

HUMAN PAPILLOMAVIRIS TYPE DISTRIBUTION
IN CERVICAL CANCER
IN INDIANA AND BOTSWANA

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DEDICATION

I dedicate this work to my wife, Fatima Zahrou. I could not have accomplished this work without her encouragement. Thank you for your constant love.

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List of Abbreviations

AD	Adinocarcinoma
BLAST	Basic Sequencing Alignment Search Tool
C-myc	Proto-oncogene
C	Celsius
CDC	Center for Disease Control
CRPV	Cottontail Rabbits Papillomaviruses
DNA	deoxyribonucleic acid
E	Early genes
EDTA	Ethylenediaminetetraacetic acid
FDA	Federal Drug Administration
FFPE	formalin fixed paraffin embedded
GS	GeneSquare Microarray Assay
HC	Digene Hybrid Capture II Assay
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HR-HPV	High Risk Human Papillomavirus
ICO	Institut Català d'Oncologia
IUSM	Indiana University School of Medicine
kb	kilobases
L	Late genes
LA	Roche Linear Array Genotyping Assay

LCR	Long Control Region
LR-HPV	Low Risk Human Papillomavirus
mL	millilitre
MMX	master mix
min	minutes
NaCL ₂	Calcium Chloride
NCI	National Cancer Institute
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
p97	HPV genome early promoter
OD	optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PVs	Papillomaviruses
S	second
SA-HRP	Streptavidin-horseradish peroxidase
SCC	Squamous Cell Carcinoma
STD	Sexual Transmitted Disease
TMB	Tetramethylene Zidine
Tris-HCL	trisaminomethane hydrochloride
ug	microgram
uM	micromole

uL

microliter

VLP

Virus-like Particles

WHO

World Health Organization

Introduction

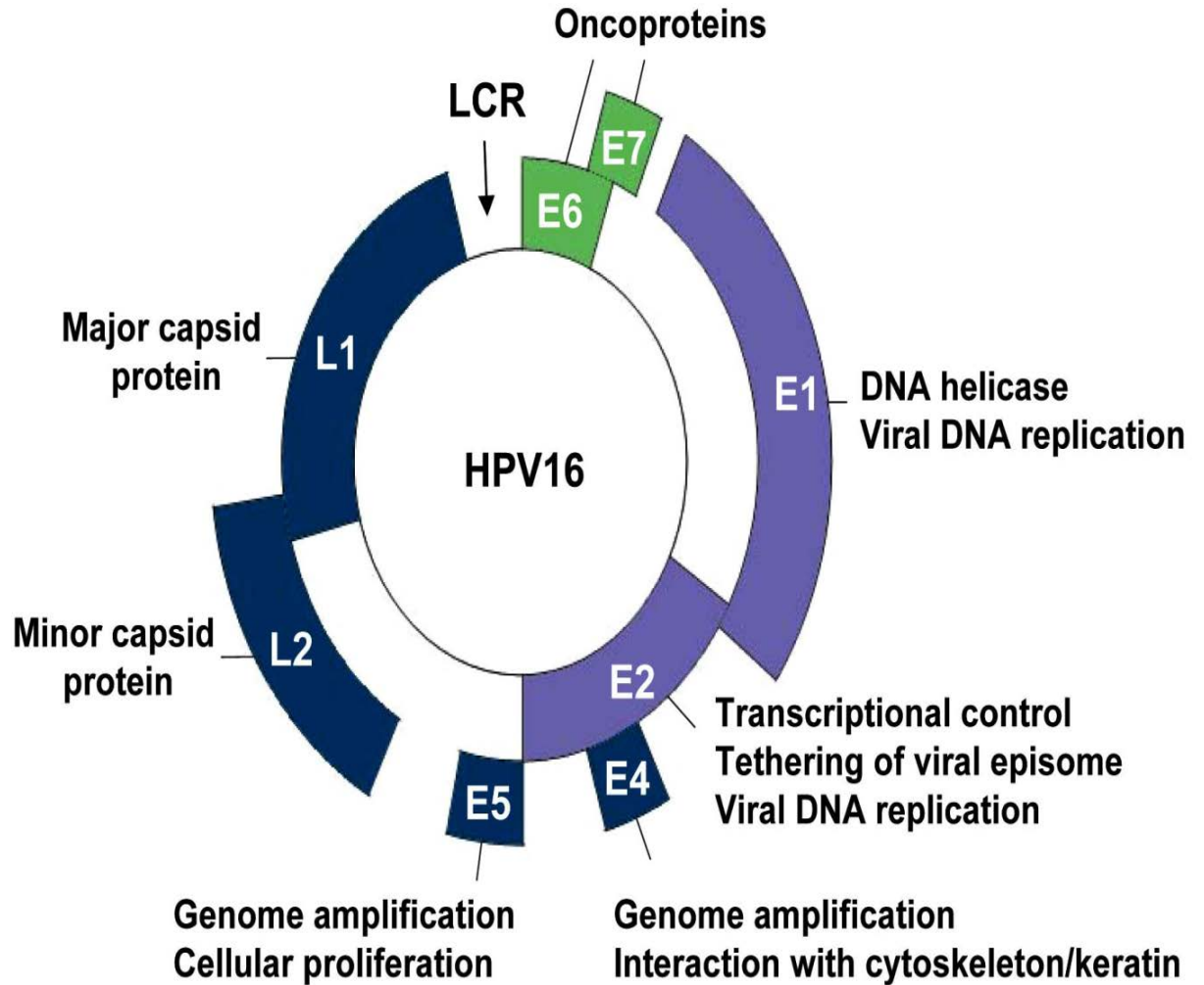
Early History of Papillomaviruses (PVs)

Research concerning PVs began in the mid-19th century. In 1842, an Italian physician, Dr. Domenico Antonio Rigoni-Stern, noticed that married women and prostitutes developed cervical cancer, but nuns did not. This was the first indicator that cervical cancer could be sexually transmitted. However, its cause was not determined at that time ¹. In 1907, Another Italian physician, Giuseppe Ciuffo, showed that cell-free filtrate from ground-up genital and non-genital warts was able to transmit both genital and non-genital warts ². In 1933, Shope and Hurst showed that soluble extracts from non-genital warts from wild cottontail rabbits contained a transmissible agent. This agent was able to cause similar warts in healthy rabbits when it was injected under their skin. Shope and Hurst correctly deduced that these warts were caused by a virus ³. In 1935, Rous and Beard showed that cottontail rabbit papillomavirus called (CRPV) could cause skin cancer in rabbits. After exposure to the extract for an extended time period, skin cancer was detected instead of warts. Since CRPV has a DNA genome, it represented the first known DNA tumor virus ⁴. In 1972, Jablonska and Orth were the first researchers to discover the connection between human papillomavirus (HPV) and skin cancer ⁵. In 1976, zur Hausen published the hypothesis that HPV instead of herpes virus plays an important role in the causation of cervical cancer ⁶. Other scientists rejected this theory ⁷. In 1983 and 1984, zur Hausen proved his theory by identifying HPV 16 and HPV 18 DNA in cervical tumors. In 2008, he received the Nobel Prize for this research ⁸. By 2006, two HPV vaccines, Gardasil and Cervarix, had been developed for the prevention of genital warts and cervical cancer.

Human papillomaviruses (HPV)

HPVs are non-enveloped DNA tumor viruses with small (approximately 8 kb) circular double stranded genome (Figure 1). Despite their small genome, they encode for 8 genes (six early (E) genes and two late (L) genes). The HPV genome is organized in three regions:

- 1) Early genes which are: E6 and E7 (oncoproteins), E1 (DNA helicases involved in DNA viral replication), E2 (Viral DNA replication), E4 (may play a role in viral escape from cornified cell envelope) and E5 (involved in genome amplification)
- 2) Late genes: L1 (major capsid protein) and L2 (minor capsid protein)
- 3) Long Control Region (LCR): non-translated region and major regulatory region for HPV life cycle.



C.M D Abramo and J Archabault 2011 Open Virol J

Figure 1: Diagram of HPV genome. Notice that this genome is composed of three regions: 1) Early genes E6, E7, E1, E2, E4 and E5. 2) Late genes L1 and L2 and 3) LCR region.

HPV classification

HPVs are divided among 4 major groups (alpha, beta, gamma and delta). HPV types in the genus alpha-papillomaviruses are associated with mucosal and cutaneous lesions in human and primates. HPV types within this genus share a conserved sequence within an open reading frame (ORF) of the E5 gene⁹. Group alpha is divided into 15 species (1-15) based on the homology of L1 sequence¹⁰. Each of these species contains many HPV types, grouped by diversity of their E6 and E7 gene sequences. More than 150 HPV types have been completely sequenced.

HPVs that primarily infect genital epithelial tissues are sexually transmitted. Infection can result in a wide range of epithelial tissue anomalies including genital warts and cervical cancer. HPV types associated with cervical cancer are known as high risk (HR) types (for example, HPV types 16, 18, 52, and 59), while those associated with genital warts are known as low risk (LR) types (for example HPV types 6 and 11).

