Expression and Purification of HPV Proteins for Early Detection of Head and Neck Cancer

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved July 2019 by
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ARIZONA STATE UNIVERSITY

August 2019

ABSTRACT

Recent studies have shown that human papillomavirus (HPV) plays a role in development of cancers, one of which is head and neck cancer. There is strong and consistent molecular evidence demonstrating that human papillomavirus (HPV) is an etiological cause of these oropharyngeal cancers. Despite the introduction of HPV vaccines, there is still an increase in human papillomavirus associated OPC (HPVOPC) and it is expected that the incidence of head and neck cancer, specifically oropharyngeal cancer (OPC) will increase. The aim of this study is to utilize human papillomavirus (HPV) seropositivity for rapid detection of HPV early specific antigen-antibodies using a lateral flow assay.

Human papillomavirus (HPV) 16 proteins of interest, E7, E6 and CE2 were expressed and purified in *E. coli* for detection of specific antibodies using lateral flow assay because viral and host factors impact the serologic responses to HPV early antigens in HPV-positive oropharyngeal cancer. 17 samples and 5 controls with already known antibody reactivity from ELISA analysis were selected for HPV serologic responses. The lateral flow strip was evaluated for its color band intensity using Image J software. Peak area was used to quantify the color intensity of the lateral flow strip. Out of the 17 samples, 11 (64.7%) showed high antibody levels to E7, 12 (70.6%) showed high Ab levels to E6 and 6 (35.3%) showed high Ab levels to CE2. Correlation coefficient between antibody detection by sight and ELISA for E7, CE2 and E6 were 0.6614, 0.4845 and 0.2372 respectively and correlation coefficient between lateral flow assay and ELISA for E7, CE2 and E6 were 0.3480, 0.1716 and 0.1644 respectively. This further proves patients or samples with HPV 16 oropharyngeal cancer have detectable antibodies to early E7, E6 and E2 proteins, which are potential biomarkers for HPV-associated oropharyngeal cancer.

DEDICATION

I dedicate this thesis to my parents for believing in me and to every person who has ever raised a prayer for me, offered a word of encouragement, celebrated with me or cried with me during this academic journey! Above all, I want to thank God for making this research possible.

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INTRODUCTION

The Human Papillomavirus is a pathogenic virus that is associated with complications such as genital warts and cancer (1). It is the cause of almost all cervical cancer and is responsible for a substantial fraction of other anogenital cancers and oropharyngeal cancers. It disrupts normal cell-cycle control, promoting uncontrolled cell division and the accumulation of genetic damage. Although most HPV infections are transient, persistent infections are a prerequisite for pre-cancerous lesions and ultimately cancer (2). In addition to cervical cancer, a substantial proportion of cancers of the vulva, vagina, penis, anus and oropharynx are due to mainly HPV16. Although no effective screening exists for these cancers, there is a possibility that they can also be prevented by HPV vaccination. The human papillomavirus vaccine (HPV vaccine) was introduced over 13 years ago and has led to an overall decline of human papillomavirus incidence, yet HPV is still a major issue in developed and developing countries. The prophylactic vaccines such as Gardasil, Gardasil 9 and Cervarix are known to be safe and quite effective in protecting against human papillomaviruses, however, this vaccine offers protection to the outer surface protein of HPV. In addition to that, no immunotherapeutic treatment has been developed for early cancer detection as well.

Even with vaccination rates and proof of decline in human papillomavirus incidence of about 29% in cervical cancer in 2011- 2014 (3), there have been numerous outbreaks of other HPV associated cancer types in recent years, coupled with the provision of vaccinations in developing countries (3). Globally, approximately 38,000 cases of head and neck cancer are attributable to HPV of which 21,000 are oropharyngeal cancers occurring in more developed countries; and approximately 8,500 cases of vulvar carcinoma, 12,000 of vaginal cancer, 35,000 of anal cancer and 13,000 of penile cancer and 530,000 of cervical cancer are attributable to HPV (4). It is now estimated that

approximately 10% of worldwide cancers are attributable to viral infection, with HPV being the virus associated with the greatest number of cancer cases, and the vast majority (>85%) occurring in the developing world. The incidence of HPV-positive head and neck cancer, specifically oropharyngeal squamous cell carcinoma (OPC), is expected to increase substantially over the next several decades due to the lack of a screening paradigm and despite the availability of a prophylactic vaccine (19).

Head and neck cancer (HNC) comprise of a diverse group of tumors, with an incidence of over 500,000 cases annually worldwide (20). The most common type or subgroup of HNC is head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide (20), which can arise from the oropharynx and, to a much weaker extent, oral cavity, nasal cavity, larynx, hypopharynx. HNSCC subgroup is predominantly characterized by oropharyngeal squamous cell carcinomas located in the lingual and palatine tonsillar regions with poorly differentiated, basaloid histopathology (22) and its pathogenesis has historically been associated with tobacco and alcohol use, but not all cases of HNSCC are associated with these high risk behaviors (20). It has become clear that HPV is one of the main causes of HNSCC. It is estimated that every year, ~30,000 oropharyngeal cancers are caused by HPV and detected in ~25% of all HNSCC. In fact, HPV is now the major cause of oropharyngeal cancer in developed countries, detected in 45–90% of cases (24). More than half of all sexually active people contract one or more types of this virus at one time, making it the most common sexually transmitted disease in the U.S. In fact, oral, head and neck cancers associated with HPV are on pace to overtake the incidence of cervical cancer by the year 2020. Oral infection with HPV16 has been detected in the majority of cases of oropharyngeal squamous cell cancer (OPC), but little is known about transmission, immunogenicity, and oncogenicity of HPV within the oropharynx (23).

Approximately 4000 cases of oral cancer and 3800 cases of laryngeal cancer per year are attributable to HPV disease. 2%-8% of healthy adults have HPV of the oral cavity, with HPV 16 being the most commonly identified serotype (approximately 95% of HPV-16 DNA). (15). Over the last few years, evidence has accumulated to support a global trend in increased OPC incidence, perhaps most striking in North America and northern Europe (21). This is evidenced by a meta-analysis of 2,099 OPCs evaluated in the US literature observed HPV DNA prevalence by polymerase chain reaction (PCR) to significantly increase from 20.9% before 1990 to 65.4% after 2000. And a separate meta-analysis including 5,396 OPCs observed increases from 40.5% before 2000 to 72.2% after 2005 (21).

HPV is a non-enveloped, icosahedral virus that is a member of the Papillomavirus genus in the *Papillomaviridae* family (5). It consists of a single double-stranded DNA molecule of about 8000 base-pairs (bp) that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers (6). It is known to infect the basal layer of squamous epithelial cells or through breaks in the epithelial surface and is maintained in the nuclei of infected basal cells (9). There are over 180 HPV genotypes, numbered sequentially, that have been cloned from clinical lesions (9). Genotypes are distinguished by different nucleotide DNA sequences, primarily those which express different ORF coding for the L1 capsid surface protein; resulting into five genera, two of which are the main phylogenetic genera: the α-HPV and the β-HPV. These correspond broadly to the mucosal infective and cutaneous infective HPV respectively (10). Different types of HPV infect different squamous epithelia or mucosa, with genital types having a specific affinity for genital skin and mucosa. Mucosal HPV can be further categorized into “low” and “high” risk types depending on whether they cause benign lesions or cause lesions that may progress to malignant tumors. The most prevalent high-risk types are 16,

18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (11). The most prevalent HR-HPV in the world today is HPV type 16 (HPV16).

