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EVALUATION OF A HUMAN PAPILLOMAVIRUS GENOTYPING ASSAY FOR CERVICAL CANCER SCREENING IN TANZANIA

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**EVALUATION OF A HUMAN PAPILLOMAVIRUS GENOTYPING ASSAY
FOR CERVICAL CANCER SCREENING IN TANZANIA**

By

Kandali S. Kapie

A THESIS

**Presented to the Faculty of
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**Under the Supervision of Professor Peter C. Angeletti
Lincoln, Nebraska**

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HUMAN PAPILLOMAVIRUS GENOTYPING ASSAY FOR SUB-SAHARAN AFRICA

Kandali S. Kapie, M.S.

University of Nebraska, 2018

Advisor: Peter C. Angeletti

Abstract: Mucosal high-risk Human papillomavirus (HR-HPV) has been identified as the primary factor in causing cervical cancer, the most common cancer in women of low and middle-income countries (LMICs). Cervical cancer screening, incorporating the HPV test shown to be more advantageous than screening by visual inspection with acetic acid (VIA) alone. However, due to resources constrain these countries could not afford the available HPV tests. We, therefore, validated a cost-effective Multiplex PCR HPV (mPCR) assay, a recently developed assay for detecting sixteen genital HPV, fourteen HR-HPV and 2 low-risk genotypes. We postulate that this HPV multiplex assay will be at least as accurate as commercially available genotyping methods to detect HPV types (16,18,30,31,33,35,39,45,51,52,56,58,59 and 66) and two low-risk (LR) type 6 and 11. The assay demonstrates high sensitivity and specificity in detecting single and multiple HPV infections. Compared to a World Health Organization (WHO) validated assay, mPCR had an almost perfect agreement in detection of the sixteen HPV genotypes, 98% (102 out of 103 per samples); coefficient of agreement (κ) value of 0.955 and 96.5% (110 out of 114 detection events); κ value of 0.923. This assay may help in assessing cervical cancer in Tanzania and other LMICs when used together with Pap smear. In the second part of the study, we evaluated a mPCR melt-off assay for detection of the sixteen HPV mPCR products. We performed a two-step experiment; PCR amplification followed by a Real-time PCR melt-off assay using SYTO 82

Orange fluorescence dye instead of Syber Green. We postulated that the assay could detect mPCR amplicons and would distinguish a few different genotypes of HPV, in a single reaction, if the amplicons had unique melting profiles. The assay was able to detect each of the sixteen HPV amplicons by their unique melting temperatures (T_m). When tested in triplicate experiments, each HPV amplicon gave consistent T_m measurements. However, we discovered that the assay could not clearly distinguish T_m peaks of more than 3 amplicons in a single sample. Instead, T_m peaks were merged together when there was less than a 1°C temperature difference of the T_m s of the amplicons. Clear T_m peaks were observed when the T_m s of the respective amplicon T_m s were at least 2°C apart. The assay could still be simplified to detect one or two of the most abundant HR-HPV genotypes found in Tanzania. We predict that this assay would be cheaper than any commercial DNA test and could be used to triage women with lesions by helping to predict their risk for developing cervical cancer hence improve management of these women.

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CHAPTER 1
LITERATURE REVIEW

Biology of Human Papillomavirus

Human Papillomavirus (HPV) is a small, circular double-stranded DNA virus of about 7900 base pairs (bp) belonging to the *Papillomaviridae* family. There are over 200 HPV genotypes; by taxonomic convention, these are distinguished by at least a 10% difference in the L1 DNA sequence (1, 2). There are about 40 different mucosal HPV, this group of viruses mainly affects keratinocytes in mucosal linings of the anogenital and oral tracts (3). The mucosal HPV group consists of high-risk (HR-HPV) and low-risk genotypes based on their oncogenic potential. So far there fifteen identified HR-HPV genotypes are 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 60 and 66; these are associated with cervical cancer while low-risk genotypes; for example, HPV 6, 11, 40, 44, 42, 43, 54, 61, 72 and 81 are commonly found in warts and low-grade cervical intraepithelial lesions (4, 5).

The prototype HPV genome has two regions, a coding, and a non-coding region. The non-coding region consists of the Locus Control Region (LCR). The LCR is the major regulatory unit for HPV gene expression; containing a keratin-dependent promoter and the origin of replication (Ori). The coding regions have eight major open reading frames (ORFs), expressed by polycistronic mRNAs transcribed from a single DNA strand (Figure 1.1). The Early ORFs encode early proteins E1, E2, E4, E5, E6, and E7. Early proteins E6, E7, and E5 are the transforming proteins; E1 and E2 are responsible for viral replication while E4 is a viral assembly protein. The HR-HPV E6 and E7 use different mechanisms for transforming infected cells. These oncogenes modify the expression and activity of many cellular proteins; such as cell proliferation and inhibition of apoptosis and delay of cellular differentiation (6, 7). HR-HPV E7 inhibits retinoblastoma (Rb) by binding to a hypo-phosphorylated form of retinoblastoma which disrupts the differentiation of human

keratinocytes (8, 9). The HR-HPV E6 protein binds to the E6AP ubiquitin ligase to target p53 for ubiquitin-mediated degradation, thus inhibiting apoptosis (6, 10). Moreover, the HR-HPV oncogenes have been shown to increase the expression of nucleophosmin, a protein that suppresses apoptosis through inhibition of PKR phosphorylation, thus increasing the copy number of the viral genome by increased keratinocyte survival time (7, 11). Late ORFs encode proteins L1 and L2, the major and minor capsid proteins respectively. These proteins play a role in viral encapsidation and entry into keratinocytes.

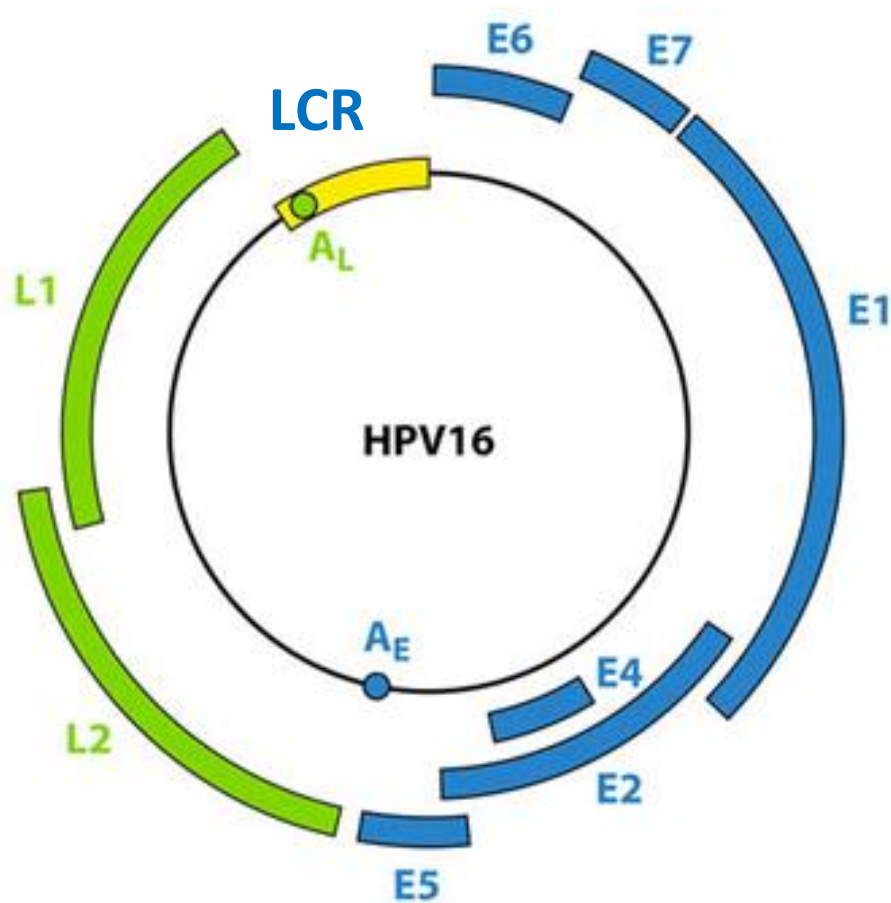


Figure 1.1: A diagram illustrating the genomic organization of HPV 16.

E1-E7 early genes (blue), L1-L2 late genes (green), The LCR (Locus Control Region) in yellow, consists of a various promoter and enhancer elements as well as the viral origin of replication (Ori) (12).

HPV Life Cycle

The HPV life cycle is tightly associated with the differentiation program of keratinocytes. HPV attaches to the receptor molecules; alpha-integrin and heparin sulfate proteoglycans (HSPGs), and enters the cell through Clathrin-coated vesicles. After infecting basal cells, the early viral gene expression cascade is activated resulting in the production of approximately 10 to 100 extrachromosomal copies of viral DNA per cell. This number is tightly maintained in undifferentiated basal keratinocyte throughout the course of the infection, by regulation of the E2 factor in infected basal cells (13). In the differentiated layers of keratinocytes, viral oncogenes E6, E7, and, E5 reactivate cell division among these cells. In the squamous layer of keratinocytes, HPV replicates by an extra-chromosomal rolling-circle mode, which increases the viral copy number to about thousands per cells.

HPV is able to evade host immune surveillance. During infection, the HR-HPV E7 protein has been shown to downregulate MHC I expression resulting in a reduction of CD8+ T cell-mediated cytotoxicity of infected cells (14, 15). Moreover, HR-HPV E7 is known to interact with and activate, DNA methyltransferase (DNMT1) (16), which leads to suppression of the immune response. Nuclear factor- κ B (NF- κ B), a vital transcription factor for immune signaling, is inhibited by the E7 protein thus interfere with many immune signal pathways (17, 18). In the absence of immune clearance, persistent HPV infection increases the probability of viral chromosomal integration, which can lead to the progression of dysplasia and finally, cervical cancer.

In the upper layers of the stratified epithelium, viral E1 and E2 are expressed. E1 and E2 bind to Ori and initiate the replication of the DNA genome (19). Also, E2 suppresses over-expression of E6 and E7 in differentiated suprabasal cells (20). Upon DNA damage, HPV

genomes can become integrated into the host genome, usually within the E2 ORF (21). This leads to loss of E2 regulation and over-expression of the E6 and E7 oncoproteins. In a normal infection, the viral genome in squamous layers is amplified to thousands of copies per cell. Late expression of capsid proteins L1 and L2 allows encapsidation of viral genomic DNA. The viral particles are then released externally as squamous cells slough off.