Low Risk HPV types (LR-HPV)

Known as non-oncogenic HPV types, these HPV types may cause benign or low-grade abnormalities of cervical cells and anogenital warts known as condylomata acuminata. HPV types 6 and 11 are found in more than 90% of condylomata acuminata¹¹.

High Risk HPV types (HR-HPV)

HR-HPV types or oncogenic HPV types are associated with intraepithelial neoplasia of the anogenital region, including cervical, vulvar, vaginal, penile, and anal cancers as well as some oropharyngeal cancers. These HPV types 16, 18, 31, 33, 35, 52, 58, 67, 56, 59, 39, 45, 66, 68, and 70 are associated with malignant mucosal lesions.

Among these HPV types, eight (16, 18, 45, 33, 31, 52, 58, and 35) are the most common HPV types found in anogenital cancers ¹². These eight HPV types are also found in 90 % of cervical cancer worldwide. The HPV types 16, 33, 31, 52, 58, and 35 are grouped as HPV species nine (designated A9); while HPV types 18 and 45 form HPV species seven (designated A7). Both species nine and seven are in the genus alpha-papillomavirus.

Proportion of cervical cancer caused by the carcinogenic HPV types		
	Proportion of cervical cancers caused	Cumulative total
HPV16	54.6%	54.6%
HPV18	15.8%	70.4%
HPV33	4.4%	74.8%
HPV45	3.7%	78.5%
HPV31	3.5%	82.0%
HPV58	3.4%	85.4%
HPV52	2.5%	87.9%
HPV35	1.8%	89.7%
HPV59	1.1%	90.8%
HPV56	0.8%	92.2%
HPV51	0.7%	92.9%
HPV39	0.7%	93.6%
HPV73	0.5%	94.1%
HPV68	0.5%	94.6%
HPV82	0.2%	94.8%
No type identified	5.2%	100%

Adapted from Schiffman et al (2007), LANCET

Table 1: Proportion of cervical cancer caused by carcinogenic HPV types. Notice that HPV 16/18 cause more than 70% of cervical cancer.

HPV life-cycle from infection to cervical cancer

Proliferative and persistent phase

The proliferative life cycle of HPV begins when viruses are deposited on the surface of the mucosal epithelium of the genitals, anus, mouth, or airways through sexual contact¹³. HPV virions then pass into the basal layer of the stratified epithelium through microscopic breaks; alternatively the virions may directly infect the columnar cells that form the basal layer of the epithelium of the transformation zone, which is the area adjacent to the border of endocervix and ectocervix¹³⁻¹⁵. The virions then enter into the epithelial cells via an endosome by binding to heparan sulphate proteoglycans in the basement membrane. Once in the epithelial cells, the virions are transported spatially through early and late endosomes. In the late endosome, the virions are uncoated and delivered into the nucleus. In the nucleus with the help of the host's transcription machinery, the HPV genome early promoter p97 is activated leading to transcription of early viral genes E6 and E7. The E6 and E7 proteins bind to the host cell cycle control proteins causing the host cell to progress to S phase, and to retard normal terminal differentiation. The first phase of viral genome replication then occurs^{16,17}. Once the host cell divides and segregates into two daughter cells, one infected daughter cell remains in the basal layer, and the other migrates away from the basal layer and starts the differentiation process¹⁸. During the differentiation process, E1, E2, E4 and E5 genes are expressed and the viral genome continues to replicate. Once the infected cell reaches terminal differentiation, the late genes L1 and L2, coding for viral capsid proteins, are expressed. The new virus particles are then packaged and released from the dead cells.

Persistence and integration phase

Persistent HPV infection with high risk types, such as HPV 16 and 18, is one of the most important events leading to the development of cervical cancer¹⁹. In addition, HPV integration is also considered an important event leading to the progression from pre-neoplastic lesions to invasive carcinoma²⁰. Our previously published data demonstrated that the integration of HPV 59 genome into the host chromosome was a major event that led to the immortalization of human foreskin of epithelial cells²¹. DNA integrates into the host cell genome within the viral E2 gene. The mechanism of HPV double strand DNA break is not yet known. However, there are some chromosomal sites known as fragile sites, where HPV integration is more likely to occur. It is hypothesized that as chromosome breaks occur, this allows for integration of viral DNA. In some cases, the HPV genome (particularly HPV 18) integrates near proto-oncogenes such as C-myc, which are associated with cellular proliferation. As a result, this integration perturbs the function of affected proto-oncogenes which may promote the development of cancer²². However, one study speculated that reactive oxygen species generated from cervical inflammation, due to HPV infection, maybe responsible²³. Moreover, integration occurs at multiple sites within the human genome; this would suggest that the integration occurs randomly²⁴.

Diseases associated with HPV

Linkage between HPV and cervical cancer was established through many epidemiological studies in which nearly all cervical cancer cases were attributable to HPV infection^{25,26}. Moreover, percentage of other non-cervical cancers was attributable to HPV infection as well, such as 12%–63% of oropharyngeal cancer, 90%–93% of anal cancers, 36%–40% of penile cancers, 40%–64% of vaginal cancers, and 40%–51% of

vulvar cancers ²⁷. In addition to cancer, HPV is responsible for all genital warts. HPV types 6 and 11 have been associated with 90 % of genital warts.

Major cervical cancer types

Major cervical cancer types comprise squamous cell carcinoma (SCC) and adenocarcinomas (AC). SCC is the most common cervical cancer histological type detected in 70% to 75% of cervical cancers ²⁸. HPV 16 type causes the majority of SCC. AC cervical cancer type makes up 5% to 10% of cervical cancers. HPV 18 is usually found in AC. Also, there are other types of cervical cancers accounting for 15% of cervical cancers, such as lymphoepithelioma and papillary squamous carcinomas.

1- Examples of normal cervix compared to cervical cancer

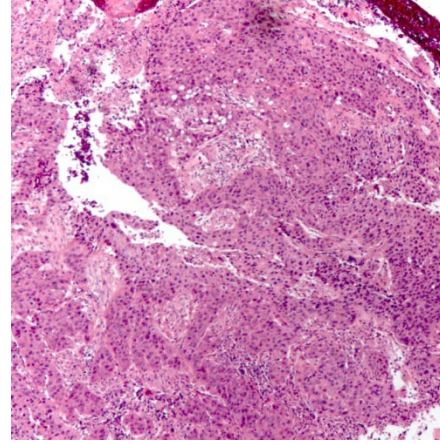
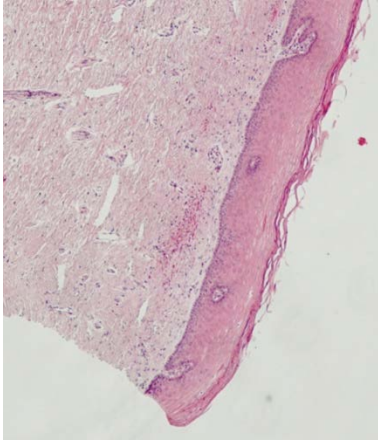


Panel (A)

Panel (B)

Panel (C)

Figure 2: Clinical samples of a normal cervix on the right in panel (A) and cervical cancer (B and C).



Panel A (Normal cervix (50 X))

Panel B (Cervical cancer (50 X))

Figure 3: Normal cervix compared to cervical cancer H&E. In Panel A there is nice separation between the normal cervical epithelium and the myometrium. However, in the cervical cancer section there is invasion of the myometrium and there is no organization to the epithelium.

HPV vaccine

Though the incidence of cervical cancer has declined in developed countries due to certain public health measures, it still has a profound impact on women living in developing countries. Implementation of HPV vaccine program in these countries can reduce the burden of HPV associated diseases, such as cervical cancer. Currently, there are two FDA approved HPV vaccines, Gardasil and Cervarix. Both vaccines contain empty HPV virus particles called virus-like particles (VLP). These VLPs are non-infectious, and are prepared from the L1 protein using recombinant DNA technology. Gardasil (Merck, Whitehouse Station, NJ) is a quadrivalent (prevents from 4 HPV types) vaccine. It is approved first for girls and women 9-26 years of age and later for males 9-26 years of age²⁹. Gardasil prevents cervical pre-cancers, genital warts and anal pre-cancers caused by HPV types 6, 11, 16, and 18. Cervarix (GlaxoSmithKline, Research Triangle Park, NC), is a bivalent vaccine, and is approved for girls and women 9-25 years of age to prevent

cervical cancer caused by HPV types 16 and 18. The CDC highly recommends that preteens be vaccinated long before the start of sexual activity. Furthermore, for optimal result, individuals should receive all three doses. It had been suggested that HPV vaccine can offer certain degree of cross-protection against HPV types that are related to HPV 16 and 18³⁰. However, cross-protection against HPV types such as HPV 31, 33 and 45 is not robust, is short lived, and the clinical importance of such cross-protection is not known³¹.

HPV research in Dr. Brown's laboratory

The World Health Organization (WHO) classifies cervical cancer as the second most common cancer in women globally³². This type of cancer is responsible for more mortality in women than any other cancer in developing countries. The outcome of hundreds of epidemiological studies helped the scientific community understand HPV infection. Such studies helped develop guidelines for cervical cancer prevention strategies, including vaccination. Several HPV epidemiologic studies performed in our lab contributed to the understanding of cervical cancer; some of these studies are summarized below.