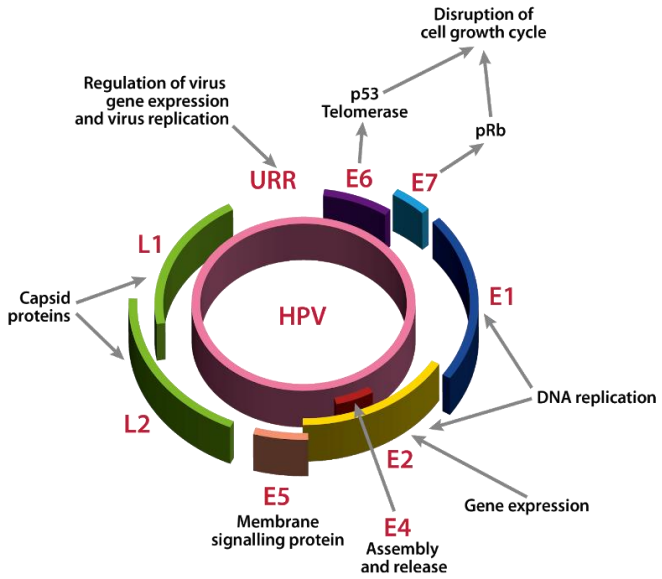


Fig 1: *An illustration of a typical HPV 16 virus genome*[33].

As one of the etiologic agents of head and neck cancer, human papillomavirus (HPV) is just such as a virus. The double stranded and circular DNA genome of HPV encodes approximately eight open-reading frames (ORFs) that are all transcribed from a single DNA strand to several structural and non-structural proteins which are L1, L2, E1, E2, E4, E5, E6 and E7 (5). For the purpose of this study, the focus is on non-structural proteins E2, E6 and E7 from high risk type 16. Regulatory protein E2 is responsible for replication cycle, particularly regulating viral gene expression and viral replication (28). HPV 16E2 is approximately 41 kDa in size and regulates E6 and E7 expression by activating the transcription of E6 and E7 oncogenes through the binding of E2 dimers to multiple copies of an inverted repeat sequence found within the viral long control region (28). For example, E2 protein activates transcription of E6 and E7 oncogenes from the P97 promoter located at the 3′ end of the HPV 16 long control region. At present, there is no model that can explain satisfactorily the effects of the E2 proteins on cell proliferation.

HPV encodes oncoproteins, E6 and E7, which degrades tumor suppressor proteins. These early proteins, E6 and E7 are basic nuclear and cytoplasmic proteins of about 18 kDa (17) and 11 kDa (26), respectively and found during the early stages of infection and can interact with a series of host cellular regulation proteins. HPV 16 protein E6 is approximately 158 amino acid in size and plays a role in promoting cell proliferation by interacting and stimulating the degradation of the tumor suppressor p53 protein via the formation of a trimeric complex comprising E6, p53 and the cellular ubiquitination enzyme E6-AP (27). E6 are expressed early in infection and interacts with p53, resulting in impairment in DNA checkpoint controls. This results in unregulated cell proliferation allowing for epithelial cells to acquire chromosomal mutations and ultimately malignant potential and genetic instability (15). E7 protein is approximately 100 amino acids in size and it possess transformative properties like E6 protein. E7 interacts with retinoblastoma (pRB), a protein responsible for DNA repair and cell cycle control (27). Inactivation of this tumor suppressor protein leads to continuous and rapid division of cells leading to lesions. E7 disrupts the interaction between Rb and E2F, resulting in the release of E2F factors in their transcriptionally active forms, thus preventing the repression of replication enzymes and inactivation pRB tumor suppressor function (27). Over the years, E6 and E7 have proven to be responsible for the development of HPV-induced carcinogenesis. Although the E6 and E7 proteins has been shown to transform cells and induce tumor, there has not been any prompt screening therapy for early detection, or any protection offered against the HPV oncogenes.

There are several clinical and molecular biology testing methods used to detect human papillomavirus infections. The pap smear test is an effective screening method for the detection of early cervical cancer. However, the Pap test is subjective, with significant interobserver variability, limited by low sensitivity and prone to high false-positive rates (18). Nucleic acid test also known as HPV DNA test can now be used to detect HPV

infection (high-risk or carcinogenic types) early. This test is done at the same time as a Pap test and is done by looking for pieces of their DNA in cervical cells and/or the virus that causes these cell changes. Understanding human papillomavirus and its role in human papillomavirus infection led to the development of prophylactic vaccines. These vaccines contain human papillomavirus L1 self-assembling virus-like particles, which induce strong neutralizing antibody responses against human papillomavirus infection (13).

One emerging concept is the production of stable HPV early proteins for routine screening primarily because it is challenging to purify HPV early proteins. In the last few years, HR-HPV DNA testing and HPV RNA testing has been included in routine screening for HPV associated cancer. However, HPV DNA test cannot differentiate a true precancerous state from self-limited HPV infection and HPV RNA is prone to degradation. Diagnostic tests based on the direct detection of the E6/E7 oncoproteins may have advantages over detection of HPV DNA or HPV E6/E7 mRNA. Until recently, most of the antibodies developed against the HPV E6 or E7 protein used either peptides or denatured proteins, as it has been difficult to purify recombinant non denatured E6 and E7 proteins suitable for antibody production (31).

HPV serology is important in the quick clinical diagnosis of HPV oral infection because it aids in identifying individuals with current or past HPV infections. In addition to that, the humoral immune response to HPV plays a significant role in the settings of natural infection, vaccination and cancer. Type-specific IgG antibodies (Abs) to the HPV proteins are induced in response to acute HPV infection, hence why HPV-OPC positive patients develop serum IgG antibodies to HPV proteins. Currently, screening for HPV-positive OPC is infeasible as a result of our current inability to detect precursor lesions and subclinical or early-stage cancers, as well as the absence of an established intervention to reduce cancer incidence or cancer mortality (35). Markers of HPV exposure may provide

an opportunity to identify high-risk individuals because case-control studies have shown that the presence of oral HPV infection or HPV serum antibodies was strongly associated with OPC. This is evidenced by a study by Zhang et al that showed HPV16 E6 seroprevalence was significantly more common in those with than without oral HPV16 infection (7.5% vs 0.7%, p=0.005) (36). Since these Abs to oncogenic E6 and E7 are rarely present in healthy blood donors, they may represent biomarkers of risk for oncogenesis

One of the goals of this study is to successfully purify the HPV non-structural proteins E2, E6 and E7. Hydrophobicity is the major factor that hinders the purification of stable proteins. It is much more prevalent in HPV E6 protein because the HPV E6 protein has a tendency to form inclusion bodies easily when produced in *Escherichia coli* (*E. coli*). It was suggested that rapid folding of the E6 protein renders exposure of strong hydrophobic amino acid residues (33). To determine the hydrophobicity regions of E6, the ProtScale tool ((http://web.expasy.org/protscale/) based on the Kyte and Doolittle hydrophobic scale was used to analyze the hydrophobicity profile of the linear polypeptide sequence of E6. The ProtParam tool (http://web.expasy.org/protparam/) was used to determine the hydrophobicity indices as the grand average of hydropathicity (*GRAVY*) values of hydrophobic peak regions. Based on figure 2, the ProtScale analysis of the HPV16 E6 protein indicated three peaks above the hydrophobic scale of 1.0. The P1 region consisted of the amino acids 31–36 (IHDIIL); the P2 region 58–62 (LCIVY) and the P3 consisted of residues 104–108 (LCDLLI). Study by Ravi et al shows that LCIVY stretch that constitute P2 region is a part of a well characterized T cell epitope and hence is an important component of the protein when used in assays for the determination of immune

response. The P3 region (a.a residues 104–109) lay in the C-terminal domain of E6 which is reported to play pivotal roles in the PDZ binding domain of the protein (34).

