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Comparative Study of HPV 16 and HPV 18 Antibody Detection in Serum, Cervical Mucus, and Oral Mucosal Transudate

Emily Lauren Blalock

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Under the Direction of Dr. Susanna F. Greer

ABSTRACT

Measuring HPV exposure relies on detection of HPV type-specific antibodies, but methods are not standardized. Additionally, there is little information on the best sample type for HPV antibody detection. This study validated pseudovirion neutralization (PVN) assay for HPV antibody detection and compared it to IgG ELISA. Both assays were applied to paired serum and cervical mucus samples. Additionally, PVN assay was utilized to evaluate the feasibility of oral mucosal transudate (OMT) samples to monitor the HPV immune response. Serum was more likely to be positive on PVN assay than on IgG ELISA ($p= 0.025$). Both assays correlated with HPV-16 DNA status. HPV-18 PVN assay results correlated with HPV-18 DNA status. Few cervical mucus samples had detectable antibodies; no correlation with HPV DNA status was seen. OMT results were unsatisfactory. PVN assay was more sensitive than IgG ELISA; serum was a more reliable indicator of HPV-16/18 antibody status than cervical mucus.

INDEX WORDS: Human Papillomavirus, Antibody detection, IgG ELISA, Pseudovirion neutralization assay

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Under the Direction of Dr. Susanna Greer

and

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LIST OF COMMON ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CIN	Cervical Intraepithelial Neoplasia
ELISA	Enzyme Linked Immunosorbent Assay
HPV	Human Papillomavirus
HSIL	High Grade Squamous Intraepithelial Lesions
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LSIL	Low Grade Squamous Intraepithelial Lesions
OD	Optical Density
OMT	Oral Mucosal Transudate
OR	Odds Ratio
PVN Assay	Pseudovirion Neutralization Assay
QC	Quality Control
SEAP	Secreted Alkaline Phosphatase
VLP	Virus-Like Particle

Introduction

Human papillomaviruses (HPV) are a significant public health problem. HPV is the most common sexually transmitted infection in the United States. Each year there are an estimated 6.2 million new HPV infections in the United States alone. It is estimated that 75% of sexually active women will be exposed to HPV in their lifetime. While the vast majority of infections are asymptotic and transient, persistent HPV infection is a significant risk for anogenital neoplasia. In particular, HPV is detected in over 99% of all cervical cancers (Walboomers, Jacobs et al. 1999). Worldwide, cervical cancer is associated with 300,000 deaths each year. (Frazer, Cox et al. 2006; Lowy and Schiller 2006; Trottier and Franco 2006; Markowitz, Dunne et al. 2007)

Papillomaviruses are a family of closely related double-stranded DNA viruses in the family *Papillomaviridae*. Papillomaviruses have been found in nearly all animals, and maintain tight species specificity. The viral particles are 55 nm in diameter, composed of a non-enveloped protein shell with a circular 8 kb genome (as reviewed in Zheng and Baker 2006). The genome is similarly arranged in all papillomaviruses into three regions: early, late, and non-coding regions. The early region encompasses over 50% of the genome and contains six or seven open reading frames (ORF) transcribed in a polycistron message from one DNA strand. HPVs have six early ORFs coding for proteins E1, E2, E4, E5, E6, and E7 (as reviewed in Zheng and Baker 2006). E1 and E2 are involved in regulating viral DNA replication and transcription. The E4 protein is produced during an active infection and is associated with cytokeratin filament collapse. Proteins E5, E6, and E7 are responsible for maintaining DNA synthesis in differentiated cells by inactivating the tumor cell repressors p53 and pRb. This is required to allow the

virus to efficiently replicate and complete it life cycle. However the action of these viral proteins also contributes to DNA instability and blocked apoptosis via the same pathways, so these proteins, particularly E6 and E7 also are the oncogenic proteins. The late region consists of 40% of the papillomavirus genome, coding for the L1 and L2 ORFs, which are the major and minor capsid proteins of the virus respectively (as reviewed in Zheng and Baker 2006). The non-coding region encompasses the remaining 10% of the papillomavirus genome. This non-coding region contains numerous transcription factor binding sites that have been associated with the RNA polymerase II. (as reviewed in Zheng and Baker 2006)

There are over 100 types of HPV; each designated a number based on the order of their discovery. At least 40 HPV types can infect the genital area. HPVs that affect the genital area can be divided further into subcategories of low and high risk. Low risk types are those rarely associated with malignant lesions. These types are associated with low grade squamous intraepithelial lesions (LSIL) of the cervix, genital warts and recurrent respiratory papillomatosis (wart-like growths in the upper respiratory tract). HPV 6 and 11 are responsible for the majority of the latter two lesions. High risk types are those associated with malignancies, but these types are also most prevalent in the general population and are associated with LSIL as well as high grade squamous intraepithelial lesions (HSIL) of the cervix. High risk HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, 69, 73, and 82. Worldwide, types HPV 16 and 18 are detected in 70% of all cervical cancers. High risk HPV has also been linked to other cancers such as vulvar, vaginal, penile, anal, and oropharyngeal cancer. (Frazer, Cox et al. 2006; Parkin and Bray 2006; Trottier and Franco 2006)

HPV is epithelioptropic, that is, infection is confined to epithelial cells. Based on papillomavirus model systems, infection is believed to be initiated in basal cells of the epithelium. Basal cells may be accessible to infection as a result of minor epithelial trauma, or at squamocolumnar junctions, such as at the transformation zone of the cervix. The basal cells are undifferentiated self-renewing cells that have stem cell-like properties. In the basal layer of the epithelium the virus is maintained as an episome at relatively low copy numbers (10-50 copies per cell) (Stanley 2006).

The virus is dependent on host cellular machinery for replication and transcription. A differentiating environment is required for completion of the viral life cycle, and high copy number viral replication is noted in differentiating keratinocytes. This feature contributes to the inability to culture HPV *in vitro*. As noted above, HPV E6 and E7 prolong cellular replication in differentiated cells that would normally not be dividing. This prolonged cell division results in a thickened epithelium, sometimes producing a wart-like growth. The L1 and L2 genes are expressed in cells near the epithelial surface and complete viral particles self-assemble in the nucleus. Virus is shed in association with cells as they exfoliate from the epithelium as part of terminal differentiation. (Stanley 2006)

The non-lytic nature of the infection and restriction of infection to the epithelial compartment results in minimal exposure of the virus to the host immune system. Nonetheless, both humoral and cellular immune responses can be detected. Cellular immunity is most important for viral clearance, in particular macrophage and T cell response directed against the HPV E2 and E6 proteins (Stanley 2006). While the innate

immunity initiates the first response to HPV infection, seroconversion, the production of antibodies, can take from 6 months to 1 year to occur (Carter, Koutsky et al. 2000). Additionally, it has been noted that the number of individuals that will never seroconvert, even though they are persistently HPV DNA positive, is less than 50 percent (Carter, Koutsky et al. 2000; Pagliusi, Dillner et al. 2006). Thus, it is estimated that 70-80% of those exposed have detectable antibodies to HPV (Carter, Koutsky et al. 2000; Pagliusi, Dillner et al. 2006). Once seroconversion has occurred, antibodies levels remain detectable for years (Scheurer, Tortolero-Luna et al. 2005). Therefore seroprevalence is a relatively specific measure of viral exposure, but has limited sensitivity.