- Distribution of human papillomavirus types in cervicovaginal washings from women evaluated in a sexually transmitted diseases clinic³³.

This study demonstrated that many HPV types were associated with cervical dysplasia, including LR-HPV types. Brown et al. also showed that HPV was common among women attending an STD clinic.

- A longitudinal study of genital human papillomavirus infection in a cohort of closely followed adolescent women³⁴.

This study found that the detection of HPV in sexually active adolescent women was extremely high, multiple HPV types were identified in this population and the persistence of this infection frequently resulted in cervical dysplasia.

- Association of condom use, sexual behaviors, and sexually transmitted infections with the duration of genital human papillomavirus infection among adolescent women ³⁵.

This longitudinal study involving only adolescent women confirmed the relationship between HPV infection, condom use and the number of sexual partners. Less frequent condom use and a greater number of sexual partners were associated with longer periods of HPV DNA detection (HPV persistence).

- Detection of specific human papillomavirus types in paraffin-embedded sections of cervical carcinomas ³⁶

In this study we were able to type HPV from paraffin-embedded sections of cervical cancer archived tissues with sensitivity similar to that found in studies using fresh tissues.

HPV 16 was the most commonly detected HPV type. Also, using DNA *in-situ* hybridization, we concluded that in most cases HPV was integrated into the host genome.

- Human papillomavirus genotypes associated with cervical cytologic abnormalities and HIV infection in Ugandan women ³⁷

In this study we found a high prevalence of HPV in cervical swabs among HIV positive Ugandan women. In addition to HPV types 16, 52 and 58 were also highly detected.

In this study we also found that HR-HPV types were associated with abnormal Pap smear in HIV women.

- Human papillomavirus infection and its association with cervical dysplasia in Ecuadorian women attending a private cancer screening clinic ³⁸

This study contributed to the understanding of HPV prevalence and distribution in swabs from Ecuadorian women. HR-HPV types 16, 52, 58 and 59 and LR-HPV types 62, 71, 72, and 83 were the most common HPV types found in this population. Furthermore, there was a strong correlation between the HPV types found in vaginal swabs and cervical swabs. However, vaginal swabs tended to have additional HPV types compared to cervical swabs.

- Human papillomavirus detection and typing in thin prep cervical cytologic specimens comparing the Digene Hybrid Capture II Assay, the Roche Linear Array Genotyping Assay, and the Kurabo GeneSquare Microarray Assay ³⁹

In this study we compared three assays for HPV detection; the Roche Linear Array Genotyping Assay (LA), the Digene Hybrid Capture II Assay (HC), and the Kurabo GeneSquare Microarray Assay (GS). We found high agreement between LA and GS.

- Natural history of multiple human papillomavirus infections in female adolescents with prolonged follow-up ⁴⁰

This prolonged follow-up study of adolescent women, provided insight into the natural history of HPV infection. Participants were followed for approximately 7 years. All study subjects tested positive for HPV at least one time during the course of the study. Additionally, most participants tested positive for HPV 16 or HPV 18 (most common HPV types in cervical cancer).

Rational

Importance of this study

The current HPV vaccines prevent infection and disease from two HR-HPV types (16 and 18). However, the prevalence of HPV types in cervical cancer may differ between geographic regions. Knowing the prevalence of these HPV types in cervical cancer from certain geographic regions may emphasize the need for a vaccine that covers a greater number of HPV types.

In this study we compared the distribution of HPV types in cervical cancer specimens from women living in either Indiana or Botswana. We selected these two regions because of many variations between Indiana and Botswana including geographical, cervical cancer incidence and HIV/AIDS prevalence.

Cervical cancer is the second most frequent cancer in Botswana with an incidence of 60 per 100,000 women per year^{41,42}. Cervical cancer is the second most deadly form of cancer in women living in Botswana, with as many as 14.8 deaths per 100,000 women per year⁴³. The most common HPV types in women with invasive cervical cancer in Botswana were HPV 16 (52.1 %), HPV 18 (10.7%), HPV 33 (9.1%), HPV 31 (4.3%), HPV 45 (3.3%), HPV 59 (2.0%), HPV 35 (1.7%), HPV 58 (1.2%), HPV 52 (0.8%) and HPV 6 (0.4%). Many factors are responsible for the high prevalence of cervical cancer in Botswana. High incidence of HIV infection is one of these factors. Botswana has a high rate of HIV/AIDS infection 23.4% among females⁴⁴⁻⁴⁶.

Cervical cancer ranks 14th of all cancers in the United States⁴⁷. Cervical cancer incidence rate was 7.4 cases per 100,000 women per year⁴¹. The mortality rate was 2.7 deaths per 100,000 women per year. This low cervical cancer incidence in Indiana is

largely due to the wide-scale implementation of cytological screening programs utilizing the Papanicolaou (Pap) test, HPV vaccination, and low incidence of HIV/AIDS. Indiana has lower HIV/AIDS infection 0.7 % among females compared to Botswana ⁴⁵.

Objectives

In this study we isolated DNA from formalin-fixed and paraffin-embedded cervical cancer specimens from Indiana and Botswana. By using this direct method, known as thin-section PCR, we were able to determine the actual HPV type contributing to cervical cancer. Most of previous studies of HPV type distribution in cervical cancers used cervical swabs to determine HPV types in cancers. Because cervical swab specimens sample a large area and can contain HPV types in addition to the types causing cervical cancer, it is difficult, and sometimes impossible to determine which type is responsible for the cancer. This has led to confusing and inconsistent results.

The aims of this thesis are to:

- Design a standardized protocol for high quality DNA extraction from archived paraffin embedded tissues (FFPE) cervical tissue
- Determine and compare HPV type distribution in cervical cancers in Botswana and Indiana.
- Evaluate the association of cervical cancer and HIV infection in women living with HIV in Botswana.

Hypothesis

- High quality DNA (less PCR inhibitors and high molecular weight amplifiable DNA) can be extracted from FFPE cervical cancer tissues and successfully amplified by PCR.
- HPV typing can be performed successfully on FFPE.
- HPV type distribution in cervical cancers will differ between Botswana and Indiana.
- Differences in HPV type distribution will be explained by HIV infection.

Materials and Methods

Acquisition of cervical cancer

FFPE cervical cancer blocks were obtained from women residing in Indiana (United States) and Gaborone (Botswana). Fifty-one cervical cancer blocks were obtained from 51 women treated at Indiana University School of Medicine (IUSM), and 214 cervical cancer blocks from 214 women treated at the STD clinic in Gaborone in Botswana. Cervical cancer tissues from Indiana were archived during 2008 through 2011 and the age of cervical cancer tissues from Botswana were collected between 2009 and 2010. These specimens had been fixed with formalin and embedded in paraffin at the local sites. Because the Botswana blocks were disintegrating from paraffin dehydration, we decided to re-embed the blocks in new paraffin prior to sectioning.

Histology and pathology classification

All sections were prepared at IUSM. Blocks were cut on a Reichert-Jung microtome at a thickness of 5 microns with a clean blade utilized for each tissue. Cut sections were then placed on positively charged slides. Twenty sections were cut for each tissue, and the first and last sections were stained with hematoxylin and eosin (H&E stain). Examination of each H&E stain was performed under light microscope (Olympus BX 41). Specimens with no or small epithelium (less than 5% of the total section) were eliminated from this study. Also, H&E stains were used for pathology classifications. All cancer precursor lesions were eliminated from this study and only invasive cervical cancers were used in this study. The invasive cervical cancers were classified as squamous cervical cancer (SCC) or adenocarcinomas (AC).

Macro-dissection

H&E stained slides were examined under light microscope for cancerous epithelium to healthy connective tissue ratio. In some sections, there was much more healthy connective tissue present than was cancerous epithelial tissue. Since the HPV genome would only be found in the cancerous epithelium tissue, a higher abundance of human DNA in healthy tissue may reduce the sensitivity of the HPV assay used. To avoid this problem, macro-dissection was used to reduce the amount of healthy connective tissue.

In each section new blades (Blades single edge from Fishers, cat# 12640) were used to Macro-dissect unstained slides. The cancerous epithelium was identified under a light microscope and separated from the surrounding healthy connective tissue as shown in Figure 4.