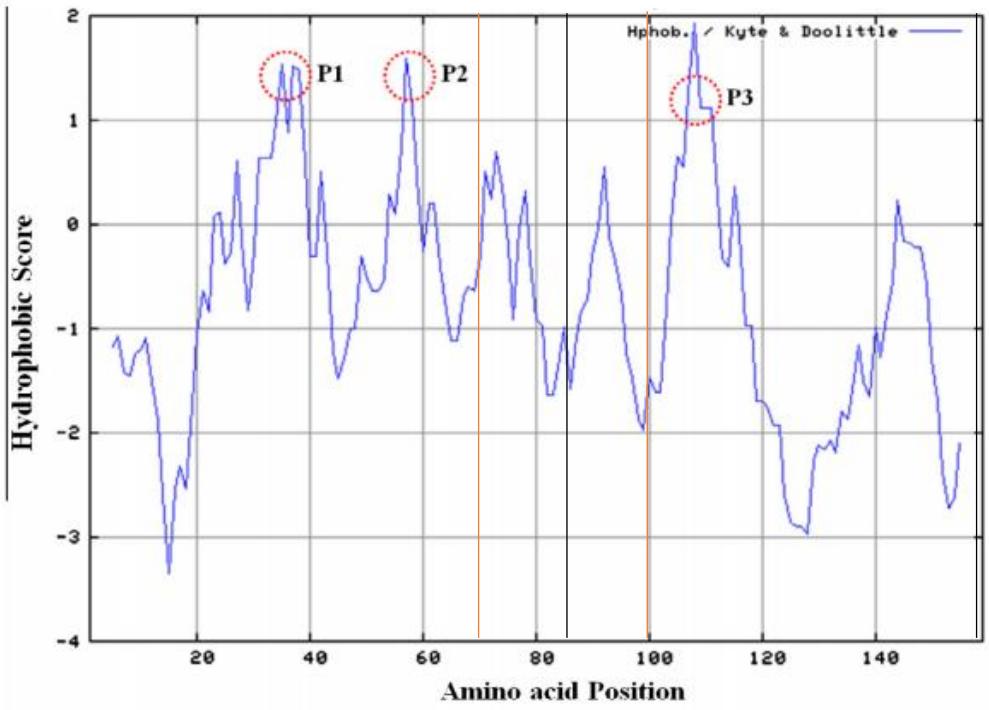


Figure 2: (A) ProtScale profile of the HPV E6 protein. P1, P2 and P3 correspond to the hydrophobic peak regions based on the Kyte and Doolittle scale. Region within the red lines shows 70 -100 and region within the black lines shows 87 – 158.

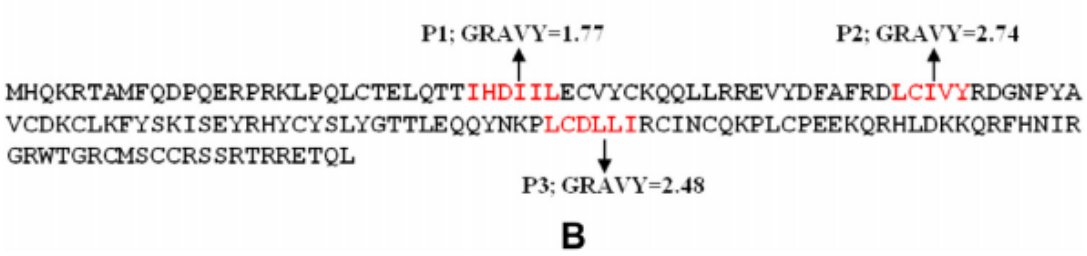


Figure 2: (B) The amino acid sequence of the HPV16 E6 gene. Highlighted in red are the amino acids that constitute the hydrophobic peak regions P1, P2 and P3. ProtParam tool was used to generate the GRAVY values for each P region (34).

We have recently overcome the technical hurdles and have purified recombinant HPV 16 E7, E6 and E2 proteins in their native and/or truncated form to measure and detect the interaction generated from antibodies in positive samples that recognize E6, E7 and E2 proteins from HPV type 16. The strategy employed was inspired from the study by Xu et al 2016, where peptide biosynthetic approach was employed and using highly

conserved epitope sequences for diagnostic antigen design (30). For this reason, we designed a conserved epitope region for E6 and expressed it as a N-terminal GST fusion protein.

Two HPV 16 E6 conserved epitope regions 70 – 100 and 87 – 158 were designed for soluble E6 protein purification. Each of these conserved epitope regions contains hydrophobic regions that are minimal as compared to the whole E6 amino acid sequence. Conserved epitope region 70 – 100 consist of no hydrophobic regions above 1.0 and conserved epitope region 87 – 158 consist of one hydrophobic region above 1.0 (GRAVY = 2.48). Region 87 – 158 of E6 were expressed as N-terminal GST fusion protein, however region 70 -100 of E6 protein failed to express. One of the plausible reasons is the omission of P2 and P3 region in the 70 – 100 amino acid sequence may have negatively influence E6 structure stability and multimerization (34).

Individual HPV16 genes such as E7, were expressed as full-length N-terminal GST fusion proteins except for the E2 protein, which was subcloned as C (CE2)-terminal fragments for optimal protein expression (23). Since these early proteins are integral to the development of cervical cancer, their gene products could potentially serve as highly specific biomarkers to identify high-grade precancerous lesions that may progress to cervical cancer if left untreated. Serum antibodies to HPV proteins, in particular the early (E) proteins E6 and E7, have been detected in several HPV-related cancers and have been found to be reliable indicators of an HPV-positive tumor, including oropharyngeal tumors.

The increasing demand for rapid testing stimulates the development of new techniques in clinical diagnosis for example lateral flow assay (LFA). This is a new and better technique for cancer detection and diagnosis because it is advantageous in terms of low cost, easy operation, friendly use, on site response, rapid and visual results based on the naked eye (25). Unlike cervical cancer, there exists no sensitive and selective screening method for the early detection of HPV OPC, hence the need for a point-of-care testing such

as lateral flow assay for early detection of head and neck cancer. Lateral flow assay will be utilized for this study because it plays an important role in detecting several compounds by obtaining results in a short time with minimal labor (25). This basis of screening is composed of a chromatographic system (separation of components of a mixture on the basis of differences in their movement through reaction membrane) and immunochemical reaction (between antibody-antigen, nucleic acid target analyte). This assay is based on the movement of sample across the membrane via capillary force and the key material in this assay is nitrocellulose membrane.

There are several types of LFA, but the type used in this study is the sandwich model. The standard structure used in this study contains a reaction membrane (usually nitrocellulose membrane), on which location containing test line and control line for target DNA-probe DNA hybridization or antigen-antibody interaction; and an absorbent pad, which reserves waste. The template of this assay consists of five lines: two control lines, IgG for positive and BSA for negative, and three test lines, each for the respective proteins, E6, E7 and E2.

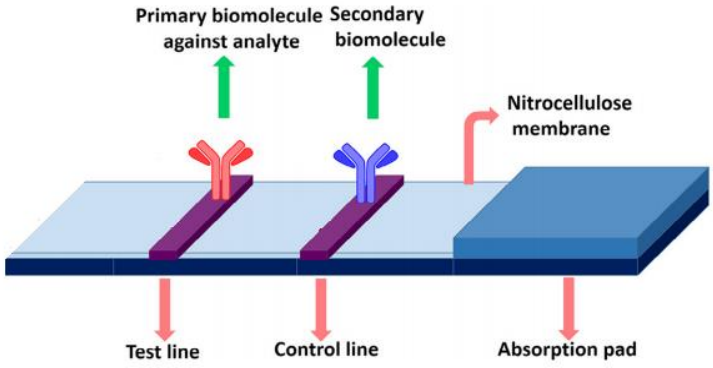


Figure 3: *The basic structure of lateral flow assay*[25]

The purpose of this study is to investigate the serologic immune responses to multiple HPV16 antigens in HPVOPC patients from multi-medical centers in the United

States [23]. In this experiment, we describe a simple sandwich protein-based lateral flow assay using HPV 16 anti-E6, anti-E7 and anti-E2 positive samples to detect purified recombinant HPV 16 E6, E7 and E2 proteins respectively. The study also describes the constructs and conditions for protein purification of HPV 16 E6, E7 and E2. ELISA results from the HOTSPOT study will be compared with our LFA data. We examined the development of HPV16 early proteins E7, E6 and E2 and its interaction with its respective HPV-16-specifc Abs in HPVOPC patients and explored the utility of the unique heterogenous serologic responses as diagnostic biomarkers for HPVOPC. The ability to detect the E2, E4 and E6 oncoproteins in clinical samples is a critical advance that will facilitate the development of diagnostic testing to distinguish benign HPV infections from precancers.