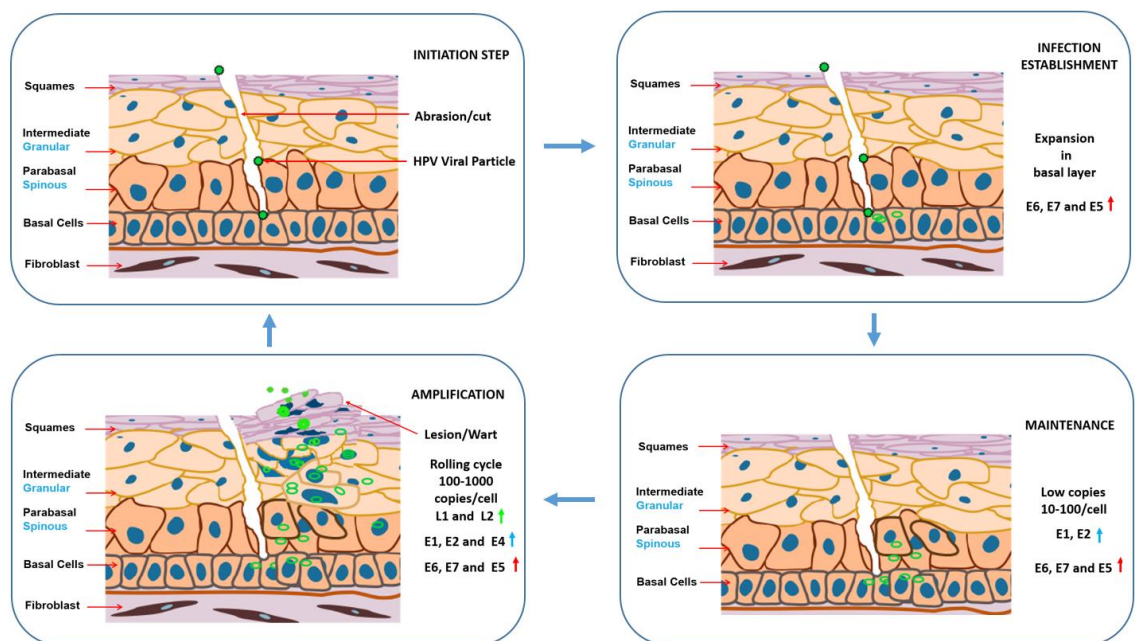


Figure 1.2: The HPV Lifecycle.

Early Proteins E6, E7, and E5 are expressed in the basal cells and as the epithelial cells differentiate the replication proteins E2, E1 and viral assembly protein, E4 are expressed. The late proteins L1 and L2 are expressed in fully differentiated cells.

HPV and Cervical Cancer Epidemiology

Cervical cancer is the fourth most common cancer in women worldwide (22). It is the most common cancer among women in Tanzania resulting in about 7,304 new cases and 4,216 deaths annually (22). HR-HPV is the main cause of cervical cancer (4). Persistent HR-HPV

infections lead to viral chromosomal integration resulting in cell transformation. Moreover, multiple HR-HPV infections have been shown to be associated with abnormal cervical cytology results (23). The period between infection and the appearance of lesions is highly variable and can vary from weeks to months (24). Despite HR-HPV infections being common in women; most of the HPV infections clear spontaneously with few cases progressing to cancer (25, 26). The time lag between the peak of HPV infection and the peak of cancer incidence is two to four decades, during this period, precursor lesions of cervical cancer may be detected by screening.

The global prevalence of HPV infection in women with normal cytology is around 11-12%, with the highest prevalence in sub-Saharan Africa at 24% (27, 28). HPV type 16 and 18 are the most prevalent HPV type in the world, accounting for 70% of all the types in cervical cancer; HPV type 31, 33, and 58 have been the nation- HPV 16 types that increase the disease risk worldwide (29).

The overall HR-HPV prevalence, as well as genotypes, vary in different world regions and age groups. The differences have been associated with cultural diversity, hormonal changes in women, and immune suppression, which varies with age and is associated with HIV (30–33). Studies done in sub-Saharan Africa have shown different prevalence trends for the most common HPV genotypes; and these differ by the country (34, 35) but HPV type 16 remains the most common type among invasive cervical cancer. A study done in Tanzania reported HPV 16, 52, 18, and 35 to be the most common genotypes in the high-grade squamous intraepithelial lesion (HSIL) (31). The study also pointed out that HPV infection prevalence increases with an increase in the severity of cervical cytology; supporting other studies done in other parts of the world (27, 29, 31, 36, 37).

HPV Detection and Genotyping Assays

DNA Tests for HPV are critical tools for cervical cancer screening and management for women with cervical precancerous lesions. HPV DNA testing is also vital to understanding the natural history of HPV and related diseases.

There are different kinds of HPV tests, based on the targets and the technique applied. The common kinds are DNA detection methods involving HPV DNA target amplification, in situ hybridization and HPV mRNA detection (38). HPV protein detection is impractical because the levels of products are very low. Since HPV seroconversion is at low-frequency in most individuals, HPV serology tests are not routinely used clinically (39, 40). The HPV DNA amplification method is analyzed by real-time methods using fluorochromes, or by Polyacrylamide Gel Electrophoresis (PAGE) or Capillary Electrophoresis (CE). In the HPV DNA hybridization method, HPV type-specific amplicon is labeled with fluorescent markers and used to probe HPV control DNAs immobilized on membrane strips. The multiplex PCR method is an HPV test which simultaneously amplifies sixteen different HPV DNAs using unique primer sets. The PCR products can be analyzed by either PAGE or CE.

Initially, HPV testing was considered in conjunction with cervical cancer screening programs. It has been performed alongside a cytological diagnosis of atypical squamous cells of undetermined significance (ASC-US), and now HR-HPV DNA testing is now considered a viable co-test in some countries (41). Although not yet recommended in the United States, HR- HPV DNA testing has been proposed as a stand-alone primary screening method for cervical cancer screening in some European countries (42). The biggest impediment to adoption of this approach is the lack of a universal risk table to

quantify the actual risk of patients developing cervical cancer when they are found to have an HR-HPV.

HPV DNA Test Validation

There has been an increase in commercial HPV DNA tests developed in the past decade, yet there are still few studies published on the performance and validation of these assays (38). The FDA accredits most of the clinically validated tests, although WHO also validated few tests. Although these bodies have suggested guidelines for validation, there is still a lack of a standardized DNA test for HPV, which is a major challenge. Variation in HPV DNA test sensitivity in detection of certain HPV genotypes is a major concern in understanding the common HPV types in a given population. A study was done to compare HPV detection between four validated assays; Hybrid Capture 2, Cobas, CLART, and APTIMA, which showed that there was only 41% agreement for HR-HPV positives by all the assays (43). This problem results in complications for women with abnormal cytology with regard to what is the appropriate follow-up. The disagreement between the methods also leaves a dilemma for positive normal cytological results as to whether there is a need for further follow-up. Variation in sensitivity with no evaluation of test performance has been shown to lead to false interpretation of results and clinically costly overtreatment of false positive individuals. In addition, there is still poor technical knowledge for effective implementation between laboratories.

Despite the presence of some data on HPV infection and its associated cancers in Sub Saharan African countries, there are still insufficient understanding of HPV related topics. Moreover, there is a need for low-cost HPV DNA testing to help triage the women who present with high-grade lesions for further management. High cost validated HPV DNA

tests are not feasible in this region due to limiting resources. Affordable, reliable, and cost-effective HPV DNA tests will enable large population studies and an increase in our understanding of the natural history of HPV in the region. Therefore, the mPCR assay is a perfect test in these circumstances. The method is simple and only costs about \$ 3 per sample.

CHAPTER 2
PERFORMANCE VALIDATION OF A MPCR ASSAY

**Performance Validation of a Low-Cost Multiplex HPV PCR Assay for Sixteen
Different Human Papillomavirus (HPV) Genotypes in Tanzanian Cervical
Screening Samples**

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Abstract

We have adapted a multiplex PCR HPV genotyping assay to fill the need for rapid and low-cost HPV detection in sub-Saharan Africa. This method allows high throughput genotyping and simultaneous detection of fourteen high-risk and two low-risk Human papillomaviruses (HPV) types, by Polymerized Chain Reaction (PCR) amplification of HPV DNA in a single reaction tube. In this study, we describe stepwise experiments to validate the multiplex HPV PCR assay for determining HPV genotypes from 104 cervical brush samples from Tanzanian women. Assay performance was evaluated by determination of intra-laboratory reproducibility, sensitivity, and specificity. A further performance was assessed by comparison with the widely accepted and validated HPV My09/My11 amplification and hybridization method. Statistics; Cohen's kappa (κ) and McNemar p values, were used to analyze inter-observer and inter-method agreement. Overall concordance between the multiplex and line blot hybridization tests was 99% (102 out of 103 per samples) with a κ value equal to 0.95; and 96.49% (110 out of 114 per detection event) with a κ value of 0.92. Inter-observer reproducibility of the assay per sample was 95.76% (103 out of 104) with κ of 0.91. These results demonstrate that the multiplex HPV PCR assay has high analytical sensitivity and specificity in detecting as many as 16 different HPV genotypes, and its simplicity and low cost makes it well suited for sub-Saharan Africa.

Keywords: Human Papillomavirus, Multiplex-PCR, High-risk HPV, Low-risk HPV, HPV Genotypes, Detection, Specificity, Validation.

Introduction

Cervical cancer is the fourth most common cancer in women worldwide (528,000 new cases), and the second most common cancer in developing countries (445,000 new cases) in 2012 (22). The disease burden is greatest among women from low and middle-income countries (LMICs) and with limited medical service resources (22, 28); who are at the late stage of disease presentation, thus causing significant treatment expenses to both, governments and families (44). Human papillomavirus (HPV) is one of the most common sexually transmitted infections (STIs) in the world. Several epidemiological studies have established that HPV is the primary cause of cervical cancer and genital warts (45–47). Persistent HPV infection with additional exposure to tobacco, oral contraceptives, and parity (48), lead to an increased the risk of cervical dysplasia, accumulation of mutations, and integration of HPV genomes into the host genome; which finally leads to the progression toward high-grade dysplasia and cervical cancer.

