due to their ability to produce prompt results in shorter times, ease of use, low cost, and little need for specialized equipment [21] with POC based FLA devices rapidly growing as strategies for qualitative and quantitative analysis[21]. Detection of HPV-DNA using first void urine provided a 22-fold increase in accuracy when compared to testing using other urine stages or entire urine sample as a whole[17] since the spontaneously exfoliated cells from the cervix with persistent infection are most likely carried away in the first void urine.

Specific aim 1: To confirm presence of HPV 18 and 16 and their viral oncoproteins E6 and E7 in the first void urine of women positively diagnosed with CIN II, CIN III or invasive cervical cancer in East Africa. First void urine of women already positively diagnosed with invasive cervical cancer will be tested using Pretect® HPV-Proofer assay that uses real-time multiplex Polymerase Chain Reaction (PCR) to amplify E6/E7 transcripts hence identify their presence in solution[12]; this assay has high sensitivity and identifies E6/E7 for HPVs 16 and 18. PCR-RFLP (restriction-fragment length polymorphism) is the preferred technique for identifying hrHPVs 16 and 18 because it is highly sensitive, specific, easy to use, simple, robust, does not require sophisticated equipment and is affordable for LMICs[12]. Specific aim 2: To design a POC test strip using nanoparticles and Lateral Flow Assay (LFA) technology, capable of identifying E6 and E7 antibodies. Different types of nanoparticles: organic (micelles, dendrimers, liposomes, hybrid and compact polymeric NPs) and inorganic (fullerenes, quantum dots, silica and gold nanoparticles)[22] and various FLA formats: sandwich format, competitive format, and multiplex detection format [21] will be analyzed for functionality, sensitivity and specificity of use in designing a strip.
Cervical cancer is the second most prevalent cancer worldwide with developing countries accounting for four-fifths of the occurrences[23]; 527,624 new cancer cases and 265,672 deaths were estimated in 2012[20], [4], with about 450,000 new cases diagnosed each year[18]. This cancer has three histological types; squamous cell carcinoma, adenocarcinoma, and adenosquamous cell carcinoma with adenocarcinoma being the most common[8]. Cervical cancer screening has led to a reduction in cervical cancer cases in most developed countries[4] because the precancers are identified and treated before invasion[14]. However, this cancer has remined largely uncontrolled in high-risk developing countries due to ineffective or lack of screening[4] since less than 5% of the population in developing countries is screened for cervical cancer compared to the 45-50% of women in the developed world[3]. Most screening methods test for presence of the human papillomavirus (HPV) DNA in the cervix, where existence of high risk HPV (hrHPV) types confirms cervical intraepithelial neoplasia (CIN), a condition from which cervical cancer emerges[6]. CIN has grades I, II and III with most of them regressing with time mostly for low grade CIN I; cervical cancer usually develops from about 10% to 20% of high grade CIN III cases. 118 different HPV types have been isolated and sequenced, and about 40 of these are known to infect the genital tract, with 12 classified as carcinogens[8] and present in CIN.

HPVs are epitheliotropic viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia[16]. HPV types 16, 18, 31, 33, 45, 52 and 58 have shown the most prevalence in cervical cancer cases hence classified as hrHPV with HPV 16 and 18 being the most frequently identified for all histological cervical cancer types[8] and are responsible for approximately 70% of cervical cancer cases[24]. In order to be accepted, a biomarker should have a positive predictive value (PPV) and negative predictive value (NPV) of at least 20% and 98% respectively. No cervical cancer biomarker has yet been identified that satisfies this criterion and detects clinically relevant precancerous lesions. Van Keer et al. recommended using more than one biomarker of different categories but which complement each other in order to target different parts of the cancer for its detection[20]. HPVs 16 and 18 and their early proteins E6 and E7 have been selected as the biomarkers for this study. E6 and E7 proteins on the HPV-DNA strand are responsible for excessive cell proliferation leading to development of precancerous lesions; E6 connects to the p53 protein, a tumor suppressor and stimulates its proteolytic degradation while E7 binds and inactivates the pRb (retinoblastoma proteins) leading to its deterioration, hence loss of control over the cell cycle[19], [25]. Currently authorized screening techniques include: the pap smear test, a first line of diagnosis that confirms presence or absence of high risk HPV-DNA usually followed by colposcopy to obtain a sample of cells from the cervix, in case of positive HPV identification in order to ascertain infection. Other screening techniques comprise visual inspection using acetic acid (VIA) and cytology smears. All these screening procedures require a well facilitated and staffed medical facility which are uncommon in developing countries. Also, the methods are invasive, uncomfortable, time consuming and expensive for people in low and middle-income countries (LMICs)[3]. There is thus a dire need for a non-invasive, affordable, point-of-care (POC) technique for cervical screening, in order to increase acceptance levels in developing countries[11]. It is assumed that by making screening of cervical cancer more readily available in such a manner, more women in developed countries will be encouraged to participate as well as take part in additional follow-up procedures in case they test positive, reducing cervical cancer prevalence.