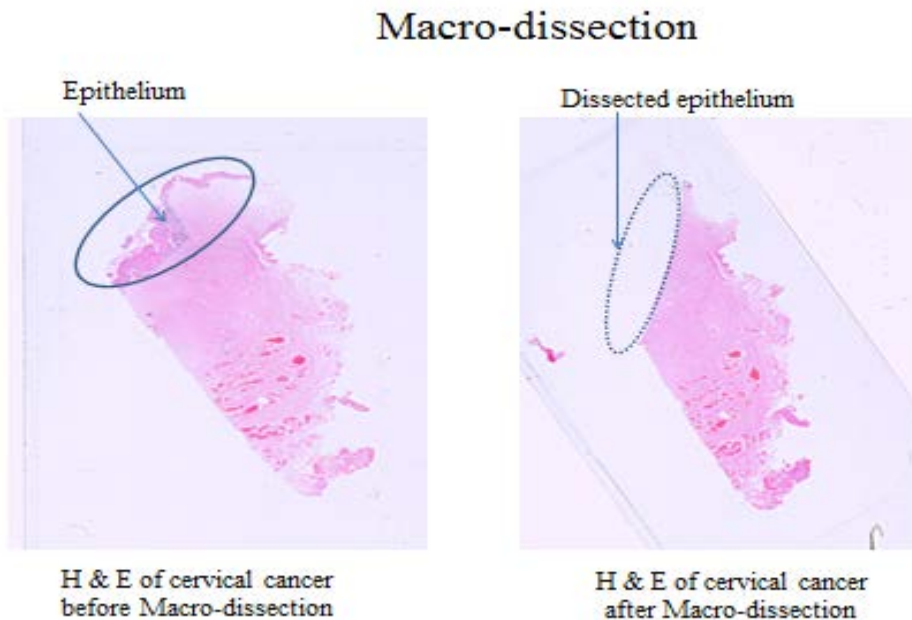


Figure 4: Macro-dissection performed on one of Botswana cervical cancer case

DNA extraction from tissue sections

Deparaffinization

Most of these protocol steps were adopted from a published protocol with some modifications³⁶. In a laminar flow hood, 4 to 6 sections of paraffin embedded slides were carefully scraped from the glass slides with new blade (sigma-Aldrich cat# 296988), using 1 mL octane (this step was adapted from Gregory Buzard online protocol)⁴⁸ (the recovered sections were then c into a labeled 2 mL sterile screw-capped tubes). Samples were rocked at room temperature for 10 minutes (min) and centrifuged at 15.000 x g for 10 min in a micro-centrifuge (Labnet Z 233 M2). The octane was then gently removed, without disturbing the pellet. Then, 500 uL fresh octane was added to the pellet and the tubes were capped and rocked for an additional 5 min to dissolve any remaining paraffin. The samples were centrifuged 19.000 x g for 5 min. The octane was again gently pipetted off without disturbing the pellet. One mL of absolute ethanol was added to the pellet; the tubes were capped and inverted to mix the pellet with ethanol, followed by centrifugation for 10 minutes at 19.000 x g. The ethanol was carefully removed and 500 uL of fresh absolute ethanol was added. The tubes were then centrifuged for 10 minutes at 19.000 x g. The ethanol was carefully removed and the open tubes were spun in a Speed Vacuum (Savant) for 20 min to dry.

Overnight Digestion

The resulting dried pellets were then suspended in 300 uL Specimen Transport Medium (STM, QIAGEN) with 200 ug proteinase K buffer (from QIAamp MinElute Media Kit). Samples were vortexed for 10 s (seconds) and incubated in a dry heat block at 55°C (degree Celsius) for 12 to 16 hours.

DNA extraction

Twelve to 16 h later, DNA was extracted from the samples using the QIAamp MinElute Media Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions.

DNA spectrophotometry

DNA concentration was determined by spectrophotometry (Beckman DU-64, Beckman Coulter, Brea, CA) and approximately 200 ng was used for each PCR reaction. For samples in which the OD 260/280 ratio was less than 1.8, additional slides were used to re-extract DNA.

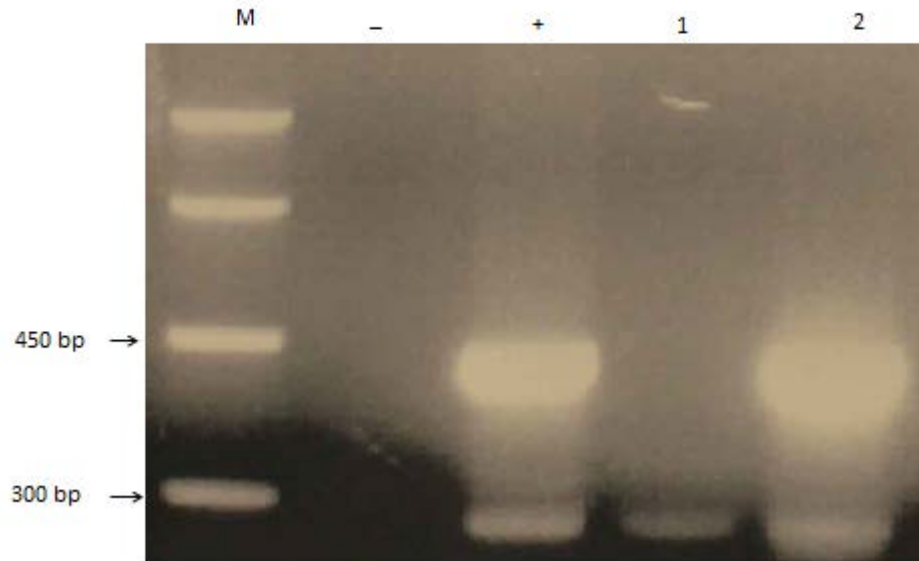
DNA Amplification and Genotyping

DNA Amplification

Using the LINEAR ARRAY HPV Genotyping Test (LA-HPV) (Roche, Indianapolis, IN, USA) HPV DNA was PCR amplified using two sets of biotinylated primers, PGMY09 / PGMY11. This Genotyping KIT specifically amplifies the polymorphic HPV L1 region of 37 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108), the resulting PCR product is approximately 450 base pairs long. Additionally, this assay includes PCR system control primers BPCO4 / BGH20 specific for human beta-globin.

DNA template (200-300 ng) was mixed with LA-HPV Master Mix (MMX) and HPV Magnesium Solution, Reactions were subjected to 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 9 min (denaturation), then 40 cycles of: 95°C for 1 min (denaturation), 55°C for 1min (annealing), 72°C for 1 min (extension), followed by 1 cycle at 72°C for 5 min

(final extension) in a Perkin-Elmer 9600 thermal cycler. Reactions were subsequently maintained at 72°C and 10 uL of PCR product was analyzed on 1.8% agarose gels as shown in Figure 5. Samples were then stored at -80°C.



M: marker, (-) negative control, (+) positive control, (1 and 2) Botswana cervical cancers cases

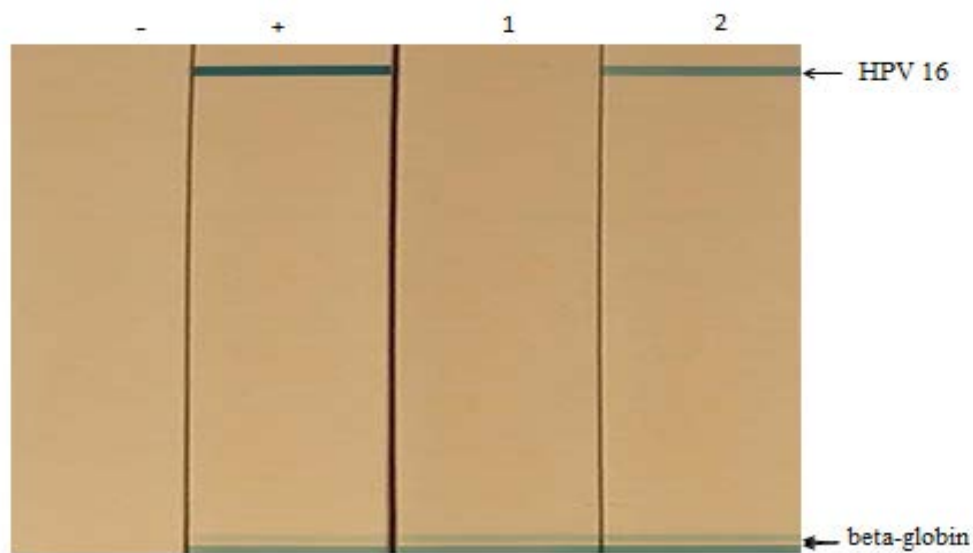
Figure 5: Agarose gel (1.8 %) electrophoresis of PCR product of HPV and beta-globin amplification

HPV Genotyping

PCR reactions, including positive and negative controls were analyzed by LA-HPV according to manufacturer's instructions. PCR products were denatured and incubated with LA-HPV Genotyping Strips. Then, biotin-labeled amplicons were hybridized to the matching sequence of the immobilized probes. Genotyping Strips were washed and incubated in Streptavidin-Horseradish Peroxidase Conjugate (SA-HRP) buffer. Genotyping Strips were washed to remove unbound SA-HRP. Genotyping Strips were incubated with peroxidase conjugate and solution containing hydrogen peroxide and

tetramethylbenzidine (TMB). SA-HRP catalyzes the oxidation of TMB to form a blue colored complex as shown in Figure 6.

Quantitative evaluation of 37 types of HPV was made by visually comparing band intensity against two concentrations (high and low) of human beta-globin control probes on each strip.



