REPEATABILITY OF SAMPLE COLLECTION METHODS AMONG MEN FROM THE *HPV DETECTION IN ASYMPTOMATIC MEN* STUDY

By

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ABSTRACT

HPV prevalence in men varies across populations and with different sampling methods. However, reliability of sampling methods in men is unknown. The goal of this study is to assess repeatability of HPV sampling among asymptomatic men. Duplicate swab samples were collected at five anogenital sites from 139 men from the *HPV Detection in Asymptomatic Men* study in 2005. HPV testing by PCR and reverse line blot genotyping for 37 HPV types was conducted on each sample independently. Anatomic site-specific HPV prevalence, concordance and Kappa statistics were calculated for any HPV, oncogenic types, and nononcogenic types. Site-specific HPV prevalence was highest at the penile shaft among detectable HPV types. Agreement was consistently observed to be the highest at the penile shaft and coronal sulcus/glans penis. No consistent predictors of discordance were identified. These results show high levels of repeatability in our HPV sampling methods.

INTRODUCTION

Human papillomavirus (HPV) is one of the most commonly occurring sexually transmitted infections among sexually active people in the United States (1). Infection with HPV is associated with cancers of the anogenital tract in both men and women, including cervical cancer in women (2) and penile and anal cancers in men (3). HPV infection is also associated with anogenital warts in both men and women (4). Prevalence of HPV infection ranges from 14% to more than 90% in women (5) and from 0-72.9% in men (6).

In women the Papinicolau (Pap) test is used to detect cellular changes on the cervix with adjunctive HPV DNA testing for the detection of HPV related cervical disease and the epidemiology of HPV infection has been established (2, 7-9). Less is known about HPV infection in men since there is no current Food and Drug Administration (FDA)-approved test to detect HPV in men and a lack of consensus on which site(s) to sample and/or specimen(s) to test for HPV exists (6). HPV infection in men is often subclinical, resulting in a large number of asymptomatic carriers (10) and a man's sexual behavior has been implicated in the HPV disease status of his female partner (11-15). However, HPV transmission between men and women is not fully understood.

Recently a quadrivalent HPV vaccine against HPV types 6, 11, 16, and 18, was approved for the prevention of HPV in women and was shown to reduce HPV incidence of these four types in young women by 90% (16). To effectively prevent the risk of HPV infection in women, in combination with the use of the vaccine, we must also understand infection in men. In order to gain a better understanding of HPV infection in men and the

male role in transmission of HPV to women, the development of sensitive and reliable HPV sampling and detection methods are necessary. A reliable test to detect HPV in men is important since men can be asymptomatic reservoirs of the virus, and unknowingly pass it on to their female sexual partner. The goal of this study is to assess the repeatability of the HPV sample collection methods used in the *HPV Detection in Asymptomatic Men* study.

Background

A. Papillomaviruses

Over one hundred types of papillomaviruses have been described and characterized to date and are classified using a complex taxonony. Of the known papillomaviruses, approximately 100 papillomavirus types infect humans and are ubiquitous throughout the environment and globally distributed (17). Papillomaviruses appear to coexist with their host over long periods of time and may have a long period of latency. A wide variety of different types can be detected on healthy skin of humans and mammals when random sites are sampled (18-20).

Infection with papillomaviruses is associated with microlesions that are not readily visible without an optical aid. However, immune suppression in humans, the most extensively studied host, is observed to result in activation of latent papillomavirus infections, or increased susceptibility to reinoculation of active infection resulting in the presence of overt lesions (17, 21-23).

B. Papillomavirus Structure

The structural molecular biology of papillomaviruses is very complex despite their small size. They contain a genome composed of double-stranded, circular DNA, close to 8 kilobases (kb) in length (24). Human papillomaviruses (HPVs) contain approximately 8 open reading frames (ORFs). The genes L1 and L2 (the late genes) encode the proteins for the viral capsid and E1 and E2 (early genes) mediate viral genome replication. Malignant progression of high-risk HPV infection is mediated by the activity of two early ORFs, E6 and E7, in which encoded oncoproteins influence the activities of cell cycle proteins p53 and pRB (25).

C. Papillomavirus Taxonomy

Papillomaviruses are classified into 16 genera, each containing distinct species and types of papillomaviruses. In determining the taxonomy of papillomaviruses the L1 ORF has been used to classify each papillomavirus into levels of family, genus, species, type, subtype and variant, since this is the most conserved gene within the genome (17). There are approximately 96 HPV types, and 22 animal papillomavirus types that have been described based on the isolation and characterization of their entire genome.