MATERIALS AND METHODS

Study design and subjects

Samples and controls used in this study were generated from the HOTSPOT (Human Oral Papillomavirus Transmission in Partners over Time) study, whose design and enrollment have been previously reported (23). Participants with HPV-OPC along with their spouses or long-term partners and healthy volunteers were enrolled into the HOTSPOT study. Only patients with incident OPC with tumor testing indicating HPV positivity were included in this analysis. 17 HPVOPC positive cases and 5 controls were randomly selected and analyzed for HPV16 E7, E6 and CE2 antibodies.

Primer Design and Constructs design for E7, E6 and CE2 protein expression and subcloning into vector pDEST 15

Primers specific for E7 gene, E6 gene that codes for amino acid region 87 – 158 and C-terminal of E2 gene were designed using attb1 site, attb2 site and delgarno

ribosomal consensus. DNA constructs of these early genes of HPV 16 representing the high-risk species were inserted in the pDONR221 vector and then transferred into pDEST 15 using LR reaction, since pDEST 15 has a N-terminal GST tag allowing effective expression of protein. Modified Bio-Rad protocol was used (29). The DNA constructs were then transformed into the E. coli BL21DE3 cells using heat shock method. These E. coli cells have the ability to express T7 RNA polymerase five times more than normal E. coli RNA polymerase. T7 RNA polymerase gene is also linked to the IPTG inducible promoter, hence the use of IPTG for induction. Below is a table that summarizes the sequences used in this experiment and its respective sizes in kDa.

Table 1: Protein type and its respective sequences and size

Protein type	Amino acid sequence	Size (kDa)
16 E7	MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDG PAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLED LLMGTLGIVCPICSQKP	11
16 E6	CYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDK KQRFHNIRGRWTGRCMSCCRSSRTRRETQL	8.7
16 CE2	NKVWEVHAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAAT HTKAVALGTEETQTTIQRPRSEPDTGNPCHTTKLLHRDSVDS APILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKCLRYRFKK HCTLYTAVSSTWHWTGHNVKHKSAIVTLTYDSEWQRDQFLS QVKIPKTITVSTGFMSI	20.6

E. coli BL21DE3 cells transformation

About 50 ng of HPV 16 E7, E6 and CE2 DNA constructs were transformed into BL21DE3 cells and left on ice for 30 minutes. The cells were then heat shocked in 42°C

water bath. The cells were then placed on ice and 250 ul Super Optimal Broth (SOC) media added and incubated for 1 hour. Using Luria Bertani agar plates containing 100 mg/ml ampicillin, the cells were then plated and left overnight in an incubator set at 37 °C. (Performed by Meredith 29).

Expression of HPV 16E6, E7 and CE2 proteins

Single colonies were picked and inoculated into a 25 ml starter culture containing 25 µl concentration (100 mg/ml) of ampicillin and 250µl of 20% (w/v) D-glucose. The pre-culture was left overnight in a 37°C shaker. The 25 ml pre-culture was then inoculated into 250 ml working volume of LB media and grown at 37°C shaker until it reached an OD_{600nm} value of 0.6 – 0.7. 250µl of 1.0 mM IPTG was also added and left for over 19 hours at 22 °C incubator. Cells were then centrifuged at 5250 rpm for 20 minutes at 4°C and the pellet lysed using lysis buffer (100 mM PMSF, 25 mg/ml DNase, 1.0M $MgSO_4$, 100 mg/ml lysozyme, 1.0M DTT, 10X Fast break and (KH_2PO_4, K_2HPO_4, NaCl, KCl pH 7.8 buffer). The mixture was subjected to short intervals of freezing and thawing four times. The lysed solution was finally centrifuged for 20 minutes at 5250 rpm at 4°C. The insoluble pellet fractions and elutions were saved for SDS-PAGE analysis (29).

HPV 16 E6, E7, CE2 purification

The supernatant from the cell lysis above was then added to the chromatography column (ECONO-COLUMN, 2.5 X 10CM, 2/PKG) with 2 ml of glutathione beads and left overnight at 4°C. Using the washing buffer (500 mM NaCI, 50 mM Tris and 1.0 mM EDTA) with DTT in ratio 1:1, the beads were washed five times and then eluted with 2 ml of elution buffer (50 mM TrisHCI) for 7 times but the beads were allowed to stay immersed in the buffer for 20 minutes before eluting. Six to seven elutions were obtained with shaking intervals of 30 minutes (29)

Characterization of HPV 16E6, E7 and CE2

A volume of 15 µl of a mixture of lysate and sample buffer was added to each well and resolved on a Bio-Rad gel set at 180 V for 45 minutes. The gel was stained with Coomassie blue 10 for visualization. The elution fractions showing the presence of protein on the Coomassie blot were combined and the overall quantities determined using the Bradford Assay (29)

HPV antibody detection in blood samples using lateral flow assay

Plasma samples were tested centrally for HPV16 E7, E6, CE2 and immunoglobulin G (IgG) antibodies by lateral flow assay in the Anderson laboratory. Proteins were expressed using *E. coli* BL21DE3 cells using vector pDEST 15. Lateral flow strip used in this study is constructed with two different pads; a nitrocellulose membrane, and an absorption pad. 420 ng of protein E7 (500 µg/ml), 840 ng of E6 (1000 µg/ml), 420 ng of CE2 (500 µg/ml), 335 ng of IgG (400 µg/ml) and 420 ng of BSA (500 µg/ml) were printed on the LFA nitrocellulose strip using Claremont Bio membrane printer/dispenser.

A volume of 50 µl of PBST (0.2% Tween) was added to the LFA strip to pre-wet. After the fluid has reached the absorption pad, 30 µl of 1: 5 diluted sample in PBS was added to the strip and incubated at room temperature for 15 minutes. The strip was washed once with 50 µl of PBST within a 5-minute interval and/or after the fluid reaches the absorption pad. 30 µl of goat anti-human IgG conjugated to gold nanoparticles was added to the strip twice within an interval of 5 minutes and left for 20 minutes to incubate. The strip is washed twice with 50 µl of PBST within a 5-minute interval. The strip is dried at 37°C for 5 - 10 minutes and analyzed for color bands intensity.

Image analysis system

The image analysis system for the lateral flow strip was composed of a 12 mega pixel dual camera iPhone, white LED light and a computer. ImageJ software (ver. 1.47, HIH, MD, USA) was used to analyze the image and to measure the maximum peak area values of the test and control zones on the detection area of the lateral flow strip. The intensity of the detection area was measured, and the peak area values of the test and control zones were calculated from the intensity result. The intensity of the control zones from all the case controls was used as an appropriate normalizing factor. To account for background interference, each protein's peak area was divided by the baseline peak area of BSA. The experimental results were expressed as the peak area of the test zone.

