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Abstract

The goal of CERVIS is to make a substantial, positive impact in the cervical cancer diagnostic space through the development of a minimally invasive, cost effective solution that enables women in low-resource settings to test for cervical cancer on a frugal and effective platform. In the developed world, there are a variety of options that can aid in early detection, including pap smears. However, due to the high cost and laboratory requirements that accompany this procedure, women in low-resource settings rarely have access to this preventative care or regular screenings for cervical cancer. Using new research about the changes in the vaginal microbiome, we aim to create a frugal, visual diagnostic screening tool for early stage cervical cancer as an alternative to the existing expensive, invasive, and clinic-dependent methods. Outcomes will be measured by partnering with a Kenyan NGO to collect data from several clinics.

Keywords

Cervical cancer, HPV, Microbiome

Introduction

Cervical Cancer is a global health issue that accounts for 7.5% of all female cancers worldwide [1]. The World Health Organization estimates that 90% of cervical cancer-related deaths occur in low and middle income countries, most of which could be prevented by HPV screening and early diagnosis [2]. Although cervical cancer is the third leading cause of cancer death in women, there is currently no low-cost, non-invasive screening method available [3]. Early detection of cervical cancer is crucial to save lives. Early detection can limit the the need for invasive procedures, significantly increase the chance for successful treatment, and reduce mortality.

Additionally, the link between Human Papillomavirus and cervical cancer has been thoroughly established. Around one-half of individuals diagnosed with HPV infections have high-risk infections. HPV 16 and HPV 18, two of the most common strains of high risk HPV cause 70% of cervical cancers [1].

The goal of the Cervical Cancer Early Response Visual Identification System (CERVIS) is to detect early stage cervical cancer using changes in the vaginal microbiome during tumorigenesis. Research on the vaginal microbiome is a relatively new field, and studies show that *Fusobacterium* spp. is undetectable in vaginal swabs from a healthy vagina but constitutes

approximately 17% of bacteria in a vaginal sample from a cervical cancer patient. *Fusobacterium* spp. is an anaerobic, gram-negative bacteria usually absent in healthy tissue but upregulated in instances of disease [4]. Fusobacterium nucleatum is associated with inflammatory diseases including appendicitis and ulcerative colitis, as well as multiple cancers, including colorectal, oropharyngeal, and cervical cancers [5].

Studies show no significant difference between the microbial populations between the vagina and the cervix [6]. For this reason, the use of vaginal sampling instead of cervical sampling was included in the device design to reduce the dependency on a trained clinician, which is unique from other cervical cancer detection methods. Women will be able to acquire their own sample in a non-clinical setting. The goal is to keep CERVIS low cost and minimally invasive to adapt to the needs of the target population in low resource areas.

The current target population is Kenya, due to the high incidence and mortality rate of cervical cancer. Cervical cancer ranks as the first most common female cancer in Kenyan women ages 15 to 44 years old with over 5,000 cases diagnosed annually. Kenya also has an extremely high incidence rate of HPV 16/18, as it is more than double that of the United States and other developed countries. Currently, 68% of Kenyan women diagnosed with cervical cancer will die from the disease, making cervical cancer the first leading cause of cancer death in women [7]. Many of these deaths could be prevented by HPV screening and early diagnosis of cervical cancer; however there is a lack of a low cost, non-invasive diagnostic for low resource settings [8].

Limitations of current methods -

Pap smears and colposcopies are the most common methods of cervical screening used in developed nations to accurately detect both precancerous and cancerous cellular processes within a swab sample of the cervix [9]. Analysis of pap smears requires a high degree of technical and clinical knowledge to identify and swab the cervix correctly and analyze sample results and diagnose accordingly. Additionally, expensive equipment to analyze the sample and the availability to a laboratory is required for proper and effective analysis. Additionally, these procedures have a high number of false positives. Finally, on a personal level, pap smears and colposcopy are invasive, painful, and costly [10].