Based upon inoculation studies, HPV seroconversion occurs via: quick onset of IgM and IgA response, rapid decline in IgM with a slow descent of IgA antibodies, and finally the steady appearance of IgG antibodies (Scheurer, Tortolero-Luna et al. 2005). A similar sequence of the IgA and IgG response is seen in cervical mucus; where presence of IgA is associated to current HPV infection and the presence of IgG was noted to be associated with lesion formation (Sasagawa, Rose et al. 2003; Bierl, Karem et al. 2005). Antibody production is important for protecting against the spread of infection and re-infection. (Lowy and Schiller 2006; Stanley 2006; Trottier and Franco 2006; Markowitz, Dunne et al. 2007)

Despite the close similarity of all the HPVs, the antibody response is type-specific. A variety of experimental and in vitro systems have demonstrated that three dimensional conformational epitopes of papillomavirus virions are required to generate antibodies that block binding and infection. While antibodies can be detected against

linear peptide epitopes, only antibodies against conformational epitopes have neutralizing properties. (Carter, Wipf et al. 2003; Orozco, Carter et al. 2005)

The L1 capsid protein, produced by recombinant protein expression systems, self assembles into structures that resemble intact virions when examined by electron microscopy. Because of this resemblance, the empty protein shells are called virus-like particles (VLPs). HPV type specific VLPs are used the target antigen in direct enzyme-linked immunosorbent assays (ELISA). In this format VLPs bound to ELISA plate are used as the target for binding of antibodies in the sample. Antibodies to a specific immunoglobulin subclass are used to bind antibodies that recognize the VLPs. The anti-immunoglobulin antibody is either conjugated to an enzyme or hapten which is used to subsequently localize an enzyme. The enzyme localization is revealed with a colorimetric or chemiluminescent substrate. The indirect ELISA is a variation of this assay that uses an HPV type-specific antibody to bind the VLPs to the microtiter plate well. The VLP ELISAs generally detect only one immunoglobulin subclass (e.g. IgG or IgA), dependent on the specificity of the detecting antibody.

The VLP ELISA has been the gold standard for HPV antibody detection. Yet, the VLP ELISA has several short comings. VLPs can be produced in a variety of expression systems including insect cells, yeast and vaccinia. Each method of production requires time consuming methods for purification of intact VLPs from the crude expression lysates. Usually ultracentrifugation, electron microscopy and protein gel electrophoresis are required to evaluate the VLP preps. While VLPs from each expression system would theoretically be equivalent reagents, they do differ in the type of non-specific antigen

carryover. VLP preparations vary between laboratories and between lots within a laboratory.

There are no commercial VLP reagents available, therefore each laboratory must undertake the production and quality control assays required for supplying the intact high-qualityVLPs that are central to the specificity of the ELISA. In addition, methods for determining the cut-off for value a positive result are not standardized and positive and negative control samples are not available. As a result the sensitivity and specificity of VLP ELISAs are difficult to validate. In addition, unless a form dilution series is performed to determine the titer, the VLP ELISA does not offer a quantitative measure of antibodies.

The competitive VLP ELISA is variation of the standard VLP ELISA assay (Wang, Christensen et al. 1997) in which the sample to be tested competes with an HPV type specific antibody for binding to the VLP. A variety of detection methods are possible. The competing antibody may be directly labeled with an enzyme, fluorescent tag or hapten, or, if produced in a different species such as mouse, detected with species-specific labeled antibodies. Samples that prevent the competing antibody from binding are considered true positives. The type specificity of the assay is improved by the competing antibody. However, particularly when monoclonal antibodies are used, some true positive samples may fail to compete because they recognize epitopes not represented in the competing antibody.

Recently a pseudovirion neutralization (PVN) assay was developed to evaluate serum antibodies (Buck, Pastrana et al. 2005). Pseudovirions differ from VLPs in that they contain DNA. In addition, like true virions, both L1 and L2 proteins are used to

make the pseudovirion. While the minor capsid protein L2 does not significantly change the surface epitopes, L2 is required to increase the efficiency of capturing DNA. The DNA in a pseudovirion is released when the particle binds to a cellular surface. This mimics the infectious process and following the DNA from the pseudovirion allows an *in vitro* assessment of the first steps of the HPV infection. The recently described assay uses secreted alkaline phosphatase (SEAP) as the reporter plasmid gene. Release of alkaline phosphatase into the tissue culture media indicates "infection". Antibodies that bind to pseudovirions prevent the particles from binding to cells. These neutralizing antibodies are type-specific and, in animal model systems, correlate with protection from infection. Any immunoglobulin that binds to the pseudovirion is detected based on a reduction in the SEAP in the media. Thus all neutralizing antibodies, regardless of immunoglobulin type are detected (e.g. IgG, IgA, etc.)

There are numerous advantages to the PVN assay. The pseudovirions are produced in cell lines transfected with expression plasmids for L1 and L2 proteins of HPV along with the SEAP plasmid. Once the transfected cell lines are prepared, the production of pseudovirus is more efficient than VLP production. Only type specific neutralizing antibodies will be detected and the detection of all immunoglobulins has the potential to improve sensitivity. Additionally, ease of pseudovirion production facilitates assaying serum dilutions providing an antibody titer as a quantitative measure. Initial studies indicate the PVN assay is more sensitive and specific than the ELISA (Pastrana, Buck et al. 2004).

Theoretically, the PVN assay could be used with any sample that contains antibodies. However to date it has only been applied to serum samples. HPV ELISA

assays in cervical mucous are compromised by the lack of references for what would constitute a positive reaction as well as by low titers. The PVN assay, by providing increased sensitivity and clear criteria for a positive result, could improve the analysis of the mucosal antibody response at the cervix.

Because the mucosal immune response is thought to be generalized, detection of antibodies at one mucosal surface (the cervix) should be linked to antibody detection at another mucosal surface (oral detection). Oral antibodies can be collected using noninvasive sampling methods, and if correlated with serum and cervical response, would have advantages for monitoring populations for exposure to HPV. Few studies have examined oral antibodies, and to date only VLP ELISAs have been used (Marais, Rose et al. 2000; Cameron, Snowhite et al. 2003). While HPV antibodies were detected in oral fluids, this sample type was much less likely to be positive than the corresponding serum sample (Marais, Rose et al. 2000).

The newly licensed vaccine against HPV has focused attention on potential clinical and public health applications of measuring antibodies to HPV. In vaccine trials serum antibodies are used to monitor the response to vaccination. Antibody titers seen after vaccination are much higher than those seen in natural HPV infection (Markowitz, Dunne et al. 2007). Currently there is no antibody correlate of protection and HPV immunoassays are not recommended for individuals. However antibody assays may play a role in post-vaccine surveillance in populations.

The aims of this study were to establish and validate the PVN assay for HPV 16 and 18 in serum, and to apply the assay to paired serum and cervical mucous collected in a in a subset of high risk adolescent females enrolled in a longitudinal study of HPV and

other sexually transmitted infections. Results of the PVN assay will be compared to the direct VLP ELISA and known epidemiologic risk factors for HPV antibodies (such as HPV DNA detection and number of sex partners). The assay will also be applied to oral fluid samples collected in the same study. Results of this study will be used to determine the optimal sample type and testing method in future studies of HPV seroprevalence and post-vaccine surveillance studies.

Methods and Materials

Study Population and Sample Collection

The study population is a subset of those enrolled in a larger multi-objective longitudinal study of sexually transmitted infections conducted at a public pediatric clinic in Atlanta, Georgia (Koumans, Black et al. 2003). Sexually active females age 13-19 years were eligible to participate if a pelvic exam was indicated; participants were excluded if pregnant or HIV infected, or treated with antibiotics in the past month. Informed consent of the adolescent and consent of a parent/guardian or the consent of adolescents aged 18 or 19 years was obtained prior to enrollment. At each visit a questionnaire was administered in private by trained interviewers (Tarkowski, Koumans et al. 2004). The questionnaire included questions on demographics, sexual and reproductive history, condom use, drug, and alcohol use. Visits were on average six months apart. The study was reviewed and approved by the institutional review boards at the Centers for Disease Control and Prevention and Emory University.