Utilizing a lateral flow assay (LFA) paper strip to detect presence of HPV-DNA in first-void urine, in order to diagnose cervical cancer fits the aforementioned requirements. LFAs, according to Geertruida et al., are prefabricated strips of a carrier material containing dry reagents, that are activated by applying the fluid sample[26]. The LFA strip usually has four components: sample application pad where the sample is placed to start assay, allowing for distribution to other components of the strip; conjugate pad, whose nature determines sensitivity of assay and where labelled biorecognition molecules are distributed; nitrocellulose membrane houses the test and control lines and is also critical in determining sensitivity of LFA; adsorbent pad which maintains flow rate of the sample, wicks the sample to the end of the strip and prevents its backflow[21], [26]. Urine has already been successfully used as a means of detection of some sexually transmitted diseases like chlamydia trachomatis and Neisseria gonorrhoea and according to a study conducted by Neha Pathak et al., detection of HPV-DNA in urine has a good accuracy for cervical cancer screening[17], with sensitivity and specificity estimated to be as high as 91.2% and 96.3%, respectively[11] for first-void urine, is non-invasive and easy to collect even in large sample volumes[20]. First void urine is generally preferred since scrapes of CIN and cervical cancer lesions are mainly concentrated there. Studies show that no emphasis should be placed on the time of day for collecting the first-void urine as samples from any time have the same effectivity[24].
Innovation
The project is innovative because it proposes a rapid home-care diagnostic technique for cervical cancer which is non-invasive, more socially acceptable and affordable for women in LMICs. A point of care diagnostic LFA strip for diagnosing cervical cancer using first void urine sample is a novel technology in this particular field; POC strips have found varying success in other fields. This technique is convenient since it utilizes urine instead of cervical extraction of samples for accurate diagnosis. Invasive diagnostic techniques and procedures are extremely discomforting, expensive, time consuming to get results and require skilled personnel and appropriate infrastructure, but are the only method currently verified for correct cervical cancer diagnosis[17]. Also, this technique seeks to identify HPVs 16 and 18 and their E6 and E7 oncoproteins as the biomarkers to diagnose the cervical cancer.

Approach
The study is going to be experimental, focused on analyzing prevalence of HPV 16 and 18 among women positively diagnosed with CIN and analyzing for the presence of these HPVs and their oncoproteins E6 and E7 in the urine of these women. The study will be longitudinal spanning a duration of two years; semi-structured interviews will be conducted with medical personnel as well as volunteers for the testing, while qualitative and quantitative data will be collected and analyzed, utilizing primary and secondary data. The first phase will consist of reaching out, meeting and addressing women positively diagnosed with high grade CIN II and III or invasive cervical cancer and getting their informed consent to carry out tests. The second phase will constitute gathering 3 sets of first void urine samples for these women. This urine will be tested for presence of HPVs 16 and 18 and their E6 and E7 oncoproteins. In the third phase, an LFA strip will be designed having the ability to recognize the HPV-DNAs and the oncoproteins in a given urine sample.

Study setting
The field of study will be in Kampala district with the data and sample collection and analysis of the samples taking place at Mulago National Referral Hospital and the microbiology department of Makerere University College of Health Sciences’ department of physiology respectively. Kampala is the capital and largest city of Uganda with a population of 1,208,544, located at 0°19′N 32°35′E, at 3,900 ft (1,189 m) above sea level[27]. Kampala was chosen because according to a survey in 1997, 40.8 per 100000 cervical cancer deaths were noted there[3], showing a high prevalence of the condition; Kampala is also the most convenient in terms of accessibility by the members. The cancer department at Mulago hospital has very skilled personnel to effectively supervise sample collection, is sanitary and spacious, giving ample room for collecting samples. About 50 women are diagnosed for cervical cancer weekly at Mulago; this increases sample population to choose from. We chose to have the tests on the samples carried out in the microbiology laboratories due to its proximity to Mulago hospital hence increased chances of safe sample travel. The laboratory is also well equipped with instrumentation and apparatus capable of aiding in carrying out the experimental procedures and tests and their analysis and has skilled and competent staff capable of operating the equipment. This microbiology department offers services of: hematology, histopathology, immunology, microbiology, molecular biology, chemical pathology, and virology.