(-) negative control, (+) positive control, (1 and 2) Botswana cervical cancer cases

Figure 6: HPV Genotyping using Roche Line Blot Assay

HIV Amplification and Detection

HIV Amplification

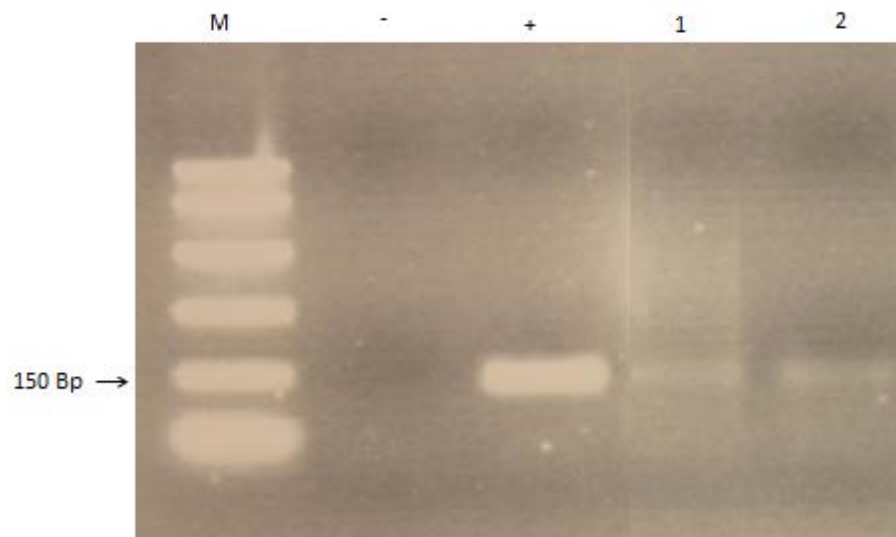
HIV was amplified by PCR from paraffin-embedded tissue using two sets of primers SK145- M (5'-GGG ACA TCA AGC CAT GCA AAT-3') and SK431-M (5'-TGC TAT GTC AGT TCC CCT TGG TTC TCT-3'). This set of primers was referenced in Ciardi's paper⁴⁹ and modified in our laboratory. We removed six nucleotides from 5' end of SK145 primer to overcome the difference in GC content between this primer set.

These primers were used to amplify specifically a portion of 142 base pairs (bp) of HIV gag gene (1360-1507). The plasmid pMDLg/pRRE was provided by Dr K. Cornetta (IUSM) in which complete HIV gag and pol genes are inserted, was used for primer optimization and as a positive control.

DNA template (200-300 ng) was mixed with 25 uL of Master Mix (MMX) FastStart kit (Roche, Indianapolis), 3 uM downstream and upstream primers and sterile double distilled water for a total volume of 50 uL. Reactions were subjected to 1 cycle at 94°C for 9 min, then 40 cycles of: 94°C for 30 S (denaturation), 55°C for 30 S (annealing), 72°C for 30 S (extension), followed by 1 cycle at 72°C for 7 min (final extension) in a Mastercycler ProS (Eppendorf, Hamburg, Germany).

HIV Detection

PCR products, including positive and negative controls were analyzed on 1.8% agarose gels as shown in Figure 7. Two PCR products were sequenced. Samples with 150 bp bands were purified from agarose gels using QIAquick kit (Qiagen, MD, USA), according to the manufacture's protocol. Purified PCR products were then sequenced in the IUSM core DNA facility. Homology of the sequenced samples was determined using the Basic Local Sequencing Alignment Search Tool (BLAST) search at NCBI.



(-) negative control, (+) positive control, (1-2) Botswana cervical cancer cases

Figure 7: Agarose gel (1.8 %) electrophoresis of HIV gag gene amplicon

Results

Statistical analysis

Beta-globin positivity among all cervical cancer specimens and HPV positivity among beta-globin-positive samples were compared between Indiana and Botswana samples. Type specific HPV detection was compared between Indiana and Botswana HPV positive samples. Prevalence of Alpha 9 and Alpha 7 types between Indiana and Botswana were also compared. Additionally, HPV detection was compared between HIV positive and negative specimens among Botswana samples. Comparisons were made using Fisher's exact tests or Chi-square tests as indicated in the specific table. All analyses were performed in the Department of Biostatistics, Indiana University School of Medicine, using SAS (Version 9.3, Copyright©2012 SAS Institute Inc., Cary, NC).

Accounting Table

Overall, 214 FFPE cervical blocks were received from the Women’s clinic in Gaborone in Botswana and 51 FFPE cervical blocks were received from Wishard Hospital, IUSM, Indiana. FFPE cervical blocks with small or no epithelium and/or pre-cancer were excluded from this study. Of the 214 FFPE cervical blocks received from Botswana, 32 were excluded from this study (26 had no epithelium and 6 were pre-cancerous lesions) (see Table 2). All 51 FFPE cervical blocks received from Indiana fit the criteria of this study (Table 2).

Study sites	Indiana	Botswana
Cases received	51	214
Cases with adequate epithelium	51	188
Cervical cancer cases	51	182
Cases with detected beta-globin	50	171
Cases with detected HPV	46	136

Table 2: Summary of cervical cancer cases from Botswana and Indiana

Histology and pathology classification

H&E sections from all cervical cancer blocks from both sites were examined by Drs. Darron R. Brown, Harvey Kramer or Helena Spartz (Indiana University School of Medicine). Sections were examined for epithelial tissue content and pathologically classified. They were classified either as SCC or AC. All 50 cervical cancer specimens from Indiana were classified as SCC, and 166 from Botswana were SCC and 5 were AC (Table 3).

Study site	Indiana (n=50)	Botswana (n=171)
¹ SCC	50	166
² AC	0	5

¹Squamous cervical cancer

²Adenocarcinoma

Table 3: Pathology classification of Indiana and Botswana cervical cancers

Macro-dissection

Macro-dissection was used to reduce healthy connective tissue in cases where more healthy connective tissue was present than cancerous epithelial tissue. Of 182 Botswana cervical cancer cases, 51 cases were macro-dissected and of 50 Indiana cervical cancer cases, 17 cases were macro-dissected.

DNA extraction from tissue sections

Adequacy of extracted DNA was determined by the amplification and detection of the human beta-globin gene. Samples were included in the analyses if human beta-globin gene was detected by reverse blot strip assay. Beta-globin was amplified in 171 of 182 (94.0%) cervical cancer specimens from Botswana, and 50 of 51 (98.0%) cervical cancer specimens from Indiana. The difference in adequate DNA extracted from Botswana specimens and Indiana specimens was not statistically significant ($p < 0.472$) as shown in Table 4.

Study site	Indiana (n=51)	Botswana (n=182)	p-value
¹ BG positive cases	50 (98.0%)	171 (94.0%)	² P<0.472

¹ Beta-globin ² Chi-Square test

Table 4: DNA adequacy results of cervical cancer cases from Indiana and Botswana.

HPV genotyping

HPV positivity

All adequate samples were tested for 37 HPV types as described in the Methods section. HPV of any type was detected in 46 of 50 (92.0%) cervical cancer specimens from Indiana, and 136 of 171 (79.5%) cervical cancer specimens from Botswana (Table 5). The difference in HPV DNA detection was statistically significant ($p=0.042$) (Table 5).

Study site	Indiana (n=50)	Botswana (n=171)	p-value
HPV-positive/BG ² positive	46 (92.0%)	136 (79.5%)	¹ P=0.042

²Beta-globin , ¹Chi-Square test

Table 5: Indiana and Botswana HPV-positive cervical cancer cases

HPV type distribution

Overall, HPV 16 was the most common HPV type in cancers from both sites, detected in 40 of 46 (80.0%) HPV-positive cervical cancer specimens from Indiana and 58 of 136 (42.9%) HPV-positive cervical cancer specimens from Botswana ($p<0.001$) (Table 6). The second most frequently detected HPV type in both sites was HPV 18, detected in 4 of 46 (8.7%) HPV-positive cervical cancer specimens from Indiana and in 32 of 136 (23.5%) HPV-positive cervical cancer specimens from Botswana. The difference in HPV 18 detection between cervical cancer specimens from Indiana and Botswana was statistically different ($P=0.029$) (Table 6).

We detected a greater number of HPV types other than HPV types 16 or 18 in cervical cancer specimens from Botswana than in cervical cancer specimens from Indiana

(Table 6 and Figure 8). We detected only 3 HPV types other than HPV types 16 or 18 HR-HPV types in cervical cancer specimens from Indiana and they were HPV types 31, 33 and 45 (Table 6). Individually, HPV types other than HPV types 16 or 18 did not differ significantly between cervical cancer specimens from Indiana and Botswana (Table 6).