Statistical Methods

Cut-off values for positive serology were established and defined as the mean of protein to BSA ratio + 3 standard deviations for each antigen observed among the set of controls. Comparison of the protein to BSA ratio between groups of antigen-antibody interaction were performed using Mann-Whitney nonparametric (GraphPad Prism version 8.1.2). Protein to BSA ratio was compared between cases and controls. Correlation data between LFA and ELISA for each protein was done using nonparametric Spearman correlation method.

- **16 E7, E6 and CE2 protein expression and purification**

The expected protein band sizes for each protein ranges from 34 - 46 kDa, including the GST protein which is 26 kDa. Highlighted box signifies protein of interest.

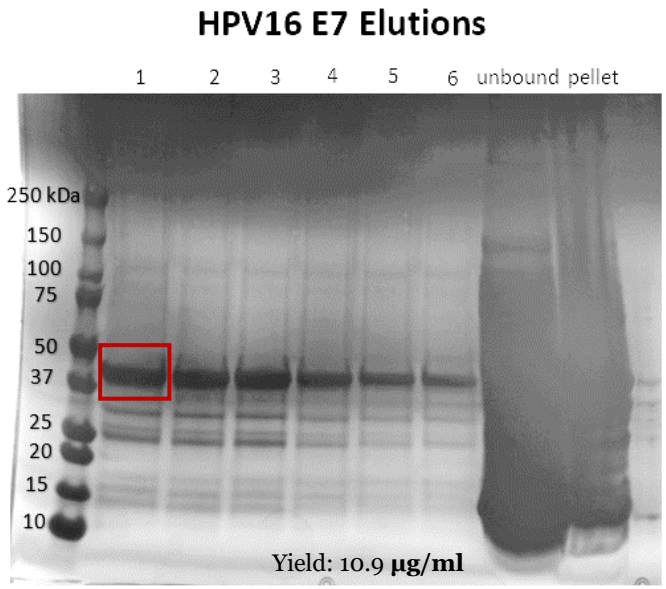


***Figure 4**: A* Coomassie stained gel *of HPV 16E7 expressed protein showing the 37 kDa size obtained from transformed E. coli BL21DE3 with the E7 constructs.*

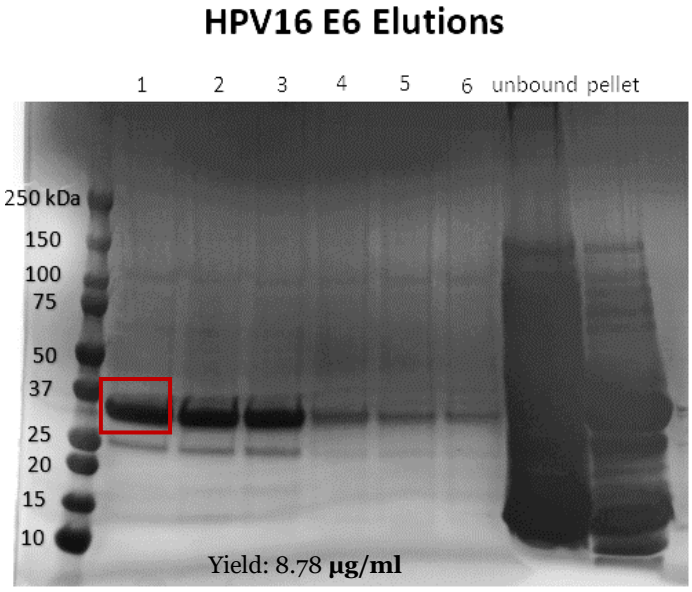


***Figure 5:** A Coomassie stained gel of HPV 16E6 expressed truncated protein showing the 34 kDa size obtained from transformed E. coli BL21DE3 with the E6 constructs.*

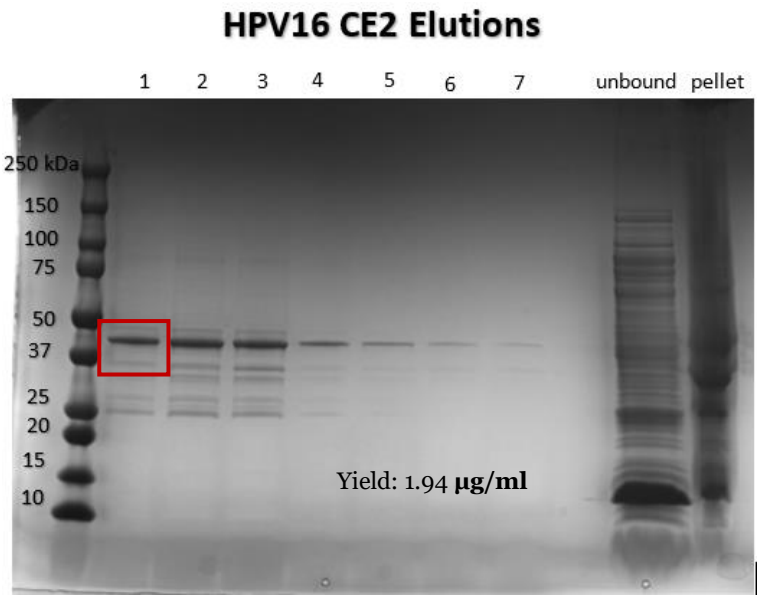
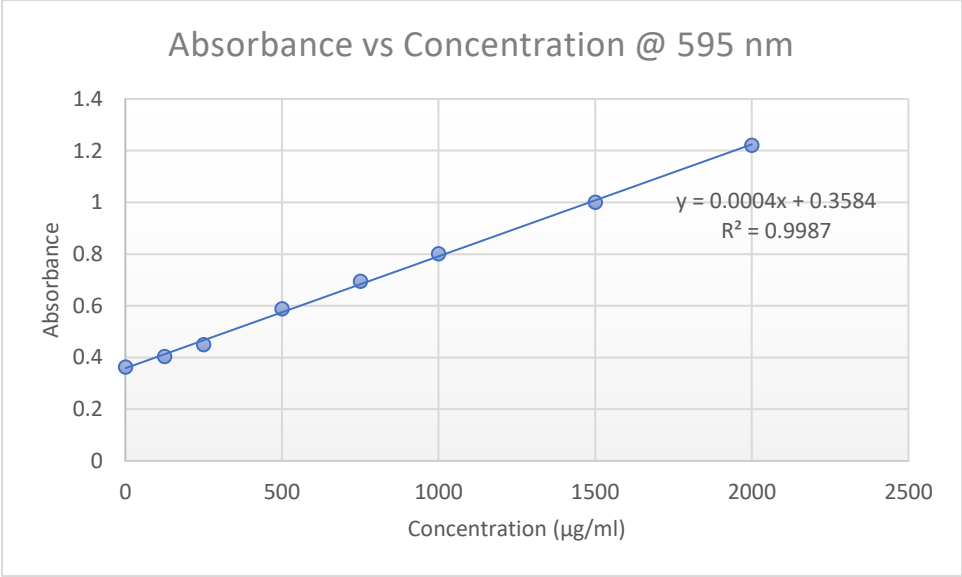


Figure 6: *A Coomassie stained gel of HPV 16CE2 expressed protein showing the 46 kDa sized obtained from transformed E. coli BL21DE3 with the E2 constructs.*

- **Bradford graph and analysis**



Graph 1: Protein Standard Curve. 2 mg/ml of Bovine Serum Albumin stock was used to make serial dilutions. Using the linear equation of the graph the concentration of proteins was calculated as shown below. $R^2>0.95$

Table 2: Concentrations obtained from using the linear equation of **Graph 1**

Protein type	Concentration (µg/ml)	Volume (ml)	Yield (µg/ml) for 250 ml
16 E7	1361.67	2.0	10.9
16 E6	1097.8	2.0	8.78
16 CE2	968.17	0.5	1.94

- **Ab detection by sight**

Antibody detection was conducted by observing the presence and absence of lines in each LFA strip for each protein before quantification. Comparison of data generated for each sample was compared with its known antibody reactivity from ELISA data.