At each visit, biologic samples were collected including peripheral blood, exfoliated cervical cells, cervical mucus, and oral samples. Oral samples were only collected as a pilot on a subset of participants. The peripheral blood was collected via

venous puncture and allowed to clot at room temperature. Serum was aliquotted and stored at -80 °C until use.

At the time of pelvic examination, two weck-cel sponges (Medtronic, Minneapolis, MN) were placed into the cervical os until the sponges were saturated with cervical mucous (at least one minute). The saturated sponges were placed into a labeled microfuge tube and placed on dry ice until transported to the laboratory for storage at -80 °C until extraction.

After collection of the cervical mucosal sample, endo- and exo-cervical samples were collected using the Cytyc plastic broom collection kit (Cytyc. Corporation, Boxborough, MA) and placed in 20 ml PreservCyt cytology fixative (Cytyc. Corporation) following manufacturer's protocol for routine diagnostic ThinPrep Pap smear (Cytyc Corporation). ThinPrep Pap smears were prepared and evaluated in the hospital cytopathology laboratory. The cytologic diagnosis, based on Bethesda classification, was recorded from the resulting clinical report. HPV detection and typing was performed on an aliquot of residual Thin Prep using L1 consensus PCR and the Roche Prototype line blot assay as previously described (Tarkowski et al. 2004). The HPV DNA results were used to select subjects and samples for this study.

Oral samples were collected using the OraSure collection device (OraSure Technologies, Bethlehem, PA). The collection pad was rubbed across the gum line twice and then placed between along the lower gum line for 2 min. The collection pad was then placed into a storage vial with storage buffer and centrifuged to collect the sample. Oral samples were stored at -20 °C until use, when they were thawed and heat inactivated at 56 °C in a water bath for 30 minutes.

Selection of Samples for Analysis

Detection of HPV 16 or 18 DNA in the cervical sample was used to indicate exposure. For comparison of humoral and mucosal antibody detection, all women with a sample positive for HPV 16 or 18 were selected (exposure group) as were an equal number of women never testing positive for these types (comparison group). The comparison group was matched to the exposure group based on age (± 6 months), race and number of sex partners. Serum and mucosal samples collected from an individual within 180 days of each other were considered to be matched samples. For the exposure group the samples had to be collected at or after the date of the positive PCR test.

Of the 312 number of women enrolled in the original study, 64 were positive for 16 or 18. Of these, 36 had serum available (24 HPV 16 positive and 12 HPV 18 positive). All but one woman also had a matched mucosal sample. Based on matching criteria, 35 women with a serum sample were available as the comparison group, 32 of whom had a matched mucosal sample. The dates of sample collection fell between 1/14/1999 and 12/12/05.

For detection of oral antibodies, all 299 available samples collected from 209 women were included. These samples included 23 from women positive for HPV 16 and 9 from women positive for HPV 18. Only 16 women contributed samples to both the oral and humoral/mucosal study.

Cervical Mucus Extraction

Tubes containing the cervical mucus sample were removed from the freezer, placed on ice and incubated 30 minutes in 300 µl of lysis buffer (10 ml M-Per Mammalian Protein Extraction Reagent (Pierce, Pittsburgh, PA) to which 0.9 g NaCl (Fisher-Scientific, Pittsburgh, PA), and 1 tablet of Complete Mini, EDTA-free Protease Inhibitor Cocktail was added(Roche, Indianapolis, IN)). The swab sticks were cut from the pad, and each pad was placed into a Spin-X centrifuge tube (Costar, Corning, NY) and centrifuged for 15 minutes at 16,000 X G. An additional 300 µl of lysis buffer was added to the top of each Spin-X tube and the tubes were centrifuged as before. The combined eluates from both spins were then aliquotted, 200 µl per tube, and stored at -80° C until use.

HPV 16 Virus Like Particle IgG Enzyme Linked Immunosorbent Assay (HPV 16 VLP IgG ELISA)

The HPV 16 VLP IgG ELISA was performed on both serum and mucosal samples as previously described (Karem, Poon et al. 2002). Briefly, a 96-well microtiter plate (Fisher-Scientific, Pittsburgh, PA) was coated overnight at 4 ° C with HPV 16 VLPs (0.1 µg/well; CDC in-house preparation). The plate was washed and blocked at room temperature. Serum and mucosal samples were diluted 1:20 and a 50 µl aliquot was added per well. Duplicate wells on the same plate were used for each sample. After a one hour incubation at 37° C, the plate was washed and detected 2 h at 37° C with a 1:1000 dilution of Goat Anti-Human IgG-Alkaline Phosphatase conjugate (Roche). Following washing, signal was developed for 45 min. at room temperature with freshly prepared substrate (Sigma 104 Alkaline Phosphatase Substrate tablets, St. Louis, MO)

and read at 405 nm. Each plate included eight control samples (four known positive and four known negative for HPV 16 based on blocking ELISA, described below). A single lot of VLP was used for all assays in this study. The average value of the optical density (OD) for duplicate wells of each sample was calculated. Serum samples with OD > 0.275, a laboratory determined cut-off value based on results with inter-laboratory control sera were considered positive. Mucosal results were handled as OD values because samples available for establishing a cut-off were not available.

HPV 16 Blocking Assay

A blocking Assay was utilized to select HPV 16 positive and negative sera to be used as pooled and individual controls. The IgG VLP ELISA was performed as described above with modifications as noted. The microtiter plate was coated with 0.5 µg HPV 16 VLP per well and blocking buffer was changed to 2% skim milk (Fisher-Scientific) in 1 X phosphate buffered saline Tween 20 (PBST; Cellgro, Herndon, VA) and block performed 1.5 h at 37 ° C. Sample diluent was changed (2% skim milk, 2% Goat Serum, 1% Insect Cell Lysate, 1 X PBST) and 50 µl volume per well was added. After 1.5 h incubation at 37 °C, 50 µl of mouse anti-HPV 16 monoclonal antibody (gift of Neil Christiansen, Hershey Medical Center) at two dilutions (1:100 and 1:200) were added to replicate wells and incubated an additional 1.5 h at 37 °C. Conjugate was changed to goat anti-mouse IgG alkaline phosphatase (Chemicon, Temecula, CA) diluted at 1:1000 in 2% skim milk in 1 X PBST and incubation time was reduced to 1.5 hours. The ability of the sample to block the mouse monoclonal from binding to the VLP was calculated as [1- (OD value of Mouse Anti-16 with serum/OD value of Mouse Anti-16

alone)] x 100% for both the1:100 and 1:200 dilutions. A value of ≥ 23% for either dilution was considered positive.

HPV 16 VLP IgA Chemiluminescent Enzyme Linked Immunosorbent Assay (ELISA)

The IgA chemiluminescent ELISA was performed as described in the IgG ELISA assay except the block consisted of 5% skim milk, 5% Goat Serum, 2% Bovine serum albumin, ((BSA) Sigma) in PBST. OraSure samples were used undiluted. A goat anti-human IgA alkaline phosphatase conjugate (Calbiochem, San Diego, CA) was diluted at 1:1250 in Conjugate Diluent (2% skim milk, 1% BSA, PBST). BM Chemiluminescence ELISA Substrate (AP) (Roche) was prepared according to manufacturer's protocol and 50 µl was added to the plate. The plate was incubated for 10 min. and was read on the Victor 2 Luminometer on the chemiluminescence setting.

Pseudovirion Neutralization (PVN) Assay

The assay was performed according to Pastrana, Buck et al. (2004). 293TT human embryonic kidney cells (Invitrogen) were plated on 96-well tissue culture plates (Costar) at 30,000 cells/ well in neutralization buffer (Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), 1% glutamax (Invitrogen), 1% nonessential amino acids (Invitrogen), 1% Hepes (pH 7.5) (Invitrogen), and 1% penicillin-streptomycin-fungizone (Invitrogen)). Tissue culture plates were incubated for 3 h at 37 ° C.