<i>HPV Type</i>	<i>Indiana (n=46)</i>	<i>Botswana (n=136)</i>	<i>p-value</i>
		High-risk Type Distribution	
HR-HPV	46 (100%)	130 (95.6%)	p=0.340 ¹
HPV 16	40 (87.0%)	58 (42.7%)	p<0.001 ²
HPV 18	4 (8.7%)	32 (23.5%)	p=0.029 ²
HPV26	0	5 (3.7%)	p=0.332 ¹
HPV31	1 (2.2%)	5 (3.7%)	p=1.000 ¹
HPV33	1 (2.2%)	7 (5.2%)	p=0.682 ¹
HPV35	0	8 (5.9%)	p=0.205 ¹
HPV39	0	6 (4.4%)	p=0.340 ¹
HPV45	3 (6.5%)	12 (8.8%)	p=0.764 ¹
HPV51	0	3 (2.2%)	p=0.573 ¹
HPV52	0	4 (2.9%)	p=0.574 ¹
HPV53	0	1 (0.7%)	p=1.000 ¹
HPV58	0	2 (1.5%)	p=1.000 ¹
HPV59	0	2 (1.5%)	p=1.000 ¹
HPV66	0	4 (2.9%)	p=0.574 ¹
HPV67	0	1 (0.7%)	p=1.000 ¹
HPV68	0	2 (1.5%)	p=1.000 ¹
HPV69	0	1 (0.7%)	p=1.000 ¹
HPV82	0	2 (1.5%)	p=1.000 ¹
		Low-risk Type Distribution	
LR-HPV	0	12 (8.8%)	p=0.039 ¹
HPV6	0	1 (0.7%)	p=1.000 ¹
HPV11	0	1 (0.7%)	p=1.000 ¹
HPV40	0	1 (0.7%)	p=1.000 ¹
HPV55	0	1 (0.7%)	p=1.000 ¹
HPV81	0	1 (0.7%)	p=1.000 ¹
HPV83	0	2 (1.5%)	p=1.000 ¹
HPV84	0	6 (4.4%)	p=0.340 ¹

¹ Fisher's exact test

² Chi-square test

Table 6: HPV type prevalence and distribution in beta-globin and HPV-positive cervical cancer specimens from Indiana and Botswana

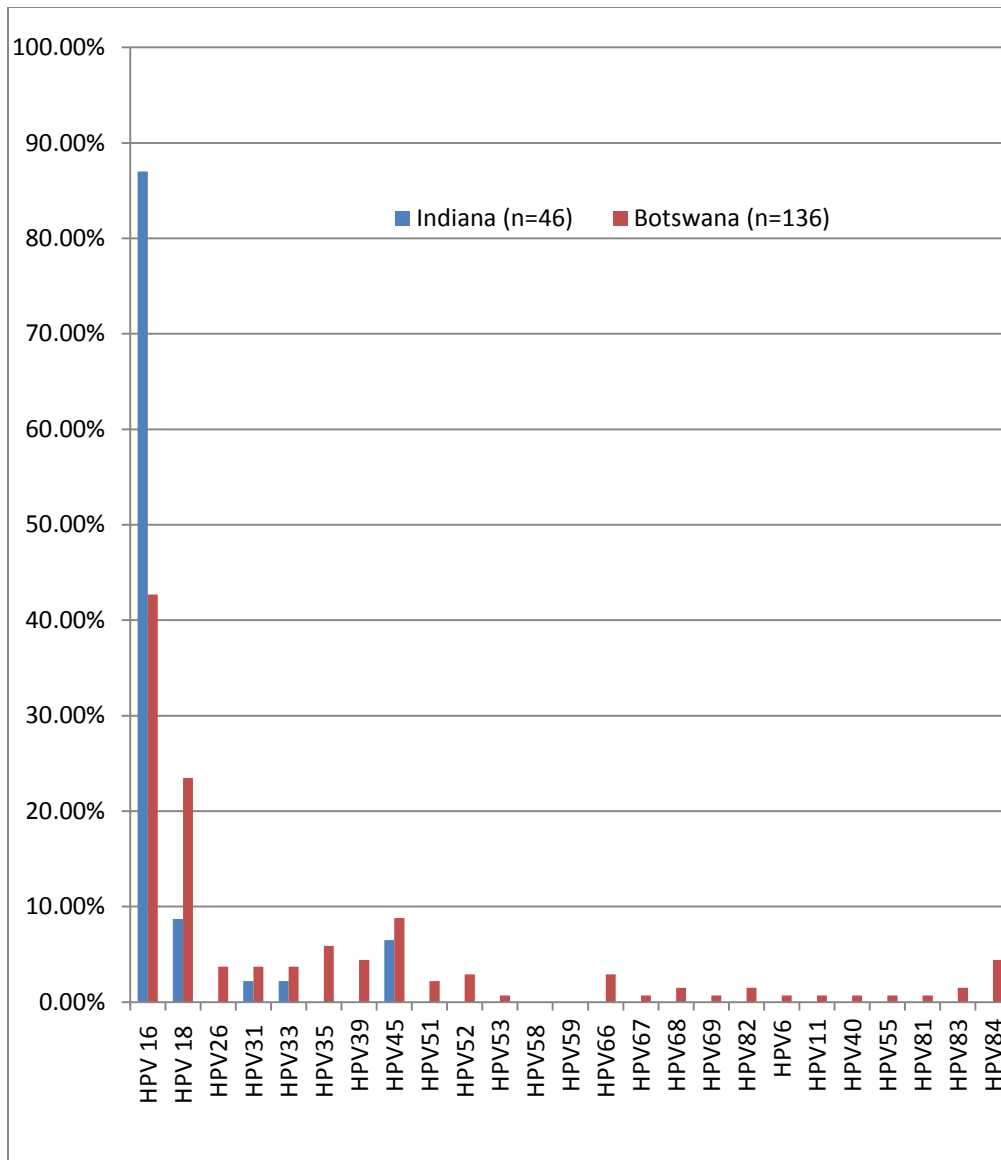


Figure 8: Figure illustrating HPV type prevalence and distribution in beta-globin and HPV-positive cervical cancer specimens from Indiana and Botswana.

HPV distribution in adenocarcinomas (AC)

A total of 5 AC specimens were obtained from Botswana; no cancers from Indiana were AC. HPV type distribution in the 5 AC specimens from Botswana were as follows (Table 7): HPV 18 in 2 of 5 (40.0%), HPV 16 in 1 of 5 (20.0%), HPV 26 in 1 of 5 (20.0%), and no HPV detected in one cancer.

HPV types	Botswana (n=5)
HPV 18	2 (40 %)
HPV 16	1 (20 %)
HPV 26	1 (20%)
No HPV	1 (20%)

Table 7: HPV type distribution in adenocarcinomas from Botswana

HPV distribution by groups

HPV types were divided into groups (non-HPV 16/18, non-HPV 16/18 HR, A9, non-HPV 16 A9, A7, non-HPV 18 A7, and LR-HPV). We conducted statistical analysis to investigate HPV distribution by groups among HPV positive cervical cancer specimens from both sites.

Non-HPV 16/18 types were detected in 5 of 46 (10.9%) cervical cancer specimens from Indiana and 65 of 136 (47.8%) cervical cancer specimens from Botswana ($p < 0.001$) (Table 8). Non-HPV 16/18 HR types were detected in 5 of 46 (10.9%) cervical cancer specimens from Indiana and 56 of 136 (41.2%) cervical cancer specimens from Botswana ($p < 0.001$) (Table 8). A9 HPV types were detected in 41 of 46 (89.1%) cervical cancer specimens Indiana and 79 of 136 (58.1%) detected in Botswana cervical

cancer ($p < 0.001$) (Table 8). Non-HPV 16 A9 types were detected in 2 of 46 (4.4%) cervical cancer specimens from Indiana and 26 of 136 (19.1%) cervical cancer specimens from Botswana ($p < 0.016$) (Table 8).

A7 HPV types were detected in 7 of 46 (15.2%) cervical cancer specimens from Indiana and 49 of 136 (36.0%) cervical cancer specimens from Botswana ($p < 0.001$) (Table 9). Non-HPV 18 A7 types were detected in 3 of 46 (6.5%) cervical cancer specimens from Indiana and 18 of 136 (13.2%) cervical cancer specimens from Botswana ($p < 0.218$) (Table 8).

HPV Groups	Indiana	Botswana	p-value
HPV	46	136	
HR-HPV ³	46 (100 %)	130 (95.6 %)	¹ P=0.340
Non-HPV 16/18	5 (10.9 %)	65 (47.8 %)	² P<0.001
Non-HPV 16/18 HR	5 (10.9 %)	56 (41.2 %)	² P=0.001
⁴A9	41 (89.1 %)	79 (58.1 %)	² P=0.001
Non-HPV 16 A9	2 (4.4 %)	26 (19.1 %)	² P=0.016
⁵A7	7 (15.2 %)	49 (36.0 %)	² P=0.008
Non-HPV 18 A7	3 (6.5 %)	18 (12.9 %)	² P=0.220
LR-HPV ⁶	0 (0.0 %)	12 (8.8 %)	² P=0.040

¹Chi-Square test, ²Fishers Exact test, ³High risk, ⁴(16, 31, 33, 35, 52, and 58), ⁵(18, 39, 45, 59 and 68),

⁶Low risk

Table 8: Distribution of Alpha 9 and Alpha 7 types in cervical cancer specimens from Indiana and Botswana

Multiple HPV types

A single HPV type was most often detected in cervical cancer specimens from both sites (Indiana and Botswana). One HPV type (vs. more than one type) was detected in 43 of 46 (93.5%) cervical cancer specimens from Indiana and 113 of 136 (83.1%) cervical cancer specimens from Botswana (p=0.603). Some cervical cancer specimens contained more than one HPV type (multiple HPV types). Multiple HPV types were detected in 3 of 46 (6.5%) cervical cancer specimens from Indiana (all 3 cervical cancer specimens were infected with 2 types) and 23 of 136 (17.0%) cervical cancer specimens from Botswana (19 were infected with 2 types, 2 infected with 3 types, 1 infected with 4 total types, and 1 infected with 7 types). When HPV multiple types were compared between Indiana and Botswana, the difference was not statistically significant (p=0.603) (Table 9).