Table 3: Detection of antibodies by presence of lines on strips

SAMPLE	E7	CE2	E6
3053	Y	Y	Y
3057	Y	Y	N
5005	N	Y	N
5017	Y	Y	N
5027	Y	Y	Y
5049	N	Y	N
7003	Y	Y	N
7008	Y	Y	N
7016	Y	Y	N
7027	Y	N	N
1050	Y	N	Y
3010	Y	Y	N
3014	Y	Y	N
3039	N	Y	N
3047	Y	Y	N
3032	Y	Y	Y
3045	Y	Y	Y

- **Data comparison between Ab detection by sight & ELISA[1,2]**

17 samples and 5 controls were used with already known Ab reactivity from ELISA (HOTSPOT study). 14 out 17 samples showed line for E7, 15 out of 17 samples showed line for CE2 and 5 out of 17 samples showed a line for E6.

Ab detection by sight

SAMPLE	E7	CE2	E6
3053	Y	Y	Y
3057	Y	Y	N
5005	N	Y	N
5017	Y	Y	N
5027	Y	Y	Y
5049	N	Y	N
7003	Y	Y	N
7008	Y	Y	N
7016	Y	Y	N
7027	Y	N	N
1050	Y	N	Y
3010	Y	Y	N
3014	Y	Y	N
3039	N	Y	N
3047	Y	Y	N
3032	Y	Y	Y
3045	Y	Y	Y

ELISA

SAMPLE	E7 AB	CE2 AB	E6 AB
3053	39.2	19.5	10.5
3057	7.1	7.6	1.3
5005	1.7	28.6	18.5
5017	8.8	7.0	1.4
5027	250.7	23.5	100.4
5049	0.8	82.3	0.9
7003	18.8	38.7	4.8
7008	50.0	53.8	12.0
7016	23.8	3.5	14.2
7027	10.6	1.1	5.1
1050	2.6	1.3	7.4
3010	19.4	29.2	7.4
3014	5.5	9.4	3.9
3039	1.2	29.1	15.5
3047	3.6	0.4	1.0
3032	24.7	21.6	8.1
3045	40.1	32.5	3.6

Figure 7: Shows data comparison between data from Ab detection by sight and ELISA.

[1]No lines were detected in the controls.

[2]Values highlighted are positive

- **Correlation data between Ab detection by sight and ELISA**

Correlation coefficient for E7, CE2 and E6 were 0.6614, 0.4845 and 0.2372 respectively.

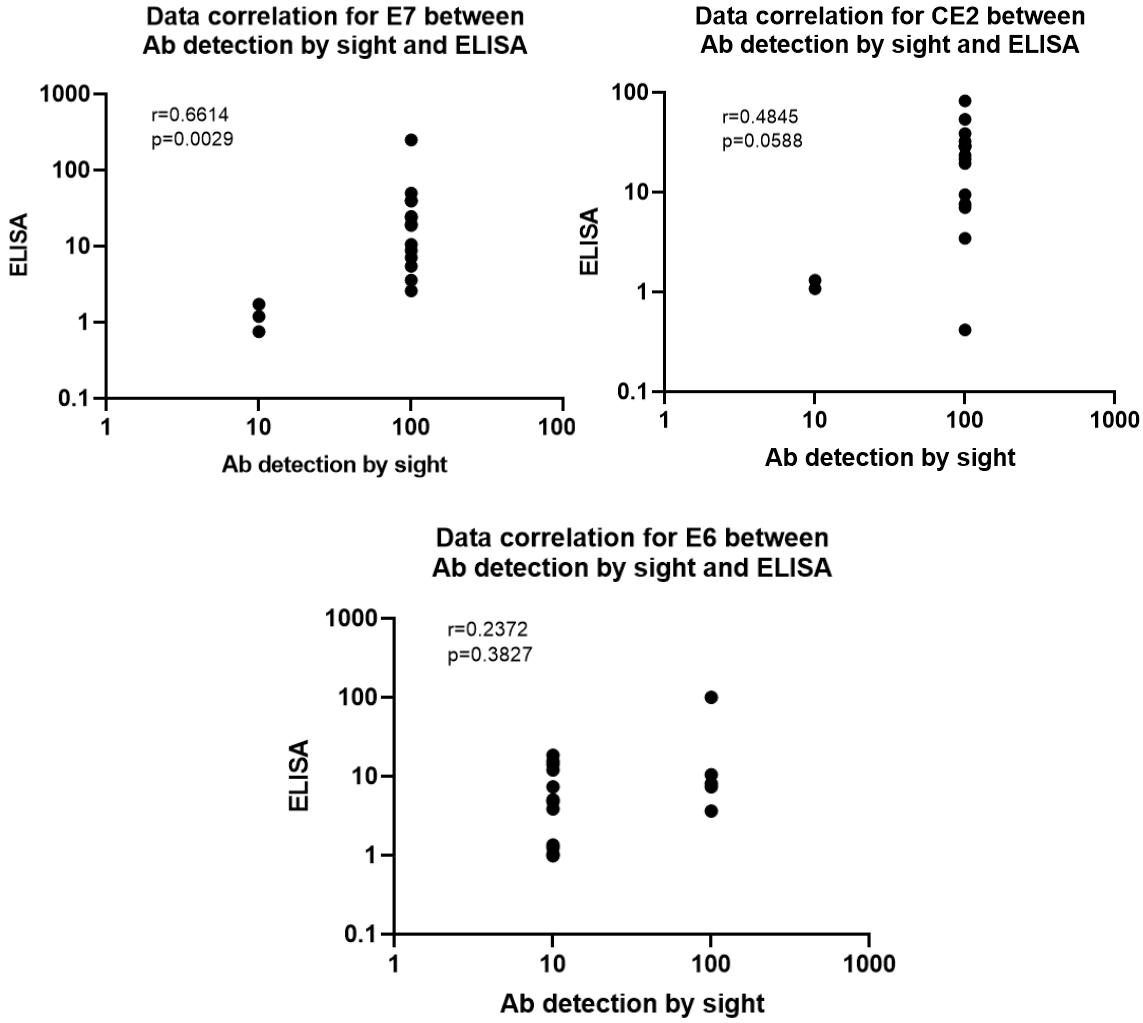


Figure 8: Data correlation for each protein between Ab detection by sight and ELISA. Numerical values were used in place of Yes and No in Ab detection by sight. 10 for No and 100 for Yes.

Image analysis

To improve the lateral flow strip sensor reading, image analysis was performed with images acquired from the strip after the end of the experiments. The figure below shows how and what measured data is generated from the lateral flow strip. As shown below, raw images contained uneven and several colors which required the conversion of raw images to 32-bit images for better data analysis. The intensity peak of each Ag-Ab response is enclosed between the peak (solid line) as shown in IgG, E7, CE2 and E6 and the background baseline (dashed line) as shown in BSA.