Serum and mucosal samples were diluted 2-fold from 1:4 to1:512 in neutralization buffer. 22 µl of sample was added to 88 µl of HPV or BPV1 pseudovirions (PV; prepared as described below) on a 96-well microtiter plate (HPV 16 PV diluted at

1:200, HPV 18 PV diluted at 1:30, and BPV1 PV diluted at 1:250 in neutralization buffer). The final sample dilutions ranged from 1:20 to 1:2560 for both serum and mucosal samples. Oral mucosal samples were diluted at 1:2, 1:4, and 1:8 in neutralization buffer and 22 µl of the sample was added to 88 µl of HPV 16 or BPV pseudovirion on a 96-well microtiter plate (PV diluted as describe above in neutralization buffer). The final dilution of oral samples was 1:5, 1:10, and 1:20.

HPV 16 pseudovirion plate positive controls consisted of pooled HPV 16 positive sera (in house samples verified on HPV 16 blocking assay) diluted at 1:180, and heparin (Sigma), diluted at 1 mg/ml. The plate positive control for HPV 18 consisted of a mouse anti-18 monoclonal antibody, 5074 (Dr. John Schiller, NIH), and a mouse anti-16 antibody (Dr. John Schiller, NIH) diluted at 1:20 was utilized as a negative control. The plate positive control for BPV1 consisted of mouse anti-BPV 1 monoclonal 5B6 (Dr. John Schiller, NIH) diluted at 1:20,000. Plate negative controls consisted of pooled HPV 16 negative sera (in house samples) diluted at 1:540, neutralization buffer, and Anti-papillomavirus mouse monoclonal (types 6, 11, and 18; Novocastra Laboratories Ltd, Tyne, United Kingdom) diluted at 1:100 for HPV 16, HPV 18 and BPV1 pseudovirion stocks. 2 2µl of each control was added to 88 µl of HPV or BPV1 pseudovirion and was added to the 96-well microtiter plate. Samples and controls were incubated 1 h. with pseudovirions on ice, and 100 µl of each mixture was then added to the cells and cultured for 72 hours at 37 °C in a 5% CO_2 tissue culture incubator.

A 50 µl aliquot of the cell supernatant was transferred into a 96-well round-bottom microtiter plate (Costar) and incubated with 150 µl of Tris buffered saline (25mM Tris HCl, 150mM NaCl, pH 7.4; TBS) 30 min. at 65 °C. After incubation, plates were

centrifuged for 5 min. at 3000 rpm and placed on ice for 5 min. A 50 µl aliquot of the supernatant was transferred to a white chemiluminescent microtiter plate (Dynex, Chantilly, VA) and assayed for SEAP activity by adding 100 µl of BM Chemiluminescence ELISA Substrate (AP) (Roche, prepared as kit indicated), covering with an aluma-seal II plate cover (Excel Scientific, Wrightwood, CA) and incubating with shaking 10 min. at room temperature. Plates were read on the Victor 2 Luminometer set at glow-endpoint 0.20 sec/well.

Preparation of L1, L2, and SEAP Plasmids for Pseudovirion Production

Recombinant plasmids (p16L1h, p16L2h, peL1fb, peL2bhb, pSheLL, pYSEAP; all received as a gift from John Schiller, NIH) were used to transform DH5-α competent E.coli cells (Invitrogen) as recommended by vendor. Transformed clones were selected based on antibiotic resistance gene carried in the plasmid and used to prepare a 3 ml overnight culture. Bacteria were lysed using detergent and boiling, and plasmid DNA extracted using the Qiagen HiSpeed Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 1 ml of ddH_2O and concentration was determined by absorbance at A_{260}.

To verify plasmid composition, PCR was performed.

Table 1. Sense and anti-sense primers utilized for PCR according to the plasmid being amplified.

Insert/ Plasmid	Primer sequence (5'-3')	Primer length	T_m (°C)	Amplicon size (bp)
16 L1	F-16L1/L2-920- AGGCCTGTACGGAAGTGTTAC	21	66.7	1675
p16L1h	R-16L1-2576 – ACAGATGGCTGGCAACTAGA	20	67.0	
	F-16L1-1182- GGTGAGCGGCCTGCAGTACAG	21	76.0	1189
	R-16L1-2353 – GTCCAGGTCGGCGCTGAA	18	74.5	
16 L2	F-16L1/L2-920 - same as above	21	66.7	1580
p16L2h	Reverse primer same as above (R-16L1-2576)	20	67.0	
	F-16L2-1001-AGAGGAGCGCCAAGAGGACCAAG	23	77.5	1404
	R-16L2-2385 – GCCAGGCTCACGTCGCTGAAG	21	78.0	
18 L1	F-18L1-785- CTAAAAGCTGCGGAATTGT	19	64.2	1611
peL1fb	R-18L1/L2 – TTCACTGCATTCTAGTTGTGG	21	64.9	
18 L2	F-18L2-717 - CCTTGGAGCCTACCTAGACT	20	63.7	1579
pL2bhb	R-18L1/L2 – same as above	21	64.9	
BPVL1	F-BPVL1-600- ACCGGACTCAGATCTCGAGAA	21	69.8	1553
pSheLL	R-BPVL1-2134- CGCGGTACCGTCGACTCTA	19	70.8	
BPVL2	F-BPVL2-2567 - CCTCAAGCGTATTCAACA	18	58.0	1923
pSheLL	R-BPVL2-4469 – TTTAATAGTGGACTCTTGTTC	21	57.3	
	F-BPVL2-3181 - GTCCATCGGCACAGACTCCTC	21	68.7	
SEAP	F-SEAP-1683- GATCTGCGATCTAAGTAAGC	20	60.4	1729
pYSEAP	R-SEAP-3391- TGTTAACTTGTTTATTGCAGC	21	61.8	

Amplicon size was verified by gel electrophoresis and restriction enzyme digestion. 5 µl of each amplicon, digested and undigested, was loaded into a 0.8% E-gel (Invitrogen) along with a DNA size standard. Gel was visualized under a UV light and compared to the DNA size standard.

Pseudovirion production. Pseudovirions were produced by transfecting 293FT (Invitrogen) cells with plasmids coding for capsids (HPV 16 L1 and L2 for HPV 16, HPV 18 L1 and HPV 18 L2 for HPV 18, and BPV L1 for BPV1 respectively) as described by Buck, Pastrana et al. (2004). Briefly, 293 FT cells were pre-plated in complete media without Geneticin (Invitrogen) or antibiotic-antimycotic in a 162 cm² flask (Corning) at 18 million cells per flask and incubated overnight at 37 °C with a humidified atmosphere of 5% CO_2. Cells were transfected with a total of 75 µg plasmid mix per T-162 cm² flask of 5% CO_2 for 4-6 h at 37 °C in CO_2 incubator. [The plasmid

mix was composed of equal parts of each required plasmid (25 μg each if three plasmids; 37.5 μg each if two plasmids) in Lipofectamine 2000 (Invitrogen).] The media was then replaced with 30 ml of fresh complete media without antibiotics and cells were incubated overnight. Cells were then split into a T-225 cm² flask (Corning) and cultured overnight in fresh media without antibiotics. The media from the flask was then removed. The cells were gently removed with trypsin and collected (Invitrogen). The media/trypsin mix was centrifuged at 800 rpm for 7 min. at 4 °C. All but 5 ml of the supernatant was discarded and the cells were resuspended in DPBS and centrifuged again as described above. DPBS was discarded and the cell pellet resuspended in lysis buffer (DPBS supplemented with 9.5mM $MgCl_2$, 0.025% Brij-58 (Sigma), 1% Benzonase (Sigma), and 0.1% Plasmid Safe ATP Dependent DNase (Epicentre, Madison, WI)) at 100 million cells per ml. The lysate was incubated at 37 °C for 24 h. Beckman polyallomer tubes (Beckman Coulter, Fullerton, CA) were prepared with an Optiprep (Sigma) gradient and the gradient was allowed to diffuse for 1.5 h. The cell lysate was chilled on ice for 10 min. A 0.17 volume of 5 M NaCl was added to chilled lysate and the mixture was iced for 10 min. The lysate was centrifuged at 4600 rpm for 15 min. at 4 °C. The supernatant was transferred to the top of the Optiprep tubes using siliconized tips (Bio Plastics INC) and centrifuged for 3.5 h. at 50,000 rpm. Fractions were dripped into siliconized microcentrifuge tubes (Bio Plastics INC). 10 μl of each fraction was run on SDS-Page (BioRad, Hercules, CA) to determine which fraction contained pseudovirion. Fractions containing pseudovirion were frozen at -80 °C until a titration assay was run on them to determine fraction concentration of pseudovirion.