Current approaches to reduction of cervical cancer depend upon cervical cancer screening methods and prophylactic HPV vaccines (32, 42, 49–51). The screening methods include visual inspection, cytology evaluation, and HPV tests. Visual inspection test consists of the naked eye inspection of the cervix after the application of 3-5% acetic acid (VIA) or Lugol's iodine (VILI) using a cotton swab. The visible changes in tissue pigmentation after solution application are classified as positive (lesion) or negative (normal) results. The Papanicolaou (Pap) test detects precancerous lesions at the cellular level by identification of abnormal or large nuclei. In this method, a pathologist examines a small sample of cervical cells under an optical microscope. Finally, HPV testing is the most objective, sensitive, and highly reproducible cervical screening approach to date. These assays test

for the presence of DNA or RNA from high-risk (HR) HPV types in cervical cells and are used in conjunction with cervical screening particularly when the Pap or VIA results are inconclusive (52). HPV testing is still mostly considered a co-test (53–55), and primary cervical screening in some European countries (56). Some limitations in using HPV testing in developing countries are the cost, laboratory infrastructure needed, and the need for trained laboratory technicians. There are several HPV testing methods available. Multiplex HPV PCR is a common method that relies on simultaneous amplification of target DNAs of different molecular weights, each corresponding to a different HPV genotype. Amplification products can be separated by polyacrylamide gel electrophoresis (PAGE), Capillary Electrophoresis (CE) or used as probes in an array hybridization. Despite the availability of FDA approved assays, the affordability of these methods is still a challenge, in low-income countries (57). In addition, although there are several HPV test kits available in the market, their reliability and validity still need to be evaluated (58, 59). In this study we adapted and validated a multiplex HPV PCR assay that detects fourteen HR HPV genotypes (16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66); and two low-risk (LR) HPV genotypes (6, 11) in a single reaction (60). HR HPV is associated with cervical cancer whereas the LR HPV types such as 6 and 11 are associated with benign genital warts, hence both are included in the multiplex PCR assay (22, 61).

Materials and Methods

Study population and specimen collection

The study protocol was reviewed and approved by the Institutional Review Boards of Ocean Road Cancer Institute (ORCI), Dar-es-Salaam, Tanzania and the University of Nebraska-Lincoln. The participant women were recruited from ORCI, Bagamoyo and

Chalınze screening clinics, and informed consents were obtained from the clients before sample collection. The women had a gynecological examination, including visual inspection with acetic acid and a conventional Pap test. Pap smear collection was performed using the concave end of an Ayer's spatula; samples were evenly spread on a glass slide and sprayed with fixative. Pap smears results were determined by three blinded cytologists according to the Bethesda classification system 2001 (62). For HPV testing, cervical cells were collected from the opening of the cervix using a cytobrush. Each cytobrush was placed in a labeled tube containing cervical lysate and stored at 4°C until DNA extraction.

DNA extraction of cervical samples

DNA samples were extracted from 200µL lysate solution of each clinical cervical sample using the Qiagen DNeasy Blood & Tissue Kit (cat no. 69504, 69506) according to manufacturer's instructions. DNA concentration of samples was determined with a NanoDrop Spectrophotometer. Cervical DNA samples were stored at -20°C until further use.

Multiplex PCR Assay

Sixteen HPV genotypes (6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) were obtained from Karolinska Institutet (International HPV Reference Center, Sweden). These plasmids were used as controls and test amplicons in the analytical experiments. The cloned PGEMT was obtained from Promega (Madison, WI, USA). The aminolevulinate synthase 1 (ALAS1) gene was cloned into the PGEMT vector and the construct was used in the analytical, sensitivity and specificity experiments. All plasmids were confirmed by

restriction digest prior to HPV genotyping experiments. PCRs were performed using a multiplex PCR kit (Qiagen Inc., Valencia, CA), according to manufacturer's instructions. A previously developed protocol, with minor modifications, was followed (60). At least 50 ng of DNA sample solution (HPV DNA plasmid or clinical sample) was used as a template for PCR amplification. Samples were incubated at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C (30 sec), annealing at 70°C (90 sec), and extension at 72°C (60 sec). PCR products were analyzed on a 6% Polyacrylamide Gel Electrophoresis (PAGE) in 1XTBE and stained with ethidium bromide. BIO-RAD ChemiDoc™ MP Imaging System captured the gel images. A positive genotyping result was called if a clearly single separate band was visualized on the gel. All HPV type detected by single band except HPV types 16 and 58 were detected by two separate bands (60).

End-point detection limits of HPV genotype-specific PCR was achieved by serial dilutions of each respective HPV target, plus 1000 ng of salmon sperm DNA as a carrier in each reaction tube. This experiment was used to optimize each HPV genotype primer set. End-point detection limits were performed for an individual or multiple HPV genotypes present in a single reaction tube. Reproducibility experiments were repeated twice within 2-3 weeks, with two blinded observers who read identical gel images.

HPV hybridization method

PCR was performed using biotin-labeled MY09/MY11 consensus HPV L1 primers, in addition to biotin-labeled human beta-globin primers, which were used as an indicator of DNA quality as previously described (63). About 50 ng of DNA was added to each 100µl PCR reaction and subjected to 40 amplification cycles. One hundred and three samples were interrogated by this method. Products were first hybridized against the cellular

control DNA, beta-globin, then against membrane-bound arrays of HPV standard DNAs. Standard DNAs included 38 different HPV types: 6/11, 16, 18, 26/69, 30, 31, 32/42, 33, 34, 35, 39, 45, 51, 52, 53, 54, 56, 57/2/27, 58, 59, 61, 62, 66, 67, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, 86/87, 90/106, 97, and 102/89. There were two separate mixtures, mix1 contained 7, 13, 40, 43, 44, 55, 74, and 91, while mix2 contained 3, 10, 28, 29, 77, 78, and 94. Negative specimens for beta-globin gene amplification were excluded from the analysis. PCR results were recorded on a 0-5 scale based on the signal intensity of dot-blots. For comparison purposes, a sample genotyped by hybridization method was considered positive only if one or more of the sixteen HPV genotypes present in the multiplex HPV PCR assay was detected; otherwise, the sample was considered negative.

Statistical analysis

All statistical analyses were carried out using SAS software, version 9.4 and Excel 2016 (Microsoft, Seattle, Washington). Agreement assessment, between methods (Multiplex HPV PCR and Hybridization) and observers, were assessed by Cohen's Kappa test. The Cohen's Kappa coefficient (κ) varies from 0 to 1, where 0-0.20 indicates slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial and 0.81-1 near perfect agreement. Cohen's Kappa tests were performed per sample or per event, where an event is considered to be a specific HPV genotyping call, including a negative call. Contingency tables were used to calculate sensitivity and specificity values with 95% confidence intervals (CI). A McNemar's test with a p-value of 0.05 is considered significant, however when there is perfect agreement, and there are "0" discordant, then a p-value of 1 is still considered significant (64). Clinical Sensitivity was calculated as the

proportion of women with high-grade lesion; atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion (ASC-H) and HSIL be positive test positive by mPCR or Hybridization method. Clinical specificity was calculated as the proportion of women who did not among those without high-grade lesions (ASC-H and HSIL).

Results

The multiplex HPV PCR assay utilizes amplified products from 16 different HPV genotypes, which map to different regions in the linear HPV genome (Figure 2.1). HPV 16 has two PCR products, a small HPV 16(L) (217 bp), and a large fragment 16(U) (397bp).

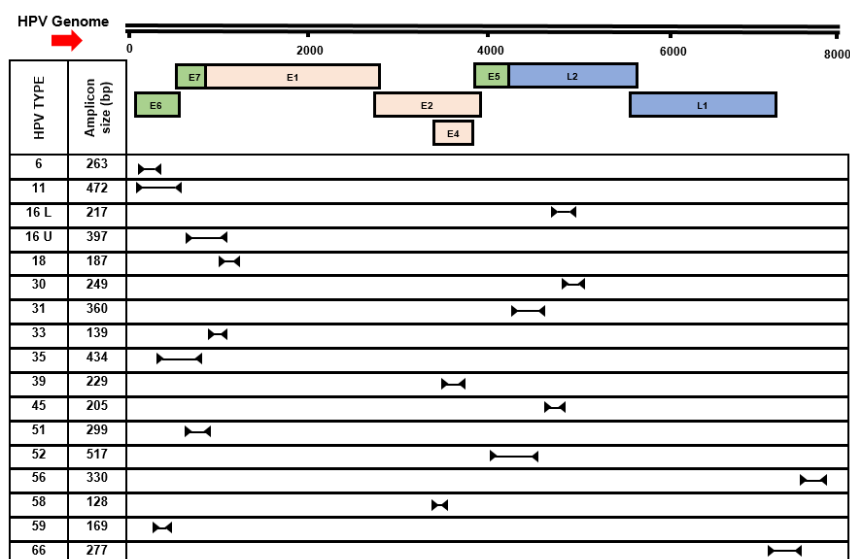


Figure 2.1: Schematic diagram of PCR products mapped on HPV genome.

Each of the 16 PCR products of the different HPV genotypes is shown mapped to different open-reading frames on the linear representation of the HPV genome.

The analytical sensitivity of detection of each of the 16 different HPV genotypes was analyzed by PAGE, as shown in Figure 2.2. The end-point detection limit of HPV genotypes 6, 11, 16(L), 18, 30, 31, 33, 39, 45 and 58 ranges from 1-10 copies; and 10-100 copies for HPV genotypes 16(U), 35, 52, 56, 59, and, 66 (Table 2.1). Real-time PCR was shown to be a more sensitive detection method than CE and PAGE as it detects one

molecule of all sixteen HPV DNA reactions. Overall, the end-point detection limits obtained with the multiplex assay are comparable with the ones obtained by CE. The end-point detection limit assays of multiplex PCR containing six different HPV genotypes (6, 16, 31, 33, 52, and 56) is shown in figure 2.3. This figure demonstrates that the number of copies detected when using the mixture was similar as those detected for their respective individual HPV genotypes; 1-10 copies for 6, 16(L), 31 and 33 and 10-100 copies for HPV genotypes 16(U) and 52 (Table 2.1 and figure 2.2). In addition, these results suggest that the multiplex HPV PCR assay possesses high specificity in detecting each of the six HPV genotypes with no material exhaustion.