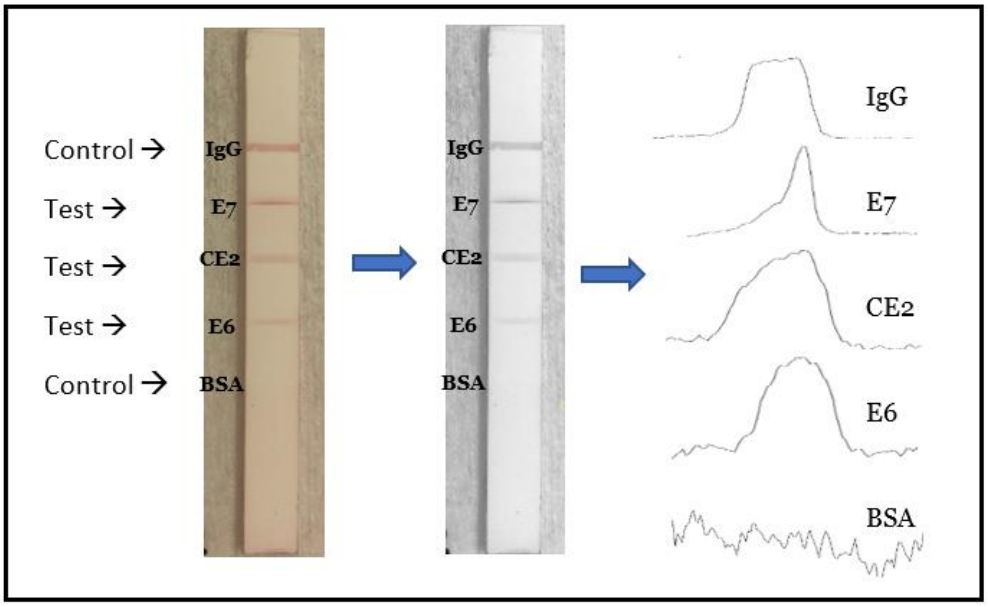


Figure 9: Image analysis procedure and result of a sample positive to all proteins.

Quantifying Lateral Flow assay (LFA strips)

Color intensity is quantified as peak area. (IgG data not shown). Each protein variable to BSA ratio was generated to account for background interference, reduce variability and normalize the test results.

LFA (Peak Area)

SAMPLE	E7 AB (INTENSITY)	CE2 AB (INTENSITY)	E6 AB (INTENSITY)	BSA (INTENSITY)
3053	24729	42290	21475	2112
3057	22946	32806	5740	3078
5005	8295	14813	5251	3524
5017	23999	24772	4029	785
5027	27321	22178	7597	6514
5049	3190	28579	4581	2634
7003	16534	26936	4778	8940
7008	22581	31575	31575	6958
7016	25504	9882	11414	8427
7027	34296	3152	3390	4526
1050	21726	1545	10070	3554
3010	25804	27145	2384	1501
3014	18703	19375	3284	1735
3039	2090	24994	8410	4252
3047	12623	20077	5672	3363
3032	10406	22853	17033	1442
3045	13592	18143	17030	1803

Lateral Flow Assay

SAMPLE	E7/BSA	CE2/BSA	E6/BSA
3053	11.7	20.0	10.2
3057	7.5	10.7	1.9
5005	2.4	4.2	1.5
5017	30.6	31.6	5.1
5027	4.2	3.4	1.2
5049	1.2	10.9	1.7
7003	1.9	3.0	0.5
7008	3.3	4.5	4.5
7016	3.0	1.2	1.4
7027	7.6	0.7	0.7
1050	6.1	0.4	2.8
3010	17.2	18.1	1.6
3014	10.8	11.2	1.9
3039	0.5	5.9	2.0
3047	3.8	6.0	1.7
3032	7.2	15.9	11.8
3045	7.5	10.1	9.4

Figure 10: Shows data conversion from peak area to protein to BSA ratio.

- **Data comparison between LFA & ELISA**

Cut-off values defined as mean of protein to BSA ratio + 3 SD for each antigen in serum of case controls. LFA cut-off values (mean + 3 SD of protein to BSA ratio) for positive serology for E7, CE2 and E6 protein are 3.5, 3.6 and 2.8 respectively. LFA values highlighted are positive and above the cut-off mark.

Lateral Flow Assay

SAMPLE	E7/BSA	CE2/BSA	E6/BSA
3053	11.7	20.0	10.2
3057	7.5	10.7	1.9
5005	2.4	4.2	1.5
5017	30.6	31.6	5.1
5027	4.2	3.4	1.2
5049	1.2	10.9	1.7
7003	1.9	3.0	0.5
7008	3.3	4.5	4.5
7016	3.0	1.2	1.4
7027	7.6	0.7	0.7
1050	6.1	0.4	2.8
3010	17.2	18.1	1.6
3014	10.8	11.2	1.9
3039	0.5	5.9	2.0
3047	3.8	6.0	1.7
3032	7.2	15.9	11.8
3045	7.5	10.1	9.4

ELISA

SAMPLE	E7 AB	CE2 AB	E6 AB
3053	39.2	19.5	10.5
3057	7.1	7.6	1.3
5005	1.7	28.6	18.5
5017	8.8	7.0	1.4
5027	250.7	23.5	100.4
5049	0.8	82.3	0.9
7003	18.8	38.7	4.8
7008	50.0	53.8	12.0
7016	23.8	3.5	14.2
7027	10.6	1.1	5.1
1050	2.6	1.3	7.4
3010	19.4	29.2	7.4
3014	5.5	9.4	3.9
3039	1.2	29.1	15.5
3047	3.6	0.4	1.0
3032	24.7	21.6	8.1
3045	40.1	32.5	3.6

Figure 11: Data comparison for each protein from LFA and ELISA.

- **Correlation data between LFA and ELISA**

Correlation data between the protein to BSA ratio values of each protein in LFA and its known antibody reactivity values from ELISA. Correlation coefficient for E7, CE2 and E6 were 0.3480, 0.1716 and 0.1644 respectively.

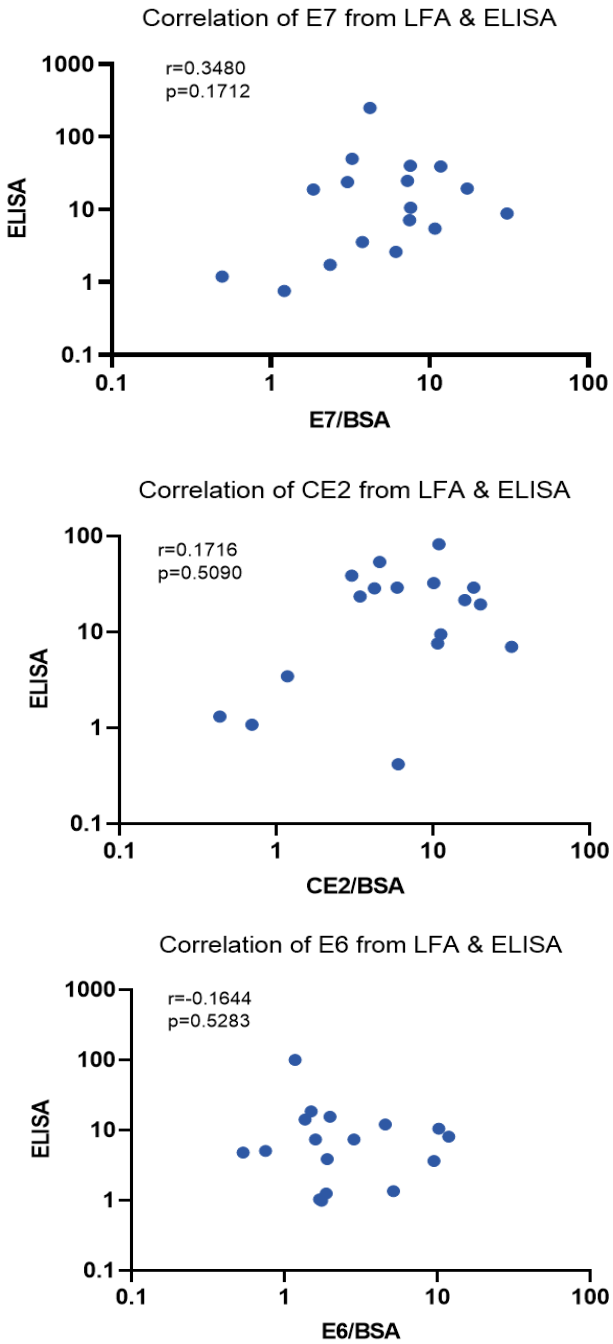


Figure 12: Data correlation for each protein from LFA and ELISA.