Study population
The study population will be sexually active women aged 21 years to 45 years. This category of women is capable of personal decision making and are most likely to have acquired HPV sexually; CIN II, III and cervical cancer tend to prevail in this particular age group. Studies show that women of ages between 21 years and 34 years may be privy to contracting HPV but are not at high risk of acquiring cervical cancer as those of ages 35-45 years[13]. This is because cervical cancer tends to metastasize a long while after HPV acquisition.
Methods

**AIM 1:** To confirm presence of HPVs 18 and 16 and their viral oncoproteins E6 and E7 in first void urine of women positively diagnosed with CIN or invasive cervical cancer in East Africa.

**Purpose.** Loose cervical lesion cells are washed away by urine and mainly found in maximum quantity in first-void urine as it carries the content of most of the washed-out cells previously resting in the cervical canal. Other stages of urine tend to dilute these cells making identification more cumbersome. This choice of analysis, though used previously to verify presence of HPV-DNAs, has not been utilized for detection of the oncoproteins E6 and E7 for women with CIN lesions or cervical cancer. Therefore, the following must be known before further proceedings: can HPVs 16 and 18 and oncoproteins E6 and E7 be detected in the urine and if so can they be qualitatively and quantitatively measured and analyzed? The *working hypothesis* is that the ability to detect the aforementioned oncoproteins with their HPVs 16 and 18 will ease detection of cervical cancer because of ease of urine accessibility as well as accuracy of detection due to the many biomarkers identified.

**Methodology.** A QIAamp viral RNA mini kit from Qiagen will be used to extract DNA from the urine[16]. PCR-RFLP (restriction-fragment length polymorphism), will then be used to discriminate HPV mRNAs and hence determine the prevalence of integrate-derived hrHPVs 16 and 18 transcripts in the cell samples. Pretect® HPV-Proofer assay will be used to detect E6/E7 in the urine. This assay has a high sensitivity and utilizes the chemistry of transcription-mediated amplification of full-length E6/E7 transcripts preempted by target capture[12].

**AIM 2:** To design a POC test strip using nanoparticles and Lateral Flow Assay (LFA) technology, capable of identifying E6 and E7 antibodies of HPV 16 and 18.

**Purpose.** POC test strips have been designed successfully using LFA and nanoparticles to test other parameters. However, none has been designed yet specially targeting testing of hrHPVs and their oncoproteins in urine. The questions asked are, Can the strip positively the identify presence the HPVs 16 and 18 as well as their E6 and E7 oncoproteins in urine and clearly display distinguishable results acquired? The ability of the strip to perform efficiently and effectively will verify its ability to detect given parameters hence diagnose invasive cervical cancer and CIN II and III.

**Methodology.** A multiplex detection format of LFA strip is the format of choice since it enables testing of multiple interdependent target species, responsible for detecting the stage of the disease[21]. This is convenient since HPV 16 and 18 need to have the ability to be detected as well as their E6 and E7 oncoproteins. The labels will be made from gold nanoparticles coated with respective antibody. Gold nanoparticles, prepared using Turkevich method, were selected owing to their fluorescent capability, affordability and colloidal stability in solution when attached i.e. not influenced by the in vivo conditions[22][28].

**Project timeline**

**Table 1: Timeline for project activities**

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<th>Pre-implementation phase (7 months)</th>
<th>Timing</th>
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<tr>
<td>Submit IRB application; mobilize team, identify subsequent activities and plan for them (research team)</td>
<td>1 month</td>
</tr>
<tr>
<td>Partner with Mulago national referral hospital to identify, reach out and address women with a positive diagnosis for cervical cancer or CIN II and III, to implore them to take part in the study as test patients</td>
<td>3 months</td>
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<tr>
<td>Survey the designated areas where sampling and testing shall take place ensuring that all standards are met and making an inventory of all available and other required items for the study (research team)</td>
<td>1 month</td>
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<tr>
<td>Finalize consent forms, data collection forms and survey instruments; obtain final IRB approval (research team)</td>
<td>1 month</td>
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<tr>
<td>Make introductory site visits to the participating clinics to sensitize them about the study and ensure they have appropriate sample collection and storage tools and equipment (research team)</td>
<td>1 month</td>
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<th>Implementation phase (12 months)</th>
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<td>Purchase and supply necessary tools and equipment for sample collection, storage and testing kits</td>
<td>2 months</td>
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<tr>
<td>Collect first void urine samples from the patients and store them at optimal conditions until testing</td>
<td>2 months</td>
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<tr>
<td>Carry out tests on collected urine samples; record and analyze resulting data</td>
<td>4 months</td>
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<tr>
<td>Make observations, deductions and further testing where necessary</td>
<td>2 months</td>
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<tr>
<td>Design, simulate and prototype POC test strip</td>
<td>2 months</td>
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<th>Post-implementation phase (5 months)</th>
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<td>Analyze process and outcome evaluation data and calculate cost of intervention (research team)</td>
<td>2 months</td>
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<tr>
<td>Study closure: initiate journal articles and R01 grant application for development of working POC test strip</td>
<td>3 months</td>
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**Project team**