HPV vaccinations in combination with cancer screenings are the greatest preventative measure against cervical cancer [11]. FDA approved vaccinations like Gardasil and Cervarix have been found to be nearly 100% effective at preventing cervical infections of HPV 16/18, the two strains most commonly associated with the development of cervical cancer. The Center for Disease Control recommends that all women through the age of 26 and all men through the age of 21 be vaccinated beginning between the ages of 11-15. While the HPV vaccine provides protection from HPV infection, it does not offer any therapeutic treatment of the virus or cervical cancer

[12]. Additionally, social stigmas prevent many from getting vaccinated [13]. The vaccine, delivered in a three-part series, must be delivered by a health care professional within a clinical setting, making it difficult for those with limited access to clinics and hospitals to receive the full dose of the vaccine. Additionally, the vaccine is costly, further creating a barrier to accessing vaccines in the developing world [13].

Visual Inspection with Acetic Acid (VIA) of the cervix with acetic acid is an effective and inexpensive screening method in poorly resourced areas [14]. Trained health workers and nurses work in mobile screening camps in low income areas to perform this diagnostic test. The test is invasive and can cause some discomfort similar to the pap smear method because of the insertion of a self-retaining vaginal speculum. After insertion, acetic acid is applied to the cervix and watched for a reaction between the suspected lesion and the acetic acid [15]. Similar to the limitations of the previous devices, VIA also requires a high degree of technical knowledge to properly administer the screening, must be done in a clinical environment, and is highly invasive. When administering the test, if acetic acid is not applied properly bubbles may still form and result in false-positives.

Roche CINtec PLUS Cytology is a qualitative immunohistochemistry (IHC) test using the p16 biomarker, which is a cancer biomarker specifically linked to Cervical cancer. The test is conducted using microscopic assessment of $p16^{INK4a}$ protein in formalin-fixed, paraffin-embedded (FFPE) cervical punch biopsy tissues. The test confirms cervical abnormalities based on hematoxylin and eosin (H&E) stained slides. This test is a more specific alternative to pap smear, but is invasive, expensive, requires a clinician, and results in high rates of false positives [16].

Table 1: A Comparison Between Current Screening Devices

Device	**Benefits**	**Limitations**
Pap smear	Gold standard in developed nations	Invasive Expensive Performed in a clinical setting Clinical and technical knowledge required for analysis
Visual Inspection with Acetic Acid (VIA)	Inexpensive Primary screening technique in low resource nations	Invasive Moderate technical knowledge required

		Low sensitivity if administered incorrectly
Roche CINtec PLUS Cytology	Specific for Ki-67 and p16 markers of HPV infection	Invasive, Multiple clinic visits Specific for ≥CIN-2

The limitations of these current screening methods highlight the need for a less invasive and frugal point-of-care diagnostic device. While this device could be used to screen for cervical cancer in a variety of settings around the world, a device, such as CERVIS, would be particularly useful for women in low resource settings with limited access to clinics, health professionals, and other higher-grade medical care.

Methods

Microbial Cultures and Conditions -

F. Nucleatum ATCC 25586 was streaked for isolation using a disposable sterile loop on Anaerobe Systems[1] Brucella Blood Agar (BRU) and Fusobacterium Selective Agar (FSA). These plates were incubated at 37 degrees celsius under anaerobic conditions for 48 hours. Anaerobic conditions were met using an anaerobic chamber, with an internal environment comprised of 90% N2, 5% CO2, and 5% H2. BRU media was chosen as a positive control, supporting the growth of *Fusobacterium spp*. [17]. FSA is an enriched selective medium for the isolation and presumptive isolation of *Fusobacterium* species, chosen to inform the formulation of CERVIS media [18]. The selective character of the FSA media is due to the presence of three antibiotics: vancomycin, josamycin, and neomycin. The concentrations of each of these antibiotics inhibit the growth of most other facultative anaerobes [19].

Optimization of Anaerobicity -

Several cytology applicators- soft bristle brush, polyester swab, and Wallach Papette cervical cell collector brush- were chosen to measure the amount of oxygen introduced into a semi-solid media in a closed glass tube. Anaerobe Systems Anaerobic Transport Media AS-911 includes resazurin, and was used to visually indicate the presence of oxygen in each sample tube. Each cytology applicator was tested under two conditions. In condition A, the cytology applicator handle was cut to fit inside the tube and upon inoculation was left in the tube. The cap was resealed after 15 seconds.In condition B, cytology brushes were inserted into the media and immediately removed. The cap of the tube was sealed after 15 seconds. Tubes under conditions

[1] Media produced by Anaerobe Systems is manufactured and packaged in a deoxygenated environment.

A and B remained sealed at room temperature for 72 hours. Color change, indicated by the redox reaction of resazurin, was measured after 24, 48, and 72 hours.