- **HPV 16 antibody prevalence.**

IgG antibodies to the HPV16 E7, CE2 and E6 antigens were measured at baseline serum from 17 HPV-OPC cases and 5 case controls. The median of all HPV16 antibodies were increased in cases compared to the controls. The prevalence of serum IgG Abs and early proteins Abs among HPVOPC cases, for HPV16 antigens is summarized in Table 4 and Figure 13 for LFA assay only. Abs to most E proteins were common in cases, including E7-Abs (64.7%) and CE2-Abs (70.6%). Abs to E6 (35.3%) were less prevalent.

Table 4: Prevalence of positive antibody response to each HPV 16 protein in LFA

Total N= 22		
	Cases	Controls
HPV 16 Ab	N = 17	N = 5
E7	11 (64.7%)	0 (0%)
E6	6 (35.3%)	0 (o%)
CE2	12 (70.6%)	0 (0%)
Any E[1]	15 (88.2%)	0 (0%)

[1]Any positive vs all negative

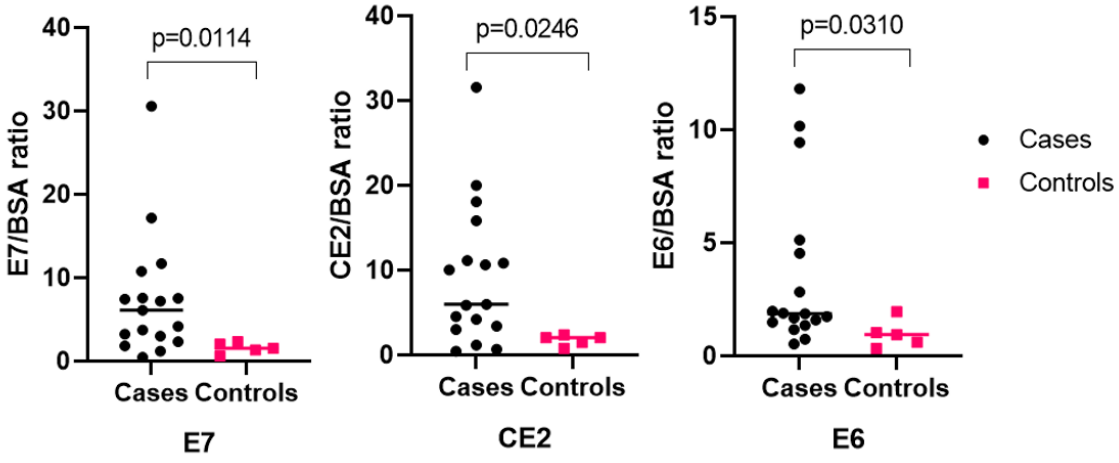
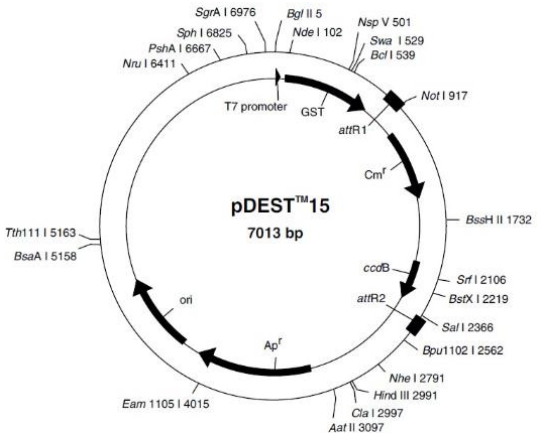


Figure 13: Detection of HPV16 antibodies in serum from 17 HPV-OPC cases and 5 controls using LFA. The black line in each group represents the median value.

DISCUSSION & CONCLUSION

HPV 16 is one of the major high-risk type that is associated with head and neck cancer, specifically oropharyngeal cancer. Due to the rising incidence of HPV-associated oropharyngeal cancers, there is an urgent clinical need for rapid diagnosis of OPC and biomarkers for early detection, diagnosis, prognosis, and monitoring of these patients, hence the goal of this study. We utilized HPV seropositivity to better understand the E7, E6 and CE2 proteins and measure its interaction with positive samples using lateral flow assay. The three early genes of interest were successfully subcloned into the Gateway expression vector, pDEST 15.



Source: https://www.addgene.org/vector-database/2354/

Subcloned genes of interest were tested for protein expression and all three proteins were successfully expressed, ensuring that all HPV early-GST fusion proteins could be used for serologic analysis. As shown in **Figure 4, 5** and **6** the Coomassie gels show the presence of GST tagged E7, E6 and CE2 proteins respectively. Despite the presence of hydrophobic regions above 1.0 scale in E6 construct, the recombinant E6 protein as well as E7 and CE2 proteins can be expressed effectively in *E. coli* with high expression levels and yield, and most importantly a large protein quantity at a low cost, thus allowing for development of low-cost point of care devices.

Here we demonstrate that IgG antibodies specific for three HPV16 early proteins (E2, E6, and E7) are common and heterogeneously present among HPVOPC cases. This study demonstrates that the majority (88.2%) of HPVOPC samples have generated an immunologic response against the virus and have detectable levels of Abs at diagnosis to

at least one HPV16 early protein (**Table 4**). Lateral flow assay was developed using early three antigens from HPV genome for serologic detection of HPV-specific Abs. As expected, we observed HPV16 serologic responses to multiple antigens. About 64.7% and 70.6% of the samples (**Table 4**) showed responses to E7 and CE2 respectively. This further explains the theory of high prevalence of E7 oncoproteins upon HPV integration. During HPV integration, E7 and E6 expression occurs and increases, which is accompanied by loss of E2 expression. However, E2 expression in OPC is not always lost upon integration (31). This explains the high detection level of E2 IgG antibodies in majority of the HPV-OPC samples. In contrast, we observed a low detection level of E6 IgG antibodies (35.3%) suggesting significant differences in pathophysiology, carcinogenesis and seropositivity of HPV among these samples. In addition to that, several reasons for low detection of E6 antibodies are different antibody levels across the samples and antibodies present in HPV-OPC samples could be specific to a different E6 epitope that was not included in our E6 construct.

Antibody detection by sight and quantified lateral flow assay results for each protein showed various correlation to its respective antibody reactivity from ELISA. There was a high correlation coefficient between antibody detection by sight and ELISA for our various proteins of interest. Correlation coefficient between antibody detection by sight and ELISA for E7, CE2 and E6 were 0.6614, 0.4845 and 0.2372 respectively. However, there was a slight decrease in the correlation coefficient for all proteins when quantified data set from LFA were compared with data set from ELISA (correlation coefficient for E7, CE2 and E6 were 0.3480, 0.1716 and 0.1644 respectively). These results highlight that LFA can easily detect antibody reactivity to HPV16 early proteins especially E7, however, optimization needs to be done to increase sensitivity and specificity to CE2 and E6.

There are limitations to this study, one of which is the lack of enough controls for serological screening. As a result of this, sensitivity and specificity could not be determined for this study. For an assay to be clinically useful, it would be necessary to have very high specificity, indicating lower numbers of false positives, and significantly high sensitivity as well. Another limitation is not determining the structure of the E6 protein as folding of protein might affect binding to its specific antibody. Studies have shown that patients seropositive for HPV 16 E antibodies had approximately 250 times the risk of HPV-positive OPC (32). This further shows serum antibodies to HPV16 early antigens as a promising biomarker that could aid in the quick diagnosis of individual with high risk for OPC. This study also proves that lateral flow assay is an ideal assay for quick HPV-OPC detection because of its stability, long shelf life, good reproducibility, low cost and rapidness. In light of this, larger prospective studies are needed to confirm our findings.

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