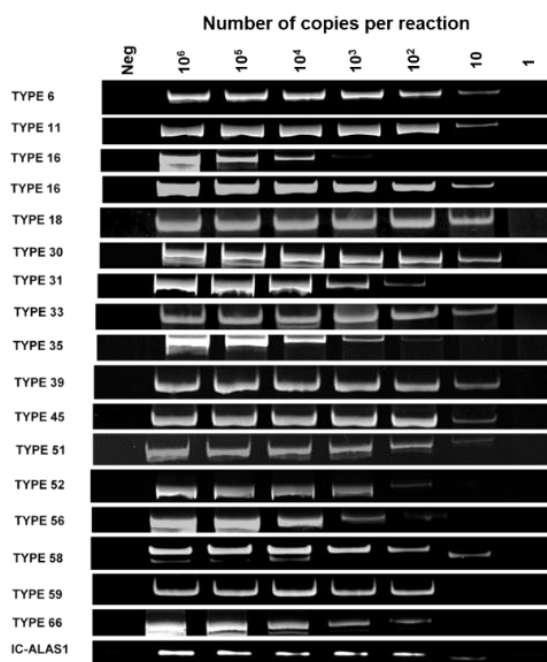


Figure 2.2:End-point detection limit of each of the 16 HPV genotypes using the multiplex PCR assay.

Tenfold serial dilutions of the internal control (IC: aminolevulinate synthase 1, ALAS1) or each of the HPV DNAs was subjected to amplification. HPV genotypes 6, 11, 16(L), 18, 30, 31, 33, 39, 45 and 58 were detected at between 1-10 copies per reaction, whereas HPV genotypes 16(U), 35, 52, 56, 59 and 66 were detected at 10-100 copies per reaction. The dilutions of PCR templates are indicated above each lane (10^6 to 1 viral copy per reaction). Neg indicates a reaction without DNA added.

Table 2.1: Comparison of end-point detection limits obtained compared by Real-Time PCR, Capillary Electrophoresis, and multiplex PCR HPV assays.

| HPV Type | Amplicon Size (Bp) | Target gene | Detection Limit (No of Molecules/Reaction) | | |
|----------|--------------------|-------------|--|---------------------------|--------|
| | | | Real-Time PCR | Capillary Electrophoresis | PAGE |
| 6 | 263 | E6 | 1 | 10-100 | 1-10 |
| 11 | 472 | E7 | 1 | 1-10 | 1-10 |
| 16L | 217 | L2 | 1 | 1-10 | 1-10 |
| 16U | 397 | E1 | 1 | 10-100 | 10-100 |
| 18 | 187 | E1 | 1 | 1-10 | 1-10 |
| 30 | 249 | L2 | 1 | 1-10 | 1-10 |
| 31 | 360 | L2 | 1 | 1-10 | 1-10 |
| 33 | 139 | E1 | 1 | 1 | 1-10 |
| 35 | 434 | E6-E7-E1 | 1 | 10-100 | 10-100 |
| 39 | 229 | E2 | 1 | 1-10 | 1-10 |
| 45 | 205 | L2 | 1 | 1-10 | 1-10 |
| 51 | 299 | E7-E1 | 1 | 1-10 | 1-10 |
| 52 | 517 | E5-L2 | 1 | 1-10 | 10-100 |
| 56 | 330 | LCR | 1 | 10-100 | 10-100 |
| 58 | 128 | E2 | 1 | 1-10 | 1-10 |
| 59 | 169 | E6-E7 | 1 | 10-100 | 10-100 |
| 66 | 277 | L1-LCR | 1 | 10-100 | 10-100 |
| IC | 100 | ALAS1 | 1 | 1 | 1-10 |

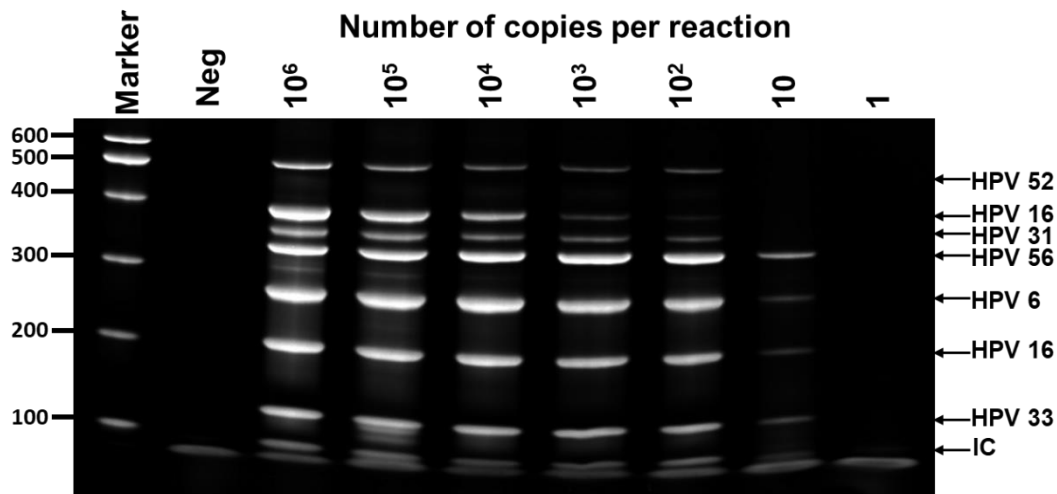


Figure 2.3: The end-point detection limit of multiple HPV templates using the multiplex PCR assay.

HPV genotypes: 6, 16, 31, 33, 52 and 56 DNA diluted by ten-fold serial dilution and subjected to multiplex PCR. The relative detection limit is indicated by the copy number per reaction above each lane (10^6 to 1 copies per reaction). Neg, negative control (without DNA). Marker, 1-kb DNA marker.

Of the 104 cervical samples used, 13.62% were negative for intraepithelial lesion or malignancy (NILM) while the remaining had atypical squamous cells of higher pathological categories (ASCUS-HSIL). Thirty-two samples out of 104 samples tested positive by multiplex HPV PCR assay. None of these samples were excluded from analysis as all samples had sufficient human DNA indicated by internal control. In testing the reproducibility of the multiplex assay the samples were retested 2 to 3 week after the initial test, gel analyses for genotype were analyzed by two respectively observers.

The agreement charts for comparison between observers' calls using the Multiplex HPV PCR assay are shown in figure 2.4. For the case of per sample comparison (Figure 2.4A), the chart shows almost exact agreement (dark gray shading) with a small partial agreement region (light gray shading) for the negative and positive results. The proportion of agreement per sample analysis was 99% (103/104 samples), and the Cohen's kappa coefficient was 0.978 (95% CI: 0.934-1.000). Regarding the per event comparison, the proportion of agreement was 97.4% (114/117 events), with a k coefficient value of 0.946 (95% CI: 0.885-1.000) (Figure 2.4B). In this case, the exact agreement regions are slightly smaller with a corresponding increment of the partial agreement regions due to an increased number of events.

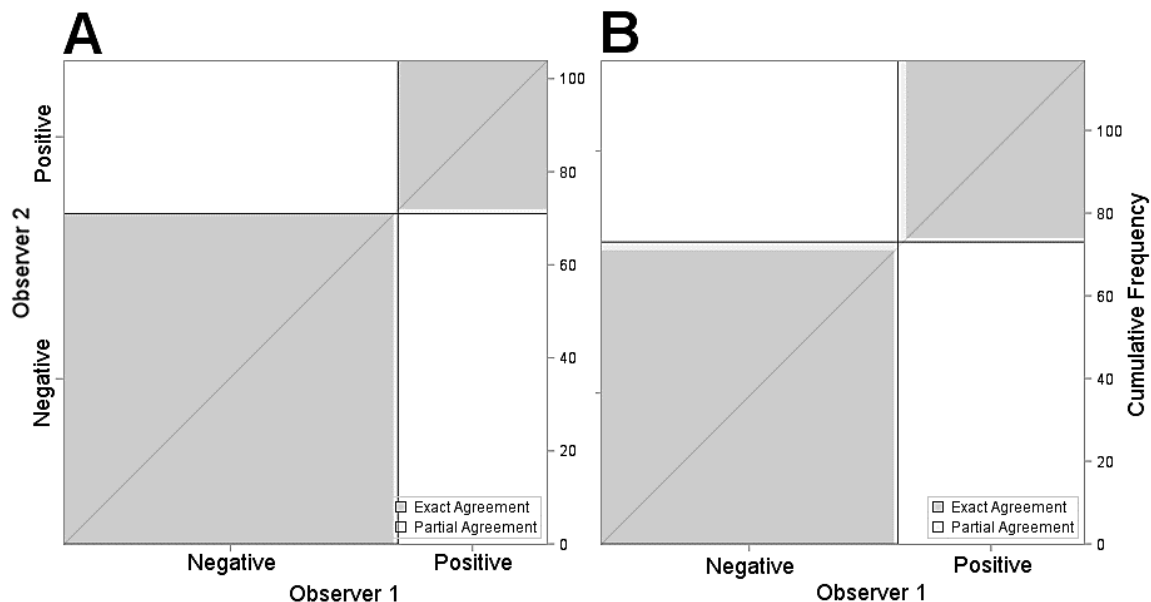


Figure 2.4: Agreement chart for intra-observer calls by multiplex HPV PCR assay. Agreement between observers per sample. B) Agreement between observers per events. Shading squares indicate exact agreement (dark gray), and partial agreement (light gray).

A total 103 of clinical sample genotype calls agreed between the observers using the multiplex PCR method in detecting any of the sixteen HPV genotypes. Figure 6 shows the agreement charts for comparison between mPCR and the hybridization method. The proportion of agreement per sample analysis was 98% (101/103 samples), and the Cohen's kappa coefficient obtained was 0.955 (95% CI: 0.891-1.000) (Figure 2.5A). The proportion of agreement of the event analysis was 96.5% (109/113 events), while the k coefficient was 0.923 (95% CI: 0.849-0.997) (Figure 2.5B). Overall, results in both cases indicate almost perfect agreement between the Multiplex HPV PCR and HPV hybridization assays.