Protein Assay

Total protein content of mucosal samples was determined using Coomassie Plus kit (Pierce) as per manufacturer's protocol. Bovine Serum Albumin Standard (BSA) (Pierce) was diluted in PBS to obtain the final concentrations of standard: 25, 20, 15, 10, 5, and 2.5 µg/ml. A blank was also run, containing only PBS. Mucosal samples were thawed on ice and then diluted at 1:50 in PBS. 150 µl of each standard and each sample was placed on microtiter plate; all samples were run in duplicate. 150 µl of Coomassie Plus Protein Assay Reagent (Pierce) was added to each well and the plate was mixed by hand for 30 sec. The microtiter plate was then incubated at room temperature for 10 min. and the absorbance was measured at 570 nm using the Victor 2 Luminometer. A standard curve was plotted based on BSA standards in order to determine protein concentrations of each mucosal sample.

Data Analysis

All data was analyzed utilizing Microsoft Office (Microsoft Corporation, Seattle, WA), SPSS 14.0 for Windows (SPSS INC, Chicago, IL), and SAS 9.1.3 for Window (SAS Institute INC, Cary, NC) software. Standard curves, averages, standard deviations, absorbance, and chemiluminescence were determined using Microsoft Excel. McNemar's Test determined the disparity between the discordant and the concordant PVN assay/ELISA results and was calculated using Microsoft Excel. Fisher's Exact Test determined the possible relationships between the PVN assay titers and HPV DNA status, the ELISA OD values and HPV DNA status, and the PVN titers and ELISA ODs; these were calculated via Simple Interactive Statistical Analysis website

(http://home.clara.net/sisa/). Kappa Test (Landis and Koch 1977) showed the strength of correlation between PVN assay's and ELISA assay's HPV antibody detection. Spearman correlation coefficient looked into the PVN assay titer and ELISA OD relationship as well as the serum and mucosal titer relationship. Both Kappa Test and Spearman correlation were determined using SPSS. Epidemiological data was analyzed in the form of odds ratios via SAS to determine if risk behaviors were associated with HPV infection.

Results

Study Population

The mean age of the 71 women in this study was 16.3 years (range, 13 -19 years); 66 (93%) were African American. They had been sexually active a median of 2 years (range 0 – 5 years) and had a median of 4 lifetime sex partners (range, 1-30 partners). Thirty three (47%) of the women had ever douched. Smoking was common in this group; 23 (32%) smoked marijuana in the last 90 days, and 11 (15.5%) smoked tobacco in the last 90 days. A summary of HPV DNA status for this group of women is in provided in Tables 1 and 1A.

Serum Samples

HPV 16 antibodies. The HPV 16 pseudovirion neutralization (PVN) assay detected the presence of HPV 16 antibodies in 41 of the serum samples (57.7%). Positive titers ranged from 40 to 2560. Seropositive women were significantly more likely to be HPV 16 DNA positive than seronegative women (19/41, 46% versus 5/30, 16.7%; $p = 0.006$).

The HPV 16 IgG ELISA detected the presence HPV 16 antibodies in 15 of the serum sample (21.1%). Seropositive women were significantly more likely to be HPV 16 DNA positive than seronegative women (9/15, 60% versus 15/56, 26.8%; $p = 0.015$).

The results of HPV 16 antibody detection in serum by the PVN assay and the HPV 16 IgG ELISA (Table 2) showed a fair correlation ($\kappa = 0.328$). The PVN assay was significantly more likely to be positive than the IgG ELISA ($p = 0.025$). All IgG ELISA positive samples were also positive in the PVN neutralization assay. The PVN neutralization titers also showed a significant correlation with the IgG ELISA optical density (OD) value (Figure 1; $p = 0.01$).

The average PVN titer of concordant positive samples (PVN+/ELISA+) was significantly different from the discordant positive samples (PVN+/ELISA-); (1536 versus 115; $p = 0.001$). While fewer women with concordant positive results were HPV 16 DNA positive than those with discordant positive results (9/15, 60% versus 10/26, 38.5%), the difference was not statistically significant. However, women with a positive serum sample by either or both assays (concordant and discordant positive) were significantly more likely to be HPV 16 DNA positive than seronegative women (PVN-/ELISA-) (5/30, 16.7%; $p = 0.004$ and 0.046 respectively).

HPV 18 antibodies. The HPV 18 PVN assay detected HPV 18 antibodies in 17 (23.9%) samples. Positive titers ranged from 40 to 2560. Seropositive women were significantly more likely to be HPV 18 DNA positive than seronegative women (6/12, 50% versus 11/59, 18.6%; $p = 0.05$).

Cervical Mucus Samples

The mean protein concentration of the mucosal samples was 803 ± 443 µg/ml (range 117-1847). No attempt was made to control for this difference in protein concentration.

HPV 16 Antibodies. The HPV 16 PVN assay detected HPV 16 antibodies in 6/67, (8.9%) mucous samples. Positive titers ranged from 20 to 40. Mucosal positive women were not more likely to be HPV 16 DNA positive than mucosal negative women (3/6, 50% versus 20/61, 32.7%; p = 0.23).

The cut-off value for a positive result in the HPV IgG ELISA has not been established for mucosal samples. However, the PVN titer showed a significant correlation with the IgG ELISA OD (Figure 2; p = 0.05). But, the mean OD for the PVN positive samples was not significantly higher than the PVN negative samples (0.16 versus 0.15).

HPV 18 Antibodies. The HPV 18 PVN assay detected HPV 18 antibody titers in 2/67 (2.9%) samples. Positive titers range from 20 to 160. Mucosal positive women were not more likely to be HPV 18 DNA positive than mucosal negative women (1/2, 50% versus 11/65, 16.9%; p = 0.13).

Epidemiological Associations with Immune Assays

Risk factors significantly associated with HPV 16/18 infection based on the serum samples (Table 3 and Table 4) were lifetime number of 2-3 (p = 0.03) and ≥ 8 (p = 0.03)sex partners, HPV 16 DNA status (p = 0.01), and HPV 18 DNA status (p = 0.03). No risk factors were significantly associated with HPV 16/18 infection based upon the cervical mucus samples (Table 5 and Table 6).

Comparison of Serum and Mucosal Samples

Paired serum and mucosal samples were available from 67 women, allowing direct comparison of humoral and local antibody response.