Number of HPV types/case	Indiana (n=46)	Botswana (n=136)	p-value
One type	43 (93.5%)	113 (83.1%)	
Two types	3 (6.5%)	19 (14.0%)	
Three types	0	2 (1.5%)	
More than three types	0	2 (1.5%)	¹ p=0.603

¹Chi-Square test

Table 9: Multiple HPV types in cervical cancer from Indiana and Botswana

HIV detection

HIV positivity

All beta-globin positive cervical cancer specimens from Indiana and Botswana were tested for HIV (see methods). The HIV gag gene segment was amplified in 2 of 50 (3.9%) cervical cancer specimens from Indiana and 54 of 171 (32.2%) cervical cancer specimens from Botswana ($p < 0.001$) (Table 10).

Study site	Indiana (n=50)	Botswana (n=171)	p-value
HIV-negative cases	48 (96.0%)	117 (68.4%)	¹ p<0.001
HIV-positive cases	2 (3.9%)	54 (31.6%)	

¹Chi-Square test

Table 10: HIV prevalence in cervical cancer specimens from Indiana versus Botswana

HIV detection and its association with HPV type distribution in cervical cancer specimens from Botswana

Analysis of the distribution of HPV-positive cervical cancer specimens between HIV-positive and HIV-negative cervical cancer specimens was done only for Botswana specimens in order to evaluate the association of HIV and HPV type distribution in Botswana. This analysis was not performed on the cervical cancer obtained from Indiana due to the low prevalence of HIV based on results of PCR assay (Table 10). Overall, 44 of 54 (81.5%) of HIV-positive cervical cancers from Botswana were HPV-positive and

92 of 117 (78.6%) of HIV-negative cervical cancers from Botswana were HPV-negative. Among HIV-positive cervical cancer specimens from Botswana, HPV 16 was detected in 20 of 44 (45.5%) and 38 of 92 (41.3%) were HPV 16-positive among the HIV-negative cervical cancer specimens from Botswana ($p=0.647$) Table 11. Eight of 44 (18.2%) of HIV-positive cervical cancer specimens from Botswana, and 24 of 92 (26.1%) of HIV-negative cervical cancer specimens from Botswana were positive for HPV 18 ($p=0.309$) Table 11. Four of 44 (9.1%) of HIV-positive cervical cancer specimens from Botswana, and 1 of 92 (1.1%) of HIV-negative cervical cancer specimens from Botswana were HPV 31-positive ($P=0.038$) Table 11.

HIV detection and its association on detection of Alpha 9 and Alpha 7 HPV types in cervical cancer specimens from Botswana

Also, Table 11 shows HPV types divided into different groups as a function of HIV status. Among HIV-positive cervical cancer specimens from Botswana, HR-HPV types were detected in 41 of 44 (93.2%). And among HIV-negative cervical cancer specimens from Botswana, HR-HPV types were detected in 89 of 92 (96.7%) ($p=0.388$). Four of 44 (9.4%) of HIV-positive cervical cancer specimens from Botswana, and 8 of 92 (8.7%) HIV-negative cervical cancer specimens from Botswana contained LR-HPV types ($p=1.00$). Among HIV-positive and HIV-negative cervical cancer from Botswana, non-HPV 16/18 ($p=0.765$), non-HPV 16/18 HR-HPV ($p=0.677$), A9 types ($p=0.201$), non-HPV 16 A9 types ($p=0.459$), A7 types ($p=0.276$), and non-HPV 18 types ($p=0.656$) did not differ significantly (Table 11).

	HIV-positive (n=44)	HIV-negative (n=92)	p-value
HR-HPV¹	41 (93.2%)	89 (96.7%)
LR-HPV²	4 (9.1%)	8 (8.7%)	p=0.388 p=1.000
Non-HPV 16/18	20 (45.5%)	45 (48.9%)	p=0.706
Non-HPV 16/18 HR	17 (38.6%)	39 (42.4%)	p=0.677
A9 Types³	29 (65.9%)	50 (54.4%)	p=0.201
HPV 16-Positive	20 (45.5%)	38 (41.3%)	p=0.647
Non-HPV 16 A9 Types	10 (22.7%)	16 (17.4%)	p=0.459
A7 Types⁴	13 (29.6%)	36 (39.1%)	p=0.276
HPV 18-positive	8 (18.2%)	24 (26.1%)	p=0.309
Non-HPV 18 A7 Types	5 (11.4%)	13 (14.1%)	p=0.656

¹HR-HPV= High-risk HPV types, ²LR-HPV= Low-risk HPV types, ³A9 types= HPV types 16, 31, 33, 35, 52, and 58;

⁴A7 types= HPV types 18, 39, 45, 59, and 68; ⁵Fisher's exact test; ⁶Chi-square test.

Table 11: HPV type distribution among HIV-positive and HIV-negative in Botswana cervical cancers

HIV and multiple HPV types

We also examined the association of multiple HPV types with HIV in cervical cancer specimens from Botswana. Multiple HPV types were detected in 5 of 44 (11.4%) HIV-positive cervical cancer specimens from Botswana, and 18 of 92 (19.6%) HIV-negative cervical cancer specimens from Botswana (p=0.232).

Discussions

Quality of extracted DNA from cervical cancer from Indiana and Botswana

The first objective of this study was to determine if high quality DNA could be extracted from FFPE cervical cancer specimens from Indiana and Botswana. Base on beta-globin positivity in specimens from Indiana (98%) and Botswana (94.2%) (Data shown in table 5) it is clear that we were able to extract high quality DNA.

Archived FFPE of cervical cancer specimens represent an easily obtainable source for HPV evaluation for many reasons: 1) FFPEs are widely available; 2) they are easy to store and 3) easy to transport. However, many challenges arise when extracting high quality DNA from FFPE tissues. DNA in FFPE tissues degrades, denatures and cross-links with cellular proteins. This is due to long incubation of tissue in formalin prior to fixation, inappropriate storage conditions (high temperature), using high concentration of formalin, and using low pH formalin^{50,51}. These factors affect primarily the size of extracted DNA fragments, which in turn affects downstream applications such as PCR. It had been reported in a number of studies that extracted DNA from FFPE specimens amplify only shorter DNA fragments (less than 200 bp)⁵²⁻⁵⁴.

In this study we were able to amplify 300 bp beta-globin fragments and 450 bp HPV L1 sequences using extracted DNA from FFPE of cervical cancer specimens from Indiana and Botswana. Our success in extracting good quality DNA was due to development of a protocol based on three steps. First, we removed paraffin in which the archived tissue was stored using octane. This step was very essential in removing PCR inhibitors from the stored tissues as Santa et al., described⁵⁵. Second, we digested the tissue with proteinase K in STM. To the best of our knowledge, this is the first time that

STM was used as buffer for proteinase K during FFPE tissue digestion. We believe that STM is very stable buffer that maximizes proteinase K activity. Also, this might play a role in removing inhibitors released during tissue digestion such as formalin. Since STM components are not published, we can't prove these speculations biochemically. Yet, we chose to use STM instead of regular digestion buffer (composed of Tris-HCL, EDTA and NaCl) mainly based on our experience. In numerous epidemiologic studies were conducted in our laboratory in which we substituted regular digestion buffer with STM, we observed that beta-globin positivity increased from approximately 92% to 99%⁵⁶. The increase in beta-globin positivity was most due to the use of STM. Third, we extracted DNA using QIAamp MinElute Media Kit. To the best of our knowledge this is the first time that this kit was used in DNA extraction from FFPE tissues. This kit had been demonstrated in isolating DNA from cervical and vaginal swabs in a number of studies conducted in our laboratory to optimize the recovery of good quality DNA and eliminate PCR inhibitors⁵⁷⁻⁵⁹. QIAamp MinElute Media Kit extracts DNA based on the silica-based membrane DNA binding mechanism. The silica-based membrane captures the DNA and allows for washes for removing most of contaminant molecules. As a result, high quality DNA was extracted. The average 260:280 ratios (quality) of extracted DNA using this kit show good values (>1.8).

HPV typing of cervical cancer specimens from Indiana and Botswana

The second objective of this study was to amplify and type HPV DNA from cervical cancer specimens from Indiana and Botswana. Overall, the prevalence of HPV DNA positivity from Indiana and Botswana was respectively 92.0% and 79.5%, which is similar to that found in other studies⁶⁰⁻⁶³. However, a significant difference in HPV

positivity was found between cervical cancer specimens from Indiana and Botswana (P=0.042). There are several possible reasons for this difference. First, integration of the HPV genome into the host genome most often leads to the disruption of the E2 gene but also can occur in HPV L1 gene²⁶. Thus, L1 cannot be amplified in such cases.