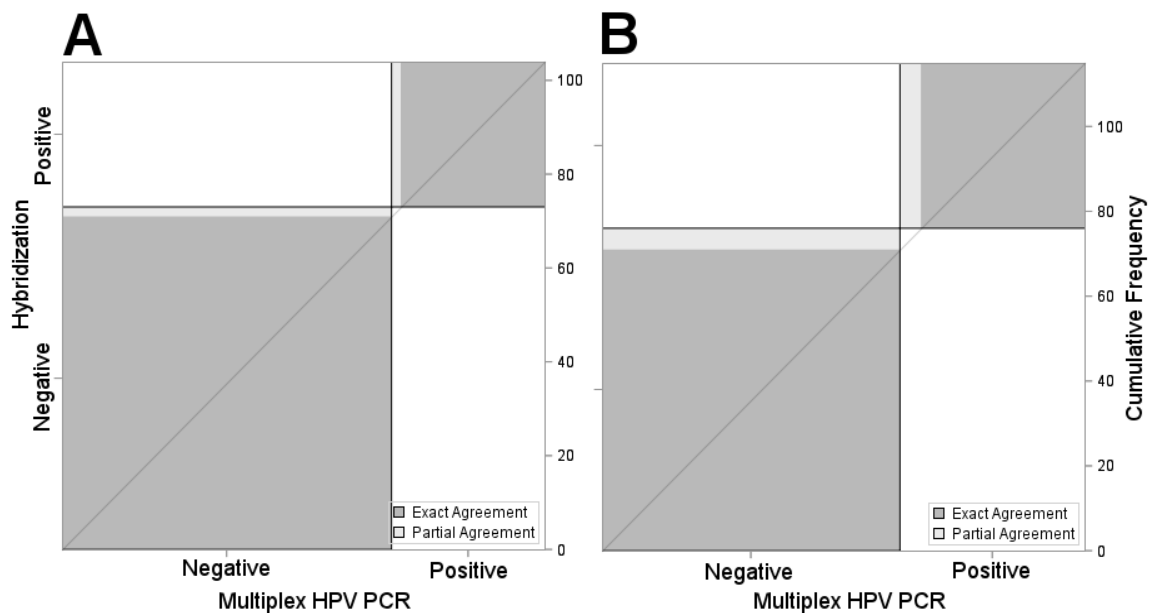


Figure 2.5: Agreement charts for comparison between HPV genotyping detection methods.

Agreement chart between detection methods per sample, B) Agreement chart between detection methods per event. Shading squares indicate exact agreement (dark gray), and partial agreement (light gray).

Figure 2.6 shows the comparison of detection of 103 cervical samples between multiplex HPV (filled circles) PCR and Hybridization (open triangles) methods. In this analysis, one of the samples was not considered because there was a disagreement between observers' calls for the multiplex assay. Our results showed that four samples (4/103) were discordant. Specifically, two cervical samples tested HPV negative by the hybridization method tested HPV types 33, and 66 by the multiplex assay. These corresponded to samples number 5 and 73, respectively. Furthermore, two additional HPV genotypes were detected in samples number 20 and 54, which were not detected by the hybridization method: HPV types 11, 18 and 66 (mPCR); and HPV 33 and 66 (mPCR), respectively figure 2.6.

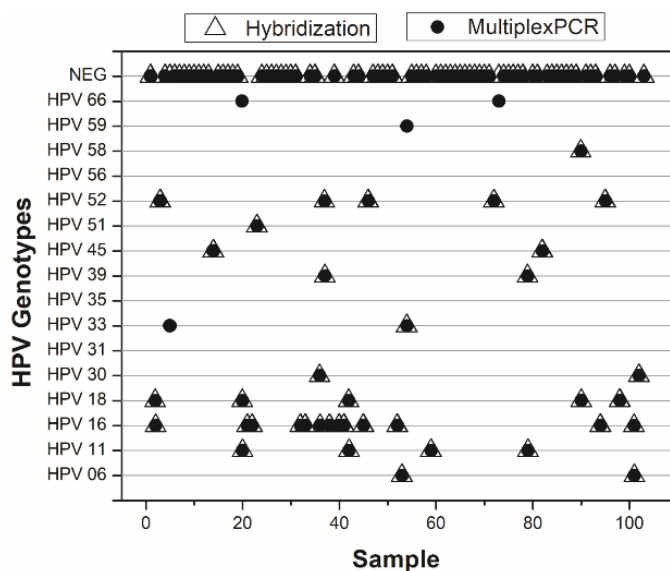


Figure 2.6: Comparison of the HPV genotypes detection efficiency between multiplex HPV PCR and HPV hybridization methods.

The different detected HPV genotypes are represented as filled circles (Multiplex PCR) and open triangles (Hybridization method). The results of 103 samples were compared. HPV negative results are also shown in the plot.

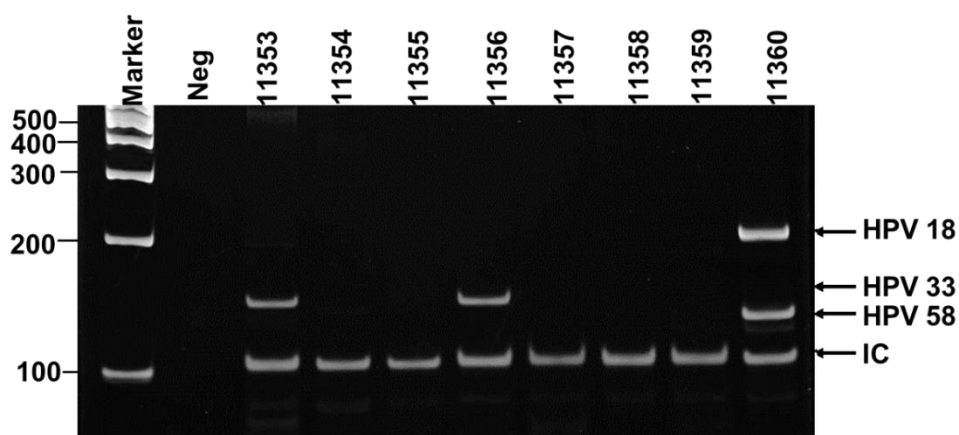


Figure 2.7: Detection of HPV DNAs in clinical samples.

Single and multiple HPV types detected by the multiplex HPV PCR assay. The internal control (IC) band was detected in all samples. Neg; negative control (without DNA). Marker, 1-kb DNA markers.

The genotyping agreement shows an almost exact agreement (dark gray) for both negative and positive there was very small partial disagreement (light gray). Moreover, the figure shows the skewing distribution of HPV infection were by many samples were negative for the sixteen HPV genotypes detected by the assay figure 2.4 and 2.5.

Correlation of detection HPV genotypes was calculated with respect to the pap-smear cytology results. Overall agreement between the methods in detecting sixteen HPV types for all abnormal cytological results was 97.78 %; κ value of 0.95 (95% CI, 0.88-1) while agreement for normal was 100%; κ value of 1. Two samples of low-grade squamous intraepithelial lesions (LSIL) and ASCUS had HPV 6 and 11 respectively.

The analytical assay sensitivity and specificity were 100% and 94.67% respectively. The clinical sensitivity and specificity of multiplex HPV PCR and Line Blot hybridization for the detection of different categories of abnormal cytology using Bethesda classification system 2001, were relative the same.

Discussions

The main goal of this study is to validate the analytical detection of HPV genotypes by the multiplex HPV PCR assay by comparing its performance to the clinical validated, WHO approved HPV (My09/My11) Hybridization method. Overall, this comparison is highly concordant, consistent, and reproducible. We chose to calculate the efficiency of detection of HPV genotype detection in HPV DNA sample and in the clinical heterogeneous samples.

The assay demonstrated high analytical sensitivity in detecting HPV DNA at very low copy number (1-10 copies per cell). of HPV DNA which is a vital criterion in studying the natural history of the HPV pathogenesis and disease diagnosis (65, 66). Agreement per

sample analysis was 99% (103/104 samples), and the Cohen's kappa coefficient was 0.978 (95% CI: 0.934-1.000) signifies an almost perfect agreement. The assay offered high specificity by being able to type each HPV present in single sample tube without exhaustion of the PCR materials irrespective of analyzing method made this assay reliable in studying the role of multiple HPV infections in cervical cancer. One study has shown, multiple HPV infections increase the risk of cervical cancer (67). Moreover, multiple HPV genotypes have found co-existence within the cervical cancer epithelium (68, 69).

Comparing the assay with Hybridization test demonstrates almost perfect agreement for the sixteen HPV genotypes tested. Moreover, the HPV type 16, 51, 35, and 18 shown to be most prevalence and substantial risk associated with anogenital squamous lesion and cancer in the Sub-Saharan African countries (31). The multiplex HPV PCR has the ability in detecting discrete genotype in the reaction, some available High- risk HPV assay report infection by HPV-16 and HPV-18, and other carcinogenic HPV genotypes (59, 70, 71) this is important in the understanding role of individual type in cervical cancer pathogenesis (68). Furthermore, the assay demonstrates high fidelity in detect low-risk HPV genotypes, as these HPV genotypes (HPV 6 and 11 included) have been showing to also be associated with cervical lesions as well as genital warts.

Despite the high agreement between observers, two or more observer are required for reliable genotype otherwise, band analysis with PAGE can be subjective. Moreover, the assay ability to detected HPV 16 from Africa samples despite variants from the region is of great value (72), however, sequences of the specific variants from the working samples were not done to determine their existence. The detection discrepancies have always been

the detection of extra genotype by multiplex PCR assay since no be further analyzed (DNA Sequencing) no conclusion can be made on sensitivity.

Although we attempt to do clinical assay analysis test by the different cytological group, further analysis requires completion of the international guidelines (73). High sensitivity and specificity in clinical samples (data not shown) suggest the assay future potential use in clinical settings. However, the sample samples size was small; some of the genotypes never appear at all in genotyping of clinical samples. A proposed clinical validation using international guidelines with large sample size might explain clearly on clinical sensitivity and sensitivity (66, 74).

Like most of the HPV DNA, amplification test mPCR showed high analytical sensitivity, however, the assay is susceptible to contamination. Proper surface cleanness and separation of the working station for DNA sample preparation, extraction, set up the PCR mixture and analyze PCR products.

Most of the cervical cancer studies focus on squamous carcinoma subtype although adenocarcinoma is a rare subtype contribute about 10-20% cervical cancer, the disease has challenges in histopathology diagnosis and is associated with poor prognosis. Few studies have been done indicating that HPV infection is highly associated disease process (36, 75); this calls further HPV test study to understand this category of cervical cancer so as to improve the knowledge on disease management. Lastly, cervical cancer increases in low-income countries, the understanding prevalence of different HPV genotypes and relationship to disease by affordable research techniques is vital (22).

CHAPTER 3
EVALUATION OF MPCR MELT-OFF ASSAY

Evaluation of a High-Resolution Melt-off Assay for Simple Detection of HPV Genotypes in Tanzania

Abstract

The aim of this study was to evaluate a post-PCR melt-off assay to detect HPV genotypes in multiplex PCR. This assay analysis utilizes the SYTO 82 nucleic acid dye of SYTO orange family, which provides a low background to detect DNAs of different molecular weights based on melting temperature (T_m). In this study, we demonstrate the ability of this melt-off assay to detect each of the sixteen individual HPV genotypes present in a sample, giving a unique and consistent melt-off temperature profile. Evaluation of the melt-off assay for simultaneous detection of multiple HPVs revealed that it was limited to 2 genotypes. This assay could be developed into a simple platform for low-cost cervical cancer screening, such as is needed in low and middle-income countries (LMICs) like Tanzania.