HPV 16 Antibodies. While there was a correlation between HPV 16 PVN titers in serum and mucosal samples (Figure 3; $p = 0.01$), the positive results of each assay correlated poorly (Table 7, $\kappa = 0.079$). Serum samples were significantly more likely to be positive than mucosal samples ($p = 0.025, \pm 5.02$). Women with HPV 16 PVN detectable antibodies in both serum and mucous samples were not more likely to be HPV 16 DNA positive than those negative in both assays (2/5, 40% versus 4/27, 14%, $p = 0.19$)

HPV 18 Antibodies. There was no correlation seen between the HPV 18 PVN titers in serum and mucosal samples (Figure 4). The positive results of each assay correlated poorly (Table 8, $\kappa = 0.055$). Serum samples were significantly more likely to be positive than mucosal samples ($p = 0.025, \pm 5.02$). Women with HPV 18 PVN detectable antibodies in both serum and mucous samples were not more likely to be HPV 18 DNA positive than those negative in both assays (1/2, 50% versus 6/49, 12%, $p = 0.14$)

Oral Samples

The HPV 16 pseudovirion neutralization assay detected HPV 16 antibodies in 11 (5.3%) samples from 209 women. Of the 16 women with serum and/or mucosal samples, 2 had a positive oral sample. None of these women also had detectable antibodies in their serum or cervical mucous samples. The OD of the HPV 16 IgA ELISA and PVN titers showed an inverse correlation (Figure 5; $p = 0.01$).

Table 2. HPV DNA status for study population.

HPV Status	Exposure Group [N= 35]	Comparison Group [N=36]	Total Study Population [N=71]
Negative (%)	0 (0)	19 (52.8)	19
Single Type (%)	10 (28.6)	11 (30.5)	21
Multiple Types (%)*	25 (71.4)	6 (16.7)	31

*The number of types ranged from 2-7, mean = 3.2

Table 2A. HPV DNA status for study population.

HPV Status	Exposure Group [N= 35]	Comparison Group [N=36]	Total Study Population [N=71]
Neg (%)	0 (0)	19 (52.8)	19
High Risk (%)	35 (100)	12 (33.3)	47
Low Risk Only (%)	0 (0)	5 (13.9)	5

Table 3. Comparison of HPV 16 antibody detection with the IgG ELISA and pseudovirion neutralization assay in serum.

HPV 16	**PVN Assay +**	**PVN Assay -**
ELISA +	**15**	**0**
ELISA -	**26**	**30**

Table 4. Univariate analysis of risk factors for detection of HPV 16/18 serum antibodies in a subset of high risk females (n = 70).

Characteristic	Total (n)	PVN+ 16/18, no. (%)	OR (95% CI)	P
Age				
≤15	7	0 (46.7)	Reference	
16	22	12 (54.5)	1.37 (0.37 - 5.1)	0.63
≥ 17	33	23 (69.7)	2.6 (0.75 - 9.2)	0.13
Age of 1st Sex				
<14	21	13 (61.9)	Reference	
14	19	11 (57.9)	1.08 (0.31 - 3.9)	0.89
15	20	11 (55)	0.85 (0.25 - 2.8)	0.78
≥16	10	7 (70)	1.6 (0.33 - 7.9)	0.55
Lifetime Number of Sex Partners				
1	9	2 (22.2)	Reference	
2-3	17	12 (70.6)	8.4 (1.3 - 55.4)	0.03
4-7	30	18 (60)	5.3 (0.93 - 29.7)	0.06
≥8	14	10 (71.4)	8.8 (1.2 - 61.7)	0.03
Marijuana use in last 90 days				
Yes	23	12 (52.2)	0.62 (0.23 - 1.7)	0.35
No	47	30 (63.8)	Reference	
Smoking in last 90 days				
Yes	11	5 (45.5)	0.50 (0.14 - 1.8)	0.29
No	59	37 (62.7)	Reference	
Douching in last 90 days				
Yes	33	23(69.7)	2.3 (0.85 - 6.2)	0.10
No	36	18 (50)	Reference	

Table 5. Univariate analysis of HPV 16 and HPV 18 DNA status for detection of HPV 16 and 18 serum antibodies in a subset of high risk females (n = 71).

Characteristic	Total (n)	PVN+ 16 no. (%)	OR (95% CI)	P
DNA Status HPV 16 ONLY				
+	24	19 (79.2)	4.3 (1.4 - 13.5)	0.01
-	47	22 (46.8)	Reference	
DNA Status HPV 18 ONLY		**PVN+ 18 no. (%)**		
+	12	6 (50)	4.4 (1.2 - 16.1)	0.03
-	59	11 (18.6)	Reference	

Table 6. Univariate analysis of risk factors for detection of HPV 16/18 mucosal antibodies in a subset of high risk females (n = 66).

Characteristic	Total (n)	PVN+ 16/18, no. (%)	OR (95% CI)	P
Age				
≤15	4	1 (6.7)	Reference	
16	19	2 (10.5)	1.6 (0.14 - 20.1)	0.69
≥ 17	32	4 (12.5)	2.0 (0.20 - 19.6)	0.55
Age of 1st Sex				
<14	21	1 (4.8)	Reference	
14	18	2 (11.1)	0.75 (0.1 -5.9)	0.79
15	19	1 (5.3)	1.8 (0.15 - 21.6)	0.64
≥16	8	2 (25)	0.3 (0.04 - 2.6)	0.28
Lifetime Number of Sex Partners				
1-3	22	2 (9.1)	Reference	
4-7	28	3 (10.7)	1.2 (0.18 - 7.9)	0.84
≥8	16	2 (12.5)	1.4 (0.18 - 11.4)	0.73
Marijuana use in last 90 days				
Yes	19	2 (10.5)	0.98 (0.18 - 5.6)	0.98
No	47	5 (10.6)	Reference	
Smoking in last 90 days				
Yes	9	1 (11.1)	1.1 (0.11 - 10.0)	0.95
No	57	6 (10.5)	Reference	
Douching in last 90 days				
Yes	33	4 (12.1)	1.3 (0.27 - 6.5)	0.72
No	32	3 (9.4)	Reference	

Table 7. Univariate analysis of HPV 16 and HPV 18 DNA status for detection of HPV 16 and 18 mucosal antibodies in a subset of high risk females (n = 67).

Characteristic	Total (n)	PVN+ 16, no. (%)	OR (95% CI)	P
DNA Status HPV 16 ONLY				
+	23	3 (13)	2.1 (0.38 - 11.1)	0.40
-	44	3 (6.8)	Reference	
DNA Status HPV 18 ONLY		**PVN+ 18, no. (%)**		
+	12	1 (8.3)	4.9 (0.29 - 84.6)	0.27
-	55	1 (1.8)	Reference	

Table 8. Comparison of HPV 16 antibody detection in matched serum and mucosal samples run on HPV 16 pseudovirion neutralization assay.

HPV 16	PVN Serum +	PVN Serum -
PVN Mucosal +	5	1
PVN Mucosal -	34	27

Table 9. Comparison of HPV 18 antibody detection in matched serum and mucosal samples run on HPV 18 pseudovirion neutralization assay.