CERVIS Media

CERVIS Media was developed as a transparent semi-solid selective media that incorporates the selective components from Anaerobe Systems' FSA media, removing blood to facilitate increased transparency. The CERVIS media was tested in parallel under anaerobic and aerobic environments. Under both conditions, bristle brush swabs were cut to fit inside the tube. *F. nucleatum* cultures were swabbed to obtain isolated colonies. Under anaerobic conditions, tubes were inoculated, sealed, and placed in the 37 degree incubator for 72 hours inside the anaerobic chamber. Under aerobic condition, *F. nucleatum* cultures grown on BRU plates were removed from the anaerobic chamber and exposed to oxygen prior to inoculation in the CERVIS media. Tubes were inoculated, sealed, and placed in the 37 degree incubator outside the anaerobic chamber for 72 hours and monitored for growth.

Results

Media Selectivity

Fusobacterium growth was observed on BRU and FSA. Small, white isolated colonies were observed after 72 hours. Larger colonies of the same morphology were observed on the BRU plate than the FSA plate.

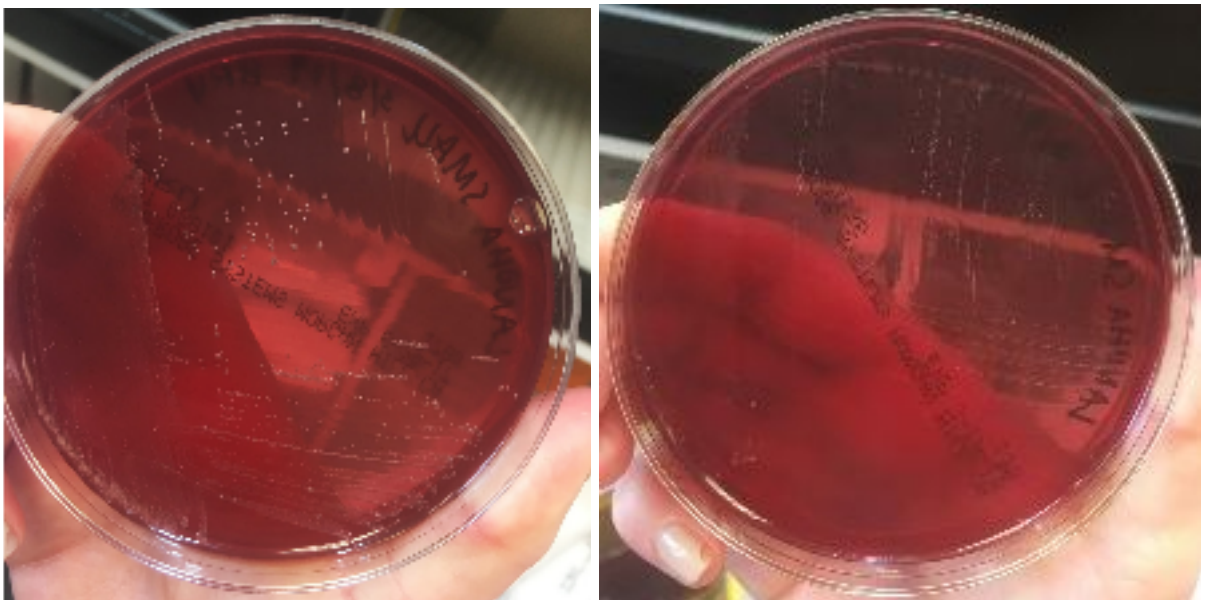

Figure 1: *F. nucleatum* Growth on BRU (left) and FSA (right)

Optimization of Anaerobicity

Table 1 shows oxygen exposure after 72 hours. Oxygen exposure was similar for all swab types under condition A. Under condition A, the polyester swab and the bristle brush introduced less oxygen into the system compared to condition B. The cervical cell brush introduced less oxygen

into the system under condition B. The cervical cell brush did not fit in the tube without size modification, raising future concerns of contamination and ease of use. The bottom of every tube does not show presence of oxygen, indicating that an anaerobic bacteria could grow there.