Introduction

Cervical cancer is the most common cancer of women in sub-Saharan Africa (22). This is likely because of the lack of cervical screening programs, limited resources and the presence of other endemic diseases such as HIV and malaria. High-Risk (HR) HPVs are the major cause of cervical cancer; this occurs by viral persistence infection and DNA integration (4, 39, 76, 77). In contrast, low-risk (LR) genital HPVs are associated mainly with genital warts and low-grade cervical dysplasia. With regional financial limitations, affordability of the HPV DNA test, a recommended co-testing method for low-grade lesion triage, is still a far goal to attain. Therefore, there is a need for cost-effective, simple assay

for detecting the clinically relevant high-risk HPV in the region. In this paper, we chose to evaluate the performance of a DNA Melt-off assay for detection of sixteen HPV types; 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 using a fluorescence melting curve method (78). This approach utilizes the established multiplex HPV PCR assay, which we validated for performance using clinical samples from Tanzania (60). The assay measures the DNA melting point T_m and dissociation kinetics of HPV DNA amplicons. The relative guanine-cytosine content (GC content,) structure, and size of the amplicons determine the melt-off signature and T_m . During the process of dissociation, the dsDNA with intercalating dye emits light until it is released from a dsDNA to a single-stranded DNA (ssDNA). Analysis of T_m and denaturing profiles, which differ by structure, length and GC content allows distinction between HPV genotypes (79, 80). In this assay, we used SYTO 82 nucleic acid dye because of its redeeming characteristics of low background when not intercalated into DNA. Previous studies showed that unlike other DNA dyes, the SYTO orange family of DNA dyes demonstrate high-quality melt curve fidelity with less interference in PCR reactions (81, 82). The dye also demonstrates a low affinity for ssDNA but binds tightly to dsDNA, and fluoresces brightly in this state (83). Also, the dye is inexpensive compared to most popular probes (84).

Methods and materials

Plasmids

Plasmids containing full-length HPV genomes of each 16 different HPV types were obtained from Karolinska Institutet, International HPV reference Center, Sweden. These DNAs samples were used for amplification analyses and for analytical experiments. The aminolevulinate synthase 1 (ALAS1) gene was previously cloned into the PGEMT, which served as a mean to quantify cellular DNA and determine assay sensitivity and specificity of multiple HPV genotypes experiments. The PGEMT easy vector obtained from Promega (Madison, WI 53711-5399 USA).

Samples

We analyzed DNA from achieving genotyped cervical samples collected from our ongoing cervical cancer screening studies in Tanzania. Isolated DNA samples were quantified spectrophotometrically, stored at -20°C until the day of use. These samples were genotyped using the multiplex HPV PCR assay.

Determine the melting behavior and structure characteristics of amplicons

the uMelt software was used to analyze melting behavior and structure characteristics of amplicons, (78). The genome sequences of all HPV genotypes were obtained through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and the Los Alamos National Laboratory HPV Database (<http://hpvweb.lanl.gov/stdgen/virus/hpv/>). Codon Code software (version 7.1. Codon Code Corporation 101 Victoria Street, Centerville, MA 02632) was used to align the

sequences. The amplicons melting domains and Tms were recorded. The same PCR reaction salt concentrations (50 mmol/L for monovalent, three mmol/L MgCl₂, at a final volume of 25 µl.) were used to predict the 18 expected PCR product Tms and folding structures.

Multiplex HPV PCR assay and melt-off analysis

The assay was performed in two steps. First, PCR amplification using the multiplex HPV PCR assay preceded the melt-off analysis. Amplification of 16 HPV plasmid DNAs or amplified products from clinical samples for HPV types 6, 11, 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 were done according to our previous protocol. A multiplex PCR kit (Qiagen Inc, Valencia, CA) was used according to the manufacturer's instructions, with minor modifications (81). At least 50 ng of DNA sample solution (HPV DNA plasmid or clinical sample) was used as a template for PCR amplification. Samples were incubated at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C (30 sec), annealing at 70°C (90 sec), and extension at 72°C (60 sec). SYTO 82 Orange was prepared by dilution of a working stock of 5 µM in molecular biology grade water. The stock solution tube was covered with aluminum foil to avoid light and store at -20°C until the day of use. One µl of the 5 µM of the SYTO 82 stock was added to each 25 µl PCR reaction in optically clear tubes. The melt-off was read by using a real-time PCR machine to denature the DNA from 60 °C to 95 °C with a 0.1 °C/s and 0.02°C/s. The melt-off assay analysis was performed using the Real-Time Quant Studio™ 3 System. Total assay time was approximately 6 h after sample receipt (30 min of sample preparation, 2h of DNA extraction, 3h of PCR amplification, and 9 min of HRMA). For sample analysis, temperature-adjusted, normalized and derivative melting curves plots were generated. To verify the quality of

products, the samples were run on Polyacrylamides Gel (PAGE). Previously genotyped clinical samples were used to compare the samples melt-off analysis and on PAGE to determine the consistency of the results.

Statistical Analysis

One-way ANOVA test was performed using SAS software, version 9.4 to determine whether the average T_m differences between the genotypes were significant. We also constructed box plots to illustrate the differences between the T_m of the seventeen-mPCR products by predicted and different experimental run.

Results

The uMelt software predicted the expected melt-off characteristics of the HPV DNAs. Unlike analysis of DNA molecular weights using gels, the T_m of amplicons varies with several characteristics noted in Table 3.1. HPV16L, 39, 66 and IC -presented low T_m s (79.67°C- 80.40°C), HPV 6, 16, 18, 30, 31, 45, 56, 59 had medium T_m s (81.95°C - 82.74°C) and HPV 11, 33, 35, 52, and 58 had high T_m s (83.3°C - 83.82°C). The predicted T_m s were a little lower than the observed T_m , but there was not statistically differences from observed T_m s.

Table 3.1: Analysis of the melt-off T_m of the individual HPV amplicon, along with predicted T_m by uMelt software.

| HPV TYPE | PRODUCT SIZE (Bp) | GC (%) | PREDICTED T_m (°C) | OBSERVED T_m (°C) | | | |
|----------|-------------------|--------|----------------------|---------------------|--------------------|--------------------|---------------------|
| | | | T_m 0 | T_m 1 0.1°C/s | T_m 2 0.1°C/s | T_m 3 0.1°C/s | T_m 4 0.02°C/s |
| 6 | 263 | 42 | 81.2 | 82.7 | 82.6 | 82.6 | 83.4 |
| 11 | 472 | 42 | 81.7, 83.0 | 83.4 | 83.4 | 83.3 | 83.8 |
| 16U | 397 | 38 | 81.0 | 81.2 | 81.2 | 81.1 | 81.2 |
| 16L | 217 | 45 | 81.7 | 82.6 | 82.6 | 82.5 | 83.2 |
| 18 | 187 | 45 | 82.0 | 82.6 | 82.7 | 82.5 | 83.3 |
| 30 | 249 | 47 | 81.8, 86.2 | 82.6 | 82.7 | 82.6 | 83.1 |
| 31 | 360 | 45 | 81.3, 84.3 | 82.6 | 82.7 | 82.5 | 83.0 |
| 33 | 139 | 49 | 83.0 | 84.1 | 84.1 | 84.6 | 83.9 |
| 35 | 434 | 45 | 83.3 | 84.1 | 83.4 | 84.0 | 84.2 |
| 39 | 229 | 41 | 77.7, 83.3 | 79.9, 82.8 | 79.8, 83.3 | 79.7, 82.7 | 82.0, 83.9 |
| 45 | 205 | 42 | 80.9 | 81.9 | 81.9 | 82.5 | 83.8 |
| 51 | 299 | 44 | 80.6, 83.8 | 82.0 | 82.0 | 82.0 | 83.6 |
| 52 | 517 | 41 | 78.2, 84.8 | 79.9, 85.7 | 79.69, 85.5 | 79.67, 85.4 | 79.9, 85.7 |
| 56 | 330 | 41 | 81.9 | 82.7 | 82.7 | 82.5 | 82.7 |
| 58 | 128 | 54 | 84.5 | 84.8 | 84.8 | 84.7 | 83.6 |
| 59 | 169 | 45 | 81.9 | 82.7 | 82.7 | 82.5 | 83.0 |
| 66 | 277 | 39 | 80.1 | 80.6 | 80.5 | 80.4 | 81.2 |
| IC | 100 | 52 | 83.2 | 80.4 | 81.9 | 80.4 | 86.7 |

In figure 3.1 and figure, 3.2 below show predicted and observed melting profile and temperatures for the different HPV genotypes. The figure, 3.2 T_m s observed the melting signatures by derivatives melt curves plots of HPV 6, 11, 18, 33, 35, 45, 56, 59 and 66 were detected as single peaks. The HPV 30, 31, 39, 51 and 52 were observed to melt in two phases; with two T_m peaks. We found high consistent T_m s of individual HPV amplicon in an experiment with the statistical test being significant. However, the melt-off curves of the each sixteen failed to reveal a unique T_m that helped to differentiate the tested genotype in a single PCR reaction as shown in figure 2. Furthermore, the predicted derivative melting

curves were almost the same as the observed amplicon's curves shown in figure 3.1 and figure 3.2.

To evaluate the specificity of the assay, we compared the number of DNA band(s) detected in the PAGE analysis with the number of peaks detected in melt-off assays. HPV 16 with two separate detectable bands, previously reported by Nishwaki et al, had two peaks from two amplicons. HPV 58 with two separate detectable bands from PAGE reported in the same previous paper presented a single melt curve peak. Some amplicon of HPV type 30, 31, 39, 51 and, 52 presented two melting curves peaks (figure 3.1 and 3.2) despite having a single band on PAGE.

We evaluated the ability of the assay to detect multiple genotypes simultaneously in a single sample. We discovered that individual peaks of Tms were not resolved when multiple HPVs were present in a given sample (data not shown). We found that only two different genotypes could be detected by melt-off assay, as long as their temperatures were at least 2°C apart as shown in figure 3.3. The figure shows normalized and derivative curves of HPV genotypes, HPV 18 (red), HPV 39 (blue) and mix of HPV 18 and 39 (green) with melting temperature of 79.72°C, 82.63°C and 79.72°C, 82.63°C, respectively. Therefore, we chose not to further characterize the assay for multiple HPV detection.