**PI Professor Moses Joloba,** MD, PhD, current dean school of biomedical sciences, Makerere University College of Health Sciences and current head of laboratories. Has won grants from WHO/TDR, SIDA-SAREC,
NIH and the European Union for TB research ranging from basic to applied and clinical. His research focus is bacterial quorum sensing, TB molecular epidemiology and drug resistance. He is the current head of laboratories and also heads the appointments and promotions committee of the department. In 2003 he established the Molecular Biology laboratory in the Department of Medical Microbiology. This laboratory focused on TB research and in 2006, molecular detection of MTC infections as a routine was introduced for the first time in Ugandan research labs. His research focus is bacterial quorum sensing, TB molecular epidemiology and drug resistance. He is also the current director of Supranational Reference Laboratory Uganda.

**PI Dr Robert Ssekitoleko**, MEng, completed his MEng Degree in Medical Engineering at Queen Mary University of London in 2007. While there, he worked on a project where he developed a device for balancing and measuring forces in the knee during Total Knee Arthroscopy. He then held a Teaching Assistant position at the same university for one year. He completed an EngD in Medical Devices sponsored by the EPSRC at University of Strathclyde, Glasgow were his interest in medical ultrasound led him to collaborate with IMSAT. He is currently a Research Fellow with University of Salford; a Lecturer in Biomedical Engineering at Makerere University from September 2013 to date and a Biomedical Engineer with Uganda Maternal and New-born Hub. He has currently been involved in researches that lead to the development of medical equipment for low resource countries like Uganda.

**Kai Sandvold Beckwith**, started a 3-year postdoc at CEMIR in March 2016. Kai holds a MSc in Nanotechnology from NTNU, and a PhD in biophysics from NTNU, with Prof. Pawel Sikorski as supervisor. During his PhD, Kai developed new micro- and nanostructured surfaces intended for high throughpt cell studies. In particular, he developed systems for surface-based transfection and cell manipulation via nanopillars and microscale cell patterning. The work involved extensive use of the NTNU NanoLab cleanroom facilities as well as advanced cell microscopy methods. At CEMIR, Kai will utilize several complementary modern microscopy methods, such as TIRF, confocal/STED, FRET, FLIM and high throughput imaging, as well as specialized fluorescent dyes and proteins, to characterize processes such as phagocytosis, phagosome maturation and signaling, and phagosome manipulation by pathogens infecting immune cells. He also has selection of recent journal publications, artistic productions, books, including book and report excerpts.

**Mr. Mugaga Julius, BSc.** He recently graduated with a BSc Biomedical Engineering at Makerere University College of health sciences. While there, he participated in many innovative research programs for example FistApp, a phone application designed to predict the percentage risk of pregnancy to have obstructed, won him awards and international acclimation; the Sesari Security Kit, a device for prompt, continuous and mobile monitoring of exhaust gases from vehicles that cause air pollution. His has experience in coming up with and executing novel research technologies that aid people in LMICs.

**Ms. Nalwoga Christine, BSc.,** is currently a student at Makerere University offering a bachelor in Biomedical Engineering. She is part of a team reaching out to teenage school girls in rural areas of Uganda, encouraging them to stay in school through sensitization about menstruation. She carries out internet-based and field visit research in order to support and validate the project ideas, acquiring data collection, analysis and assembly skills. She also interacts and counsels the students, improving her interpersonal skills.

**Environment**

This study will be conducted in two environments; Mulago National Referral Hospital, cancer department and the microbiology laboratories at Makerere University College of health science department of physiology. The microbiology laboratory is well equipped with state-of-the-art equipment capable of accommodating tests of every nature on biological samples, with proper investigation and analysis tools for results obtained. Mulago hospital is the country’s main national referral hospital. It thus provides a large database of patients and cases from which to select the best candidates for the study. In addition, most of the staff are research oriented having

*Ethics statement: All the research strategy and procedures will be approved and carried out in accordance with the standards of Makerere University, School of Medicine, Research and Ethics committee. The committee will go through the proposed methods to see if they can be workable with and will not violate human rights nor cause harm to the society and environment in general. The samples will be collected in a well sanitized and private environment and labelled using ID numbers as opposed to names to preserve patient privacy.*
References