Supporting this hypothesis, in a comparison of MY09/11(target L1 gene) consensus PCR and type specific PCR (target E6/E7 genes), Depuydt et al. shown an increase of 10% in HPV DNA detection when type specific E6/E7 PCR was used to test HPV DNA negative specimens from MY09/11 consensus PCR⁶⁴. Since the LA-HPV used in this study tests only for L1 gene, however, the HPV DNA negative result in cervical cancer specimens from Botswana could be contributed to the loss of HPV L1 gene during integration in host genome.

Second, it is possible that the integrity of extracted DNA from samples from Indiana exceeds that of specimens from Botswana, which may have been subjected to prolonged fixation in formalin. Lastly, it is possible that Botswana cervical cancer specimens contain a lower HPV viral load than Indiana cervical cancer specimens, so that the sensitivity of the PCR/typing assay may not detect HPV in as many Botswana specimens.

Our success in amplifying and typing HPV DNA from cervical cancer specimens from Indiana and Botswana validates a method for HPV DNA detection from archival cervical cancer specimens. This adds another method that detects HPV DNA directly from cervical cancer specimens instead of relying on data obtained from cervical swabs. As discussed above, cervical swabs may contain many HPV types that may not represent the types causing the cervical cancer.

Comparison of HPV type distribution in cervical cancer specimens from Indiana and Botswana

To investigate the impact of geographical variation in the distribution of HPV types in Indiana and Botswana, we compared cervical cancer specimens from Indiana and Botswana.

Our results showed that HPV 16 was most commonly detected HPV type in cervical cancer specimens from Indiana. HPV 16 prevalence in cancers from Indiana was 87%, a higher figure compared to the international average for HPV 16 detection in invasive cervical cancer, which is approximately 53%⁶⁵. This international average of HPV 16 prevalence was established based on a meta-analysis study by Clifford et al. in 2007, in which cervical cancer data from 130 studies conducted in Africa, Asia, Europe, North America and South America was analyzed⁶⁶. Clifford et al. showed that HPV 16 prevalence in cervical cancers from North America was approximately 55%. This meta-analysis included studies that utilized exfoliated cells instead of cervical cancer tissues, and used a variety of PCR methods. In contrast, our study utilized FFPE cervical cancer specimens with a validated HPV typing method, and likely provides a good estimate of the prevalence of HPV 16 in cervical cancers from Indiana.

HPV 16 was also the most frequently detected HPV type in cervical cancers from Botswana. The overall frequency of HPV 16 detection in cancers from Botswana was 42.7%. The prevalence of HPV 16 reported by Odida et al. (47.8%) was similar to what we demonstrated in cancers from in Botswana⁶⁷. Similar to our study, the analysis by Odida et al. used FFPE cervical cancer specimens with a validated HPV typing method.

HPV type 18 was the second most frequently detected HPV type in Indiana. Our results showed that HPV 18 prevalence was 8.7%. This result is in accordance with the international average for HPV 18 detection in invasive cervical cancers in North America, which is approximately 12%^{66,68}. The prevalence of HPV 18 detected in cancers from women living in Indiana was lower than that found in cervical cancers from Botswana. We found that HPV 18 was the most common HPV type detected in cervical cancer from Botswana, which is 23.5%. This result was similar to that reported by Odida et al. in the Uganda study mentioned above (22.8%)⁶⁷. However, the overall prevalence of the two major HPV types combined (HPV types 16 and 18) detected in cancers from Indiana, (93.5%), was significantly higher than combined frequency of HPV 16 and 18 in cervical cancers from Botswana (61.8%).

After HPV 16 and 18, HPV 45 was the third most common HPV type in cervical cancers from Indiana and Botswana, detected in 6.5% of cervical cancers from Indiana and 8.8% in cervical cancers from Botswana. Our results are consistent with previous studies, which showed that HPV 45 was detected 6% in cervical cancers from North America and 10% in cervical cancers from Africa^{69,70}. In addition to HPV types 16, 18 and 45, we detected only two additional HPV types, which are HPV 31 and 33, in cervical cancer from Indiana with a low prevalence of 2.2%. However, other investigators found additional HR-HPV types other than HPV types 16, 18, 45, 31 and 33. For example de Sanjose et al. identified other HR-HPV types such as HPV types 34, 39, 51, 52, 53, 56 and 58 in North America⁷⁰. These additional HR-HPV types contributed small percentage to the overall HPV found in North America. We did not detect these additional HR-HPV types in cervical cancer from Indiana in our study. This

may be due the fact that our results were based on relatively small number of cervical cancer cases from Indiana or we used in this study different DNA extraction and HPV detection methods.

Since HPV types 16 and 18 were less often found in cervical cancers from Botswana, what HPV types were more abundant in these specimens? We examined the distribution of non-HPV 16/18 HR types in cervical cancers. Overall, non-HPV 16 A9 family members (HPV types 31, 33, 35, 52, and 58) were significantly more frequently detected in cancers from women living in Botswana. In contrast, non-HPV 18 A7 family members (HPV types 39, 45, and 68) were found with the same frequency in cancers from women living in Indiana or Botswana.

Our findings have shown different HPV type distribution patterns in Indiana compared to Botswana. This has implications with regard to HPV vaccine recommendations. The available HPV vaccines, which protect against HR-HPV types 16 and 18, may provide higher protection from cervical cancer to women from Indiana than to women in Botswana.

HIV infection and HPV type distribution in Botswana

What factors could contribute to these differences in HPV type distribution between Indiana and Botswana? HIV infection is one possible factor, as HIV infection increases the relative risk of cervical cancer⁷¹⁻⁷⁴. Because HIV infection is prevalent in women living in Botswana (see the section on rationale), HPV detection was compared in HIV-positive and HIV-negative cervical cancer specimens from Botswana. Due to the low HIV positivity in Indiana specimens, we focused our analysis on Botswana specimens only. Our results show that the distribution between HPV types and groups of

HPV types was similar in women who were HIV-negative and HIV-positive, except for HPV 31, which was found more frequently in HIV-positive women than HIV-negative women from Botswana. Therefore, our study did not support the hypothesis that HIV infection is a major factor that influences distribution of HPV types in Botswana.

However, it cannot eliminate completely HIV as a factor driving some differences. This could be attributed to some limitations of this study including small size samples and unavailability of HIV serological data from Botswana. Due to the low number of HIV positive cervical cancers from Indiana, our ability to compare HPV type distribution among HIV positive specimens from both sites was limited.

Also, due the lack of serological data from Botswana, we developed a unique method to amplify HIV pro-viral DNA from paraffin-embedded tissues to determine the HIV status of these women (see method section). However, the sensitivity of this method has not been determined. Therefore, a larger cervical cancer study with high HIV prevalence in which serologic data for HIV-positivity is known, and could reveal the true impact of HIV infection on type differences in cervical cancers. Other factors may be considered in future studies include geographical variation, nutritional factors and co-infections with certain pathogens commonly found in Botswana.

Other limitations to the current study should also be considered. The archived paraffin-embedded cervical cancers we used in this study may reduce the sensitivity for HPV detection compared to fresh cervical cancer tissues, as has been shown in a study by Odida et al.⁶². Moreover, our results are specific to cervical cancer in Indiana and Botswana, and cannot necessarily be generalized to other populations.

Conclusions

- High quality DNA was extracted from FFPE cervical cancer specimens from Indiana and Botswana.
- The prevalence of HPV types 16 and 18 was significantly higher in cervical cancers from women living in Indiana compared to cervical cancers from women living in Botswana. Oncogenic types other than HPV 16 or HPV 18 were more frequently detected in cervical cancers from women living in Botswana.
- Multiple HPV types were not frequently identified in individual cases of cervical cancers from either country.
- HIV status appeared to have a modest effect on HPV type distribution in cervical cancers from women living in Botswana, but the test utilized to determine HIV status in these women has not yet been validated.
- The current HPV vaccines, which protect from HPV16 and HPV 18, may provide protection from cervical cancer to a higher degree in women living in the U.S. compared to women living in Botswana. Therefore, even if widely used in a vaccination program, a substantial percentage of women from Botswana may still remain at risk of cervical cancer from the other HR-HPV types. Second generation HPV vaccines that provide protection against oncogenic HPV types in addition to HPV 16 and HPV 18 may be better suited for Botswana. Further studies are needed to determine if the findings described here are generalizable to other sub-Saharan countries.

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Curriculum Vitae

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Detection of HPV 16 in cervical carcinomas by a novel FISH assay. Janine T. Bryan¹, Kathrin U. Jansen¹, Frank Taddeo¹, Carolee Welebob¹, Dee Marie Skulsky¹, Bingfang Huan², Barbara M. Frain³, Brahim Qadadri³, and Darron R. Brown³. Merck Research Laboratories¹, West Point, PA, NAXCOR², Palo Alto, CA, and Indiana University School of Medicine³, Poster presentation in 21st International Papillomavirus Conference, February 2004, Mexico City, Mexico.

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