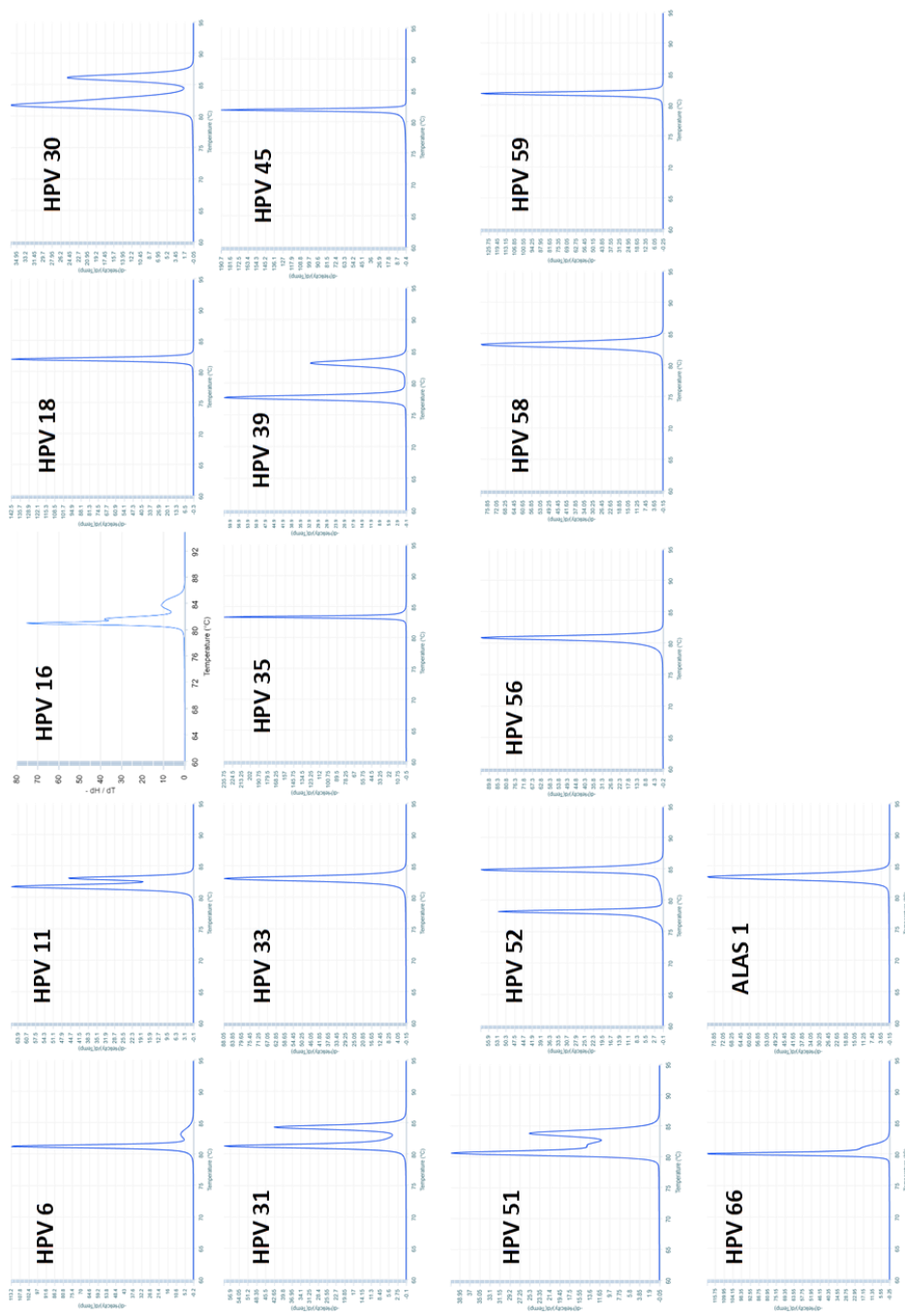


Figure 3.1: Predicted Melting curves of the mPCR sixteen HPV genotypes and Aminolevulinatase 1 (ALAS1), used as an internal control amplicon.

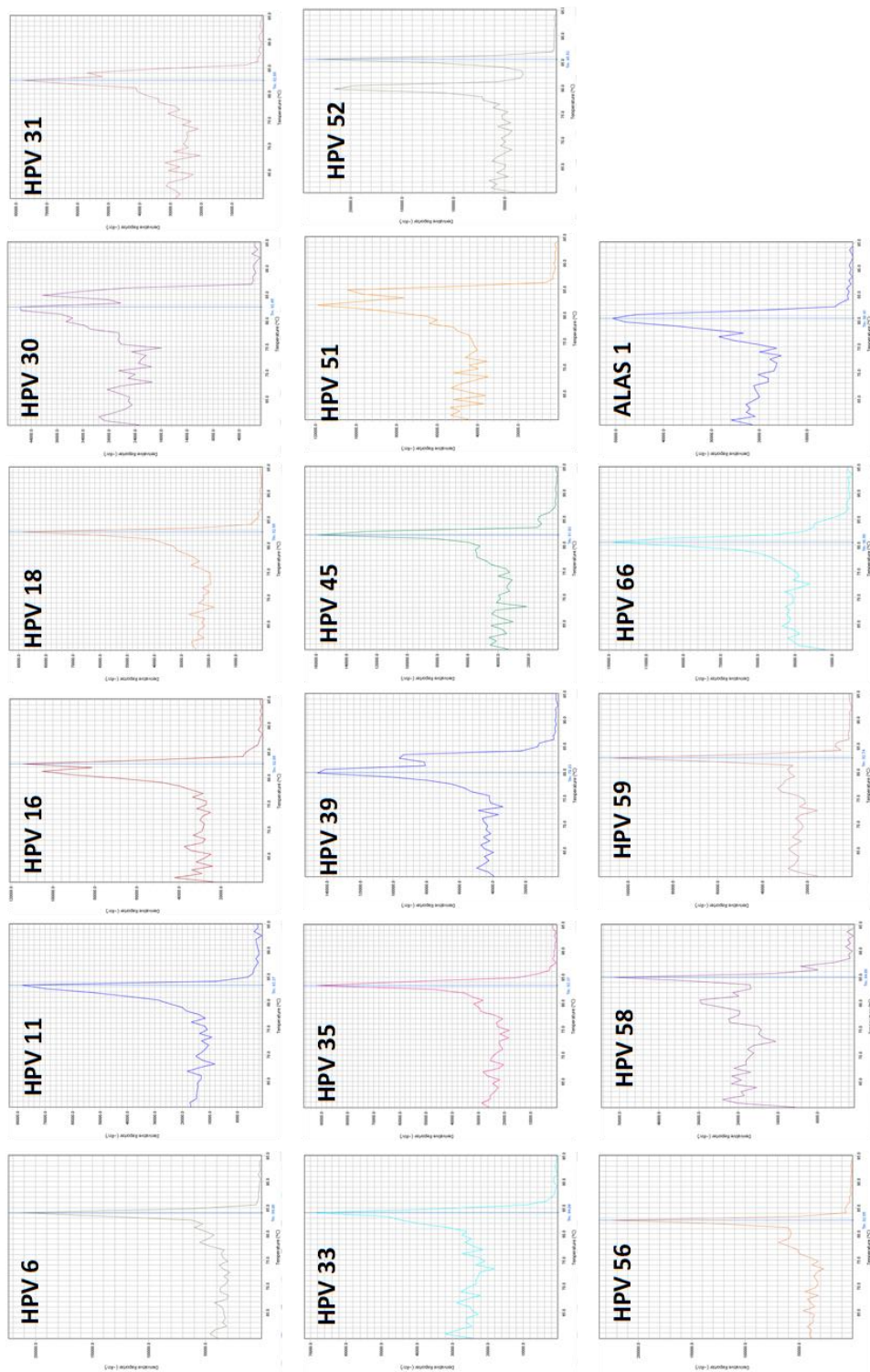


Figure 3.2: The end-point detection limit of multiple HPV templates using the multiplex PCR assay.

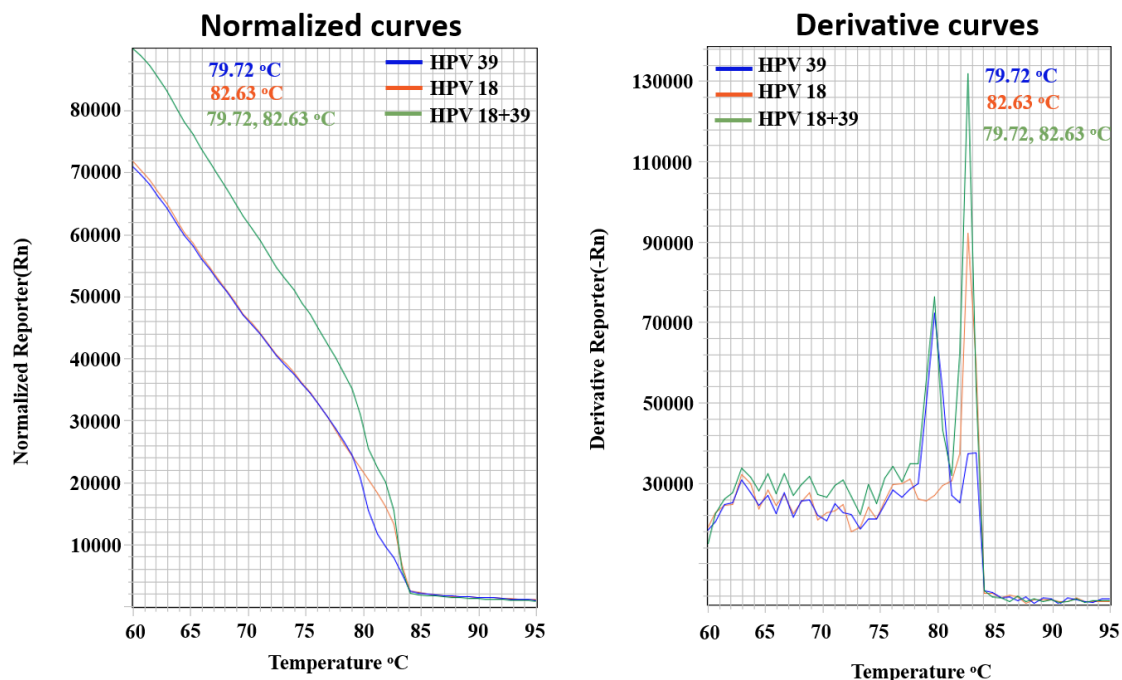


Figure 3.3: Normalized and derivative curves of multiple HPV genotypes. HPV 18 (green), HPV 39 (blue) and Mix of HPV 18 and 39 (red) with Melting temperature 79.72°C, 82.63°C and (79.72°C and 82.63°C) respectively.