Table 2: Aerobicity Results

Polyester Swab	Bristle Brush	Cervical Cell Brush
 **A B**	 **A B**	 **A B**

CERVIS Media

F. nucleatum growth was observed in CERVIS media under both anaerobic and aerobic conditions. After 24 hours, regions of concentrated turbidity were observed surrounding the bristle brush in the bottom of the tube signifying microbial growth. Although growth was visible after 24 hours, tubes were left in the incubator for 72 hours, checked periodically after 24, 48, and 72 hours respectively. After 48 and 72 hours, growth was more well defined. Similar patterns of growth were observed in both anaerobic and aerobic conditions.

Table 3: *F. Nucleatum* Growth Observed in CERVIS Media

24 hours	48 hours	72 hours
		

Discussion

A. Prototype

The results above informed the final design prototype. The prototype includes the glass anaerobic tube, CERVIS semi-solid selective media, and a sterile soft-bristle brush. This prototype is designed for ease of use and limits the need for expensive laboratory materials. Semisolid media was determined as the best choice for the device because it allowed for easy visualization in a tube while still preventing the diffusion of oxygen when the swab is inserted.

B. Sources of Funding

This project was primarily funded by the Santa Clara University School of Engineering, which was supplemented with additional funds through the Xilinx grant. These funds were used to fund the laboratory materials required to conduct research inside the anaerobic chamber. Materials required for the development of the media and the tube were donated by Anaerobe Systems at zero cost to the research team. Additional funding is currently being pursued through the formation of a corporate partnership with a local molecular diagnostic company. Grant funding through the Massachusetts Medical Society (MMS), the American Medical Women's Association (AMWA), and the Adell & Hancock Fund Scholarships are also being investigated as additional sources of funding to travel to the country of interest to further explore the needs of the target population.

C. Limitations

One of the primary limitations that comes along with the development of this device is the difference that exists between in vitro and in vivo results. While we can test *fusobacterium* spp. growth from a culture, it must also be tested with *fusobacterium* spp. from an infected cervix to fully understand how the device can be used. The field of vaginal microbiome research is new and it has only recently gained more attention. There are limited studies about variability and changes in the microbiome. In order to develop a highly accurate and reliable device, it is essential to realize that this variability exists. Without some of this knowledge it is difficult to be able to predict the variability that the device might encounter. For the purpose of the preliminary experiments conducted, the swabbing procedure was held constant. It is unlikely that these same conditions will be met after the device is implemented.

Additionally, the *Fusobacterium* spp. Requires an incubation temperature of 37 degrees celsius. Further tests will investigate the minimal temperature requirements and the associated optimal growth period to attempt to eliminate this criteria. To initially combat this limitation, alternative incubation methods will be explored to expedite the pilot test including the potential use of portable neonatal incubators and/or placing one incubator for multiple subjects located in a central community space [20].

C. Next steps

Observing *Fusobacterium* spp. growth in CERVIS medium proved difficult because these are non-motile organisms. To improve the visibility of these microbes in CERVIS, the formula should be altered to include an indicator system. During the next phase of the project, the primary focus will be on developing a secondary, differential test to confirm the presence of fusobacterium in the patient's vaginal microbiome. *Fusobacterium* spp. produces hydrogen sulfide in the presence of cysteine [21]. Ammonium iron sulfate reacts with hydrogen sulfide creating a black color change. An iteration of CERVIS media will be developed to include cysteine and ammonium iron sulfate to trigger the more obvious black color change, making it easier to visually identify the presence of *Fusobacterium* spp. [22].

Further steps with this project will also work to incorporate a test for high risk HPV. This would allow the device to not only have the capabilities of detecting early stage cervical cancer, but also the ability to pick up on high risk HPV infections that are strongly linked to the development of cervical cancer [23]. Studies show the potential of using other species found in the vaginal microbiota as an indicator of HPV infection. *Sneathia* spp. has been described as a possible microbiological marker of high risk HPV strains associated with the development of cervical cancer and warrants consideration as a secondary biomarker to increase test specificity in future device iterations [24].

Partnering with a local NGO in Kenya will help further advance this project to develop an optimal device deployment and pilot testing strategy. Human subjects research standards and protocols will be taken. To see the maximum impact, an educational component teaching women about their anatomy, the risks of cervical cancer, and the importance of screening will be developed with the help of local community members for ensured cultural relevance and incorporated into the device implementation strategy. This program will extend to both patients and providers to increase screening uptake by the women living in the community and minimize the risk of false positive results. This is an important step in navigating cultural barriers and ensuring that the device is implemented in the most effective and ethical way.

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