HPV 18	PVN Serum +	PVN Serum -
PVN Mucosal +	1	1
PVN Mucosal -	16	49

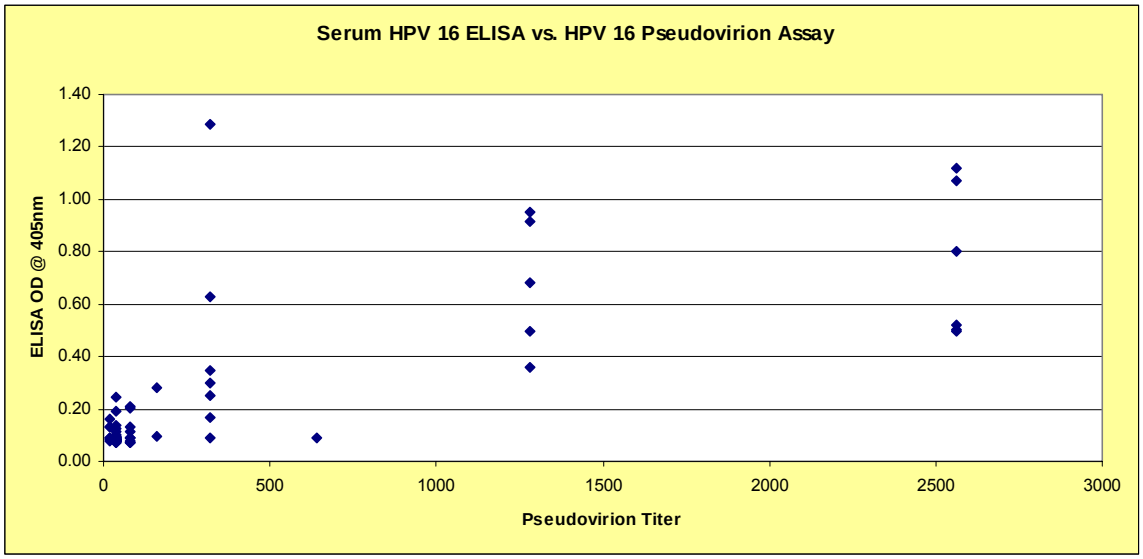

Figure 1. Comparison of ELISA OD value vs. pseudovirion neutralization assay titer of serum samples.

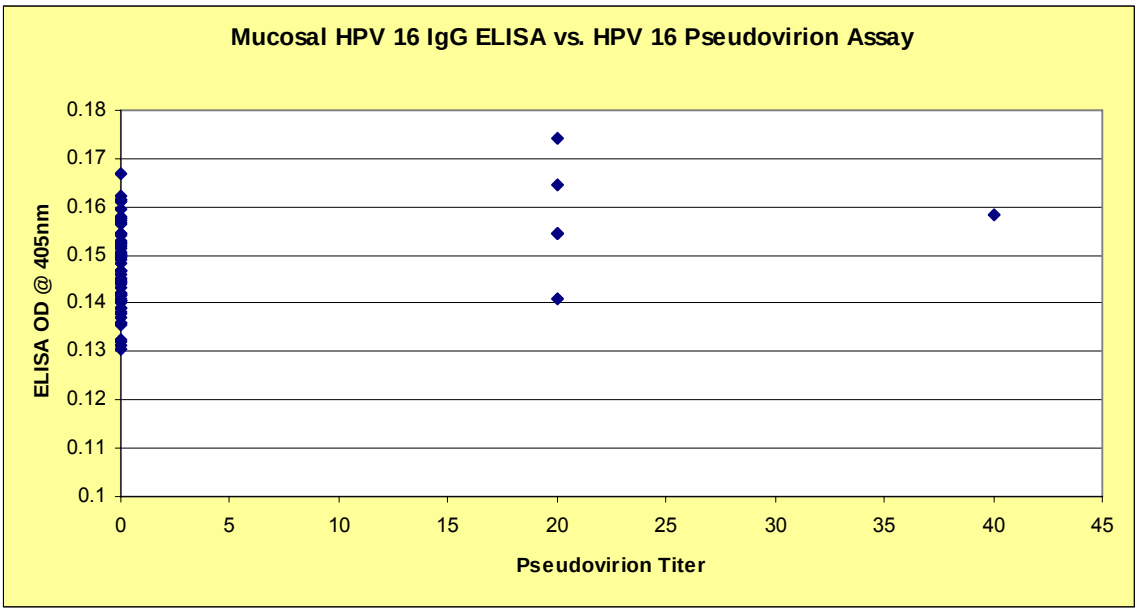

Figure 2. Comparison of IgG ELISA OD vs. pseudovirion neutralization assay titer in mucosal samples.

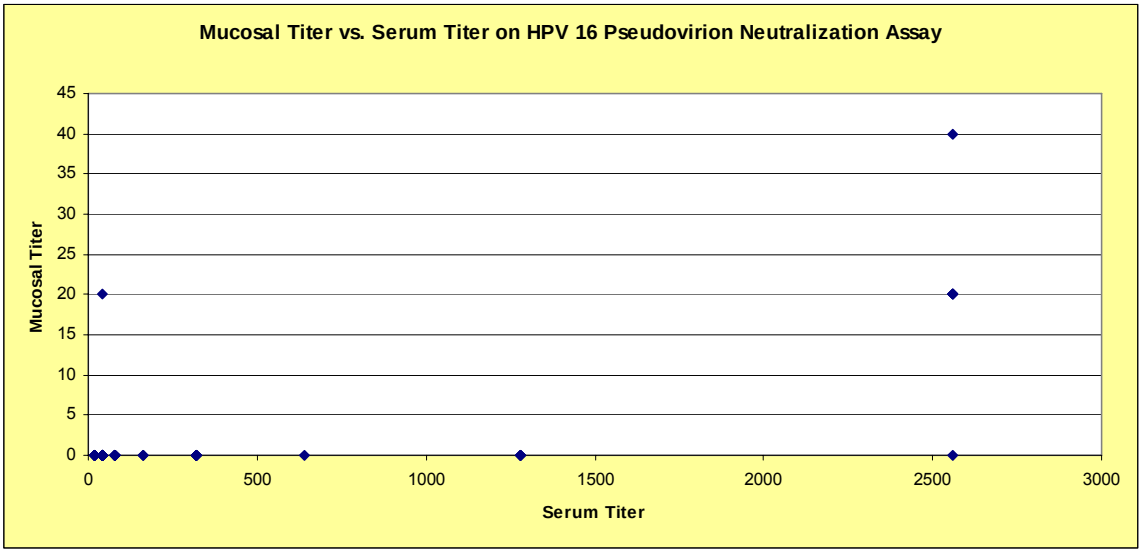

Figure 3. Comparison of mucosal titer and serum titer on HPV 16 pseudovirion neutralization assay.

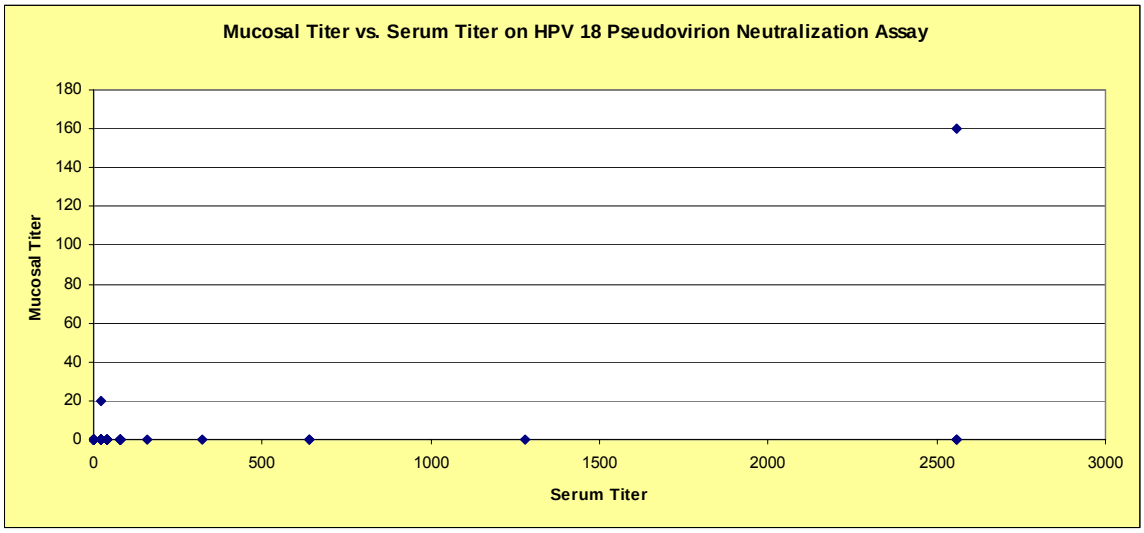

Figure 4. Comparison of mucosal titer and serum titer on HPV 18 pseudovirion neutralization assay.