Figure 3.4 below, shows the derivative curves of some clinical samples; in sample 11318 and 11292, the curves and Tms correlate with to the predicted melting profile figure 3.1 and 3.2. In sample, 13172 and 11333 melting curves the melting profiles could not distinguish individual genotypes present in the multiple infected samples. The Tm peaks summed up to the individual genotypes Tms. These results were confirmed by analysis of the DNAs on PAGE (figure 3.4 right panel); demonstrating sample 11318 negative control (ALAS1), 11292 infected by HPV 16 shown by two separated bands. Sample 13172 tested positive for HPV type 16 and 59 while sample 11333-tested positive for HPV types 16, 31, 51, 58 and 59.

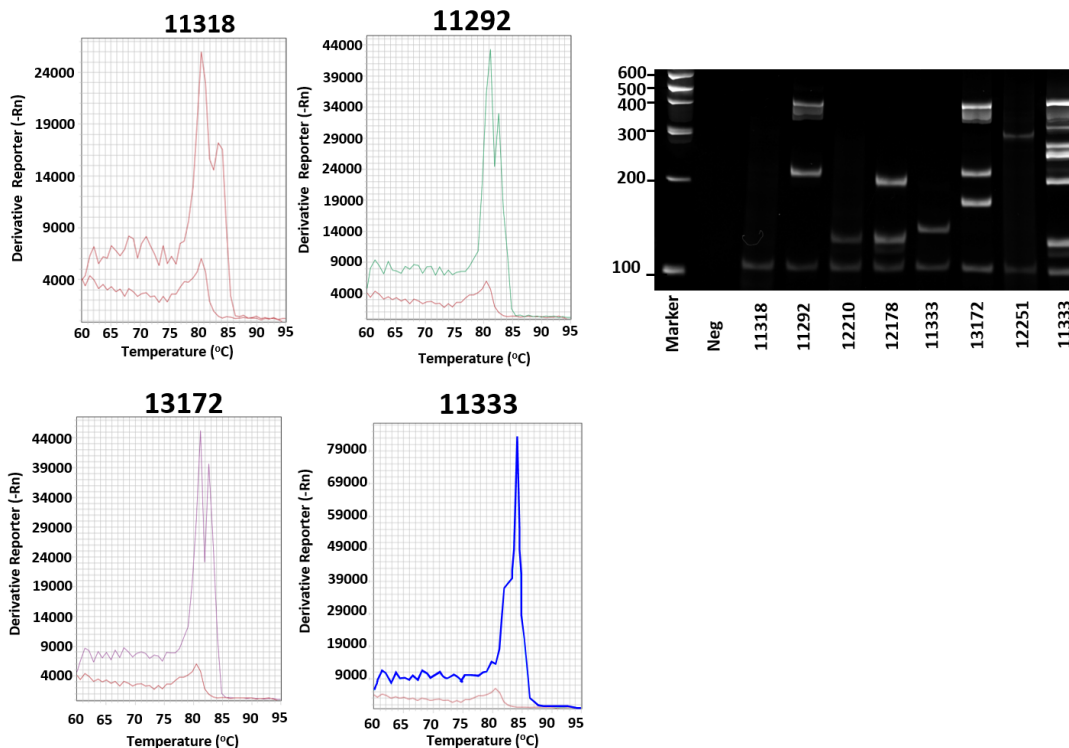


Figure 3.4: Derivative melting curves of four clinical samples 11318, 11292, 13172 and 11333; each shown with Non-Template Control, NTC in (lower curve in red). The lower right panel shows post-melt-off an analysis by PAGE run to confirm the genotypes in the samples; Marker, 1-kb DNA marker, Neg; negative control (NTC) and 8 clinical samples. The four sample analyzed by PAGE as Negative clinical for Sixteen HPV genotypes (11318), HPV 16 (11292), HPV 16 & HPV 59 (13172), HPV 16,31,51,58 and 59 (11333).

Discussions

The aim of this study was to evaluate the melt-off assay as a simple one-step alternative method to detect HPV genotypes amplified by the mPCR assay instead of performing PAGE to separate the amplicons band based on their molecular weight. Our objective was to minimize the expense of using multiple fluorescent probes, thus, we limited detection to a single fluor, SYTO 82. The multiplex PCR method had already proven to be sensitive and reliable for detection of HPV genotypes at low cost, the use of the melt-off assay could further simplify and reduce cost.

The assay demonstrated high fidelity detection of the T_m in triplicate analyses of individual HPV amplicon. Although the assay presented the possibility of a simplified means to detect multiple HPV, we found that it lacked the resolution necessary to discern individual T_m s of each genotype. However, when multiple genotypes were constructed with T_m of 2°C apart between each genotype the assay detects the multiple HPV genotypes. Therefore, the melt-off assay was shown to be less efficient than desired, the due merging of peaks and biphasic melting patterns. Moreover, the melting temperatures for most of the PCR products were only different by 1°C in T_m , which is too close. As a result, multiple HPVs appear as one peak instead of separate multiple peaks. However, we can still make the assay to detect the only HPV16 and/or HPV 31.

Further optimization of this assay will be needed, for the assay to detect multiple HPV genotypes. One possibility is by designing the assay primers that suit dye-based detection. Primer dimers also affect assay specificity because of the nonspecific DNA-binding of primers. Optimization of primers for GC content and proper T_m will ensure the amplicons give reliable melting curves with the single peak(85). Previous studies have shown that amplicon length could influence the sensitivity of genotyping; design of short length amplicons is preferred to give better resolution of genotypes (79, 86). The amplicons for this assay were long hence the assay was slightly less sensitive(85).

Considering the simplicity of detection of one or two HPVs with this approach along with the fact that the most important contributors to cervical cancer are usually only HPV16, and 31, this assay could still be adapted to monitor the two most abundant HPVs in cervical cancer in Sub-Saharan Africa. In essence, the best use of limited clinical resources would be to triage patients who are HPV16 or HPV31 positive and are positive for visible cervical

lesions by visible inspection with acetic acid (VIA). This is true because these are the cases, which are most likely to progress squamous cell carcinoma (SCC). As a whole, HPV 16 is the most clinically important HPV genotype in Tanzania, since it is prevalent in about 15% of randomly screened patients and is most common in invasive cervical cancer in Saharan Africa and the world (29, 31, 34).

We anticipate testing for HPV16 and 31 by real-time PCR along with VIA would provide excellent information for clinical decision-making. We believe that this simplified low-cost genotyping approach would provide the most clinically relevant information, which when combined with VIA, could better inform treatment.

CHAPTER 4

CONCLUSIONS

Cervical cancer has been shown to be of high prevalence in LMICs, accounting for about 80% of cervical cancer-related deaths in these countries (22). Poor healthcare infrastructure and difficulty in initiating and sustaining an HPV immunization programs for adolescents account for the increased cervical incidence in LMICs (87). Other setbacks in combating cervical cancer include the affordability and cost-effectiveness of the screening tools and HPV DNA test kits.

Although Tanzania has introduced a prophylactic HPV vaccination into its routine immunization program, it will take a long time to achieve a significant reduction in cervical cancer. In order for the HPV vaccine to be effective, the vaccination levels must reach about 80%, to effectively halt transmission from person to person. In addition, the cultural diversity and public acceptability of the vaccination program are continuing challenges for Tanzania (88, 89).

Other diseases such as HIV and malaria compete for scarce national resources, and thus, progress on all diseases suffer. Studies have shown that cervical cancer is common in sub-Saharan Africa due to the presence of comorbid conditions and diseases like HIV and other sexually transmitted diseases. Despite these findings, there have been few large population studies on HPV and HIV in Tanzania because of poor financial resources and unsustainable HPV testing tools. Large population studies could have been achieved if there were a feasible cost-effective HPV test.

The validated mPCR HPV assay ensures cost-effective and extremely reliable HPV detection, which promises a better understanding of HPV and its natural history in Tanzania. The test uses simple molecular methods and detects fourteen high-risk and common two low-risk HPVs in a single reaction. Our multiplex PCR assay proved to

have high fidelity in the detection of sixteen HPV genotypes with high sensitivity and specificity. The detection of single and multiple HPV DNAs within each sample was in exact agreement with the WHO validated Hybridization method. In the future, a large-scale clinical validation study of this assay should be done; along with FDA approved test and inclusion of international validation HPV DNA test guidelines.

In the past decade, the HPV DNA assay has been incorporated into the cervical cancer-screening program, in several developed countries. Although it is not a routine practice in the Tanzanian cervical cancer program, studies have pointed out the advantages over Pap smear or VIA alone. In Tanzania, VIA has been the routine standard cervical screening method due to its lower cost; however, the method is less sensitive than HPV DNA tests. HPV DNA testing could be used as co-test in triage women having lesion by VIA. We anticipated that mPCR or a version of the DNA melt-off assay might be a better molecular HPV test to triage VIA-positive women than VIA alone. Since the HPV DNA test is more sensitive than VIA, testing of the VIA positive patients with a simple and cost-effective mPCR assay could reduce the number of VIA positive patients who unnecessarily undergo biopsy (90). With those considerations, we evaluated the mPCR melt-off assay as a simple tool for detection of the most prevalent HR-HPV in the LMICs. The advantage of this approach is that it would cost less since no gel electrophoresis would be required and would be faster. In our studies, we found that each PCR product on its own gave consistent Tms after analysis in triplicate experiments. However, we found that it was not possible to distinguish multiple HPVs due to lack of resolution. Thus, the assay might be best used to detect the most prevalent and most important HPV genotypes in cervical cancer in Tanzania, for

example; HPV 16 and 31. The objective of this approach is to provide simple, inexpensive and quick results to advise clinical decision makers on VIA positive cases, to more efficiently utilize limiting cervical screening resources.

Apart from cervical cancer, HR-HPV has been also associated with other malignancies like anal, vulvar, penile and oropharyngeal carcinomas (45, 91, 92). Therefore, these two methods, mPCR, and mPCR melt-off analysis will provide a better understanding of HPV associated malignancies. Low-risk HPVs, type 6 and 11, are notorious for their role in condyloma acuminatum (large genital wart). Therefore, evaluation of the two assay and future development of simple, cost-effective assays is critical for the cervical cancer screening services and level of risk analysis of cervical cancer as well as HPV related the diseases.

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