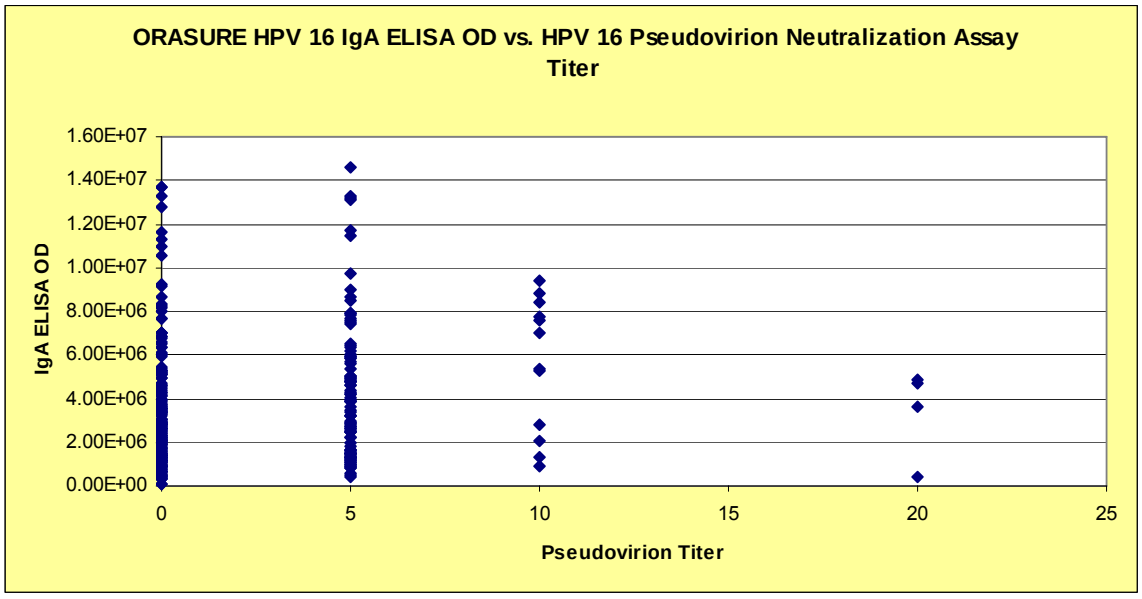

Figure 5. Comparison of HPV 16 IgA ELISA OD and HPV 16 pseudovirion neutralization assay titer in oral samples.

Discussion

This is one of the largest studies to compare the results of serum antibody detection using the PVN assay with the IgG VLP ELISA and the first to apply the same comparison to cervical mucosal samples. This study design thus allows for the direct comparison of two assay platforms as well an investigation of both the local and humoral immune response. In addition, both assays were applied to oral fluid samples to determine if this non-invasively collected sample would be an accurate reflection of serum antibodies, and thus suitable for population monitoring.

While the serum PVN has been published, this assay requires considerable technical time and expertise to establish in a laboratory. The CDC laboratory received the required constructs and training from Dr. Schiller's laboratory and had established the assay for serum samples. The first part of my thesis project was to establish and validate the assay in cervical mucous samples, an application not previously described. The validity of the serum and mucosal PVN assays in my hands was established based on expected performance with QC samples, controls for non-specificity (BPV PVN controls) and results in dilution series.

In this study of 71 serum samples, detection of HPV 16 antibodies the HPV 16 PVN assay was more sensitive and specific than the HPV 16 IgG VLP ELISA; as all ELISA positive samples were PVN positive and the ELISA assay failed to detect antibodies in 26 PVN positive samples. This is in basic agreement with a previous study (Pastrana, Buck et al. 2004), although the absence of ELISA+/PVN- negative samples is somewhat unexpected. Differences in the assay format account for some of the discrepancy. The PVN assay is able to detect total immunoglobulin from both recent

(IgM, IgA) and past (IgG) HPV 16 infections, while the ELISA detects only IgG. Additionally, the ELISA assay may recognize non-neutralizing and cross-reactive antibodies not detected in the PVN assay. As the samples tested are derived from a young population, there may be an unusually high number of recent exposures that explains the particular advantage of the PVN assay. The validity of the PVN assay for HPV 16 and 18 is supported by the epidemiologic association with sexual exposure (lifetime numbers of sex partners) and HPV DNA status.

Establishing the cut-off value is a particular vulnerability of the ELISA format. In the absence of known controls, obtained primarily based on known epidemiologic status (such as samples from virgins with unlikely exposure to HPV, or HPV DNA positive) or verified by an independent assay, a cut-off value cannot be established. This was the case for the HPV 16 ELISA in cervical mucous. To compare HPV 16 antibody detection in cervical mucus via IgG VLP ELISA and the PVN assay, only the correlation of ELISA OD and PVN titer could be made. While there was a significant correlation, few samples were positive in the PVN assay and there was no difference in the ELISA ODs of the positive and negative group. Likewise there were few mucosal samples positive in the HPV 18 PVN assay. As anticipated based on these limitations of the mucosal assays, the HPV 16 and 18 PVN assays in matched serum and mucosal samples were poorly correlated. In addition, unlike results with the serum assays, the mucosal antibody assays did not correlate with HPV DNA status. The relative lack of success with the mucosal samples may be due to using the same dilution as that used for the serum samples. If titers are significantly lower in cervical mucous, the starting dilution may have prevented

accurate detection. Future studies should examine less dilute mucous samples in order to provide more definite results.

As expected, while there was a correlation between a woman's HPV DNA status and serum PVN assay antibody results both in terms of titers and positive reactions, exceptions were noted. It has been noted previously that antibodies to HPV 16/18 infection can persist although the virus itself has been cleared (Pastrana, Buck et al. 2004; Einstein, Studentsov et al. 2007). Thus, even though a woman may have tested HPV 16/18 DNA negative, she may have been previously exposed to HPV and seroconverted. In addition, not all exposed women develop antibodies.

Results of antibody testing in the oral samples were disappointing. The PVN assay for HPV 16 was positive in only 5.3% of the oral samples. This low detection of oral antibodies versus serum antibodies correlates with other studies (Marais, Rose et al. 2000; Cameron, Snowhite et al. 2003). However, the inverse correlation of HPV 16 PVN titers with ELISA OD in these samples suggests the possibility of a non-specific substance reacting with the HPV 16 pseudovirus. This was also the case with the oral HPV 18 PVN assay (data not shown); even after heat inactivation of the oral samples, the positive antibody reaction could not be diluted out. This non-specific substance could be a component of the proprietary OraSure collection fluid, or occur as a result of deterioration due to long term freezer storage in this media. The PVN assay may be successful if alternative collection methods, similar to those used in the studies cited above, were used. In future studies a new set of oral samples should be collected and run on a competitive assay in addition to PVN assay.

This study demonstrates a favorable comparison between the results of the PVN assay and the VLP ELISA in both serum and mucosal samples. This is in part due to ability to definitively establish a positive result based on neutralization. The improved sensitivity of the PVN versus ELISA may not be generalizable to all populations, as the study focused on a unique population of young highly exposed women. In this study serum was a more reliable indicator of HPV 16 and HPV 18 antibody status than cervical mucus or oral samples. Improvements in assay conditions, specifically less dilute samples and alternative collection methods may improve results. Because oral samples would represent a convenient sample for epidemiologic monitoring of HPV vaccine uptake in the population, this sample is worth further exploration. As vaccination produces significantly higher titers than those that occur with natural exposure, the lower sensitivity of oral sampling may be overcome.

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