HPV types are mostly encompassed by three genera: Genus-Alpha, Genus-Beta, and Genus-Gamma. While types from Genus-Beta and Genus-Gamma produce cutaneous lesions in humans, HPV types from the Genus-Alpha cause cutaneous lesions and mucosal lesions in humans (17). The Alpha-papillomaviruses include the genital papillomaviruses which are sexually transmitted. Of the more than 50 Alpha-

papillomaviruses, 13 are considered to be high-risk, or carcinogenic, meaning they are capable of causing pre-malignant, or malignant lesions in humans, and are associated with high-grade squamous intraepithelial lesions (SIL) and anogenital cancers, including cervical and anal carcinomas. The high risk types include HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 (26). All other genital types in Genus-Alpha are low-risk types and cause non-cancerous lesions and are associated with anogenital warts and mild dysplasia.

D. Epidemiology of HPV

HPV is the most commonly occurring sexually transmitted infections among sexually active people in the U.S (1) and an estimated 6.2 million Americans acquire new HPV infections annually (6). Infection with HPV has been strongly linked to cancers of the anogenital tract: anal and penile cancers in men, cervical cancer in women, and their precursor lesions (27). In addition HPV causes anogenital warts in both men and women (4). Invasive cervical cancer in women is one of the most commonly occurring cancers caused by HPV and is estimated to account for approximately 12% of all cancers worldwide (28). In the US it has been estimated that in 2006, more than 9000 new cases of invasive cervical cancer will be diagnosed and over 3000 deaths will occur as a result of this disease (29).

E. HPV in Women

Among women in the U.S., the reported prevalence of HPV infection ranges from 14% to more than 90% (5). Prevalence estimates for HPV are reported to be the highest among women attending sexually transmitted disease (STD) clinics and among college students (30-37) , while the lowest prevalence estimates are observed among the general population (38, 39). In women, the Pap test is used for the detection of cellular changes on the cervix with adjunctive HPV DNA testing to screen for cervical cancer and genital HPV infection. Sensitive tests for the detection of HPV DNA are still under evaluation (40, 41).

With the use of current screening methods, invasive cervical cancer is a very preventable disease. The technology exists to treat and manage pre-neoplastic lesions associated with HPV infection. However, due to costs related to screening and treatment, and incomplete participation in screening programs, this disease has not been completely eliminated. Annual costs of repeat Pap screening in the U.S. exceed $150 million and the costs of colposcopy, biopsy, and treatment add an additional $600 million (42).

F. HPV in Men

Less is known about genital HPV infection in men than in women because it has not been extensively studied and there exists a lack of consensus in regard to sampling sites to test and sample collection methods to use (43). Often, HPV infection in men will have no signs or symptoms. However, HPV prevalence estimates in men range from 0-

72.9% and vary widely based on sampling techniques, processing methods, and anatomic sites or specimens sampled (6).

In a systematic review of the literature Dunne *et al*. (6) outline the variation in sampling techniques employed in studies of HPV prevalence in men. The utility of samples and specimens collected in men is evaluated and it is noted that the best anatomic sites for sampling include the glans, corona, prepuce, and shaft of the penis, taking into account the convenience of sampling, adequacy of the sample, and detection of HPV DNA. Furthermore the specimens least useful include urine, semen, and swabs of the urethra. Utility was influenced by the difficult, and sometimes uncomfortable, collection of these samples and limited ability to detect HPV DNA in urine.

At present, a FDA-approved screening test for HPV and the management of cervical cancer exists in women (44). For men there is no FDA approved method available to test for HPV and no therapy has been identified to eradicate infection. Also, screening for HPV infection in men is not recommended since infection is common, no FDA approved test is available, and HPV infection in men is of limited clinical importance (6). However, the male role in the sexual transmission of genital HPV is not completely understood and few studies have evaluated infection with HPV among men and their female sexual partner (37, 45).

G. HPV in Sexual Partners

In a study of HPV infection among asymptomatic couples attending an STD clinic, it was reported that co-occurrence of type specific HPV infection among partners

was higher than expected by chance alone (37). Furthermore, men's sexual behavior and HPV infection has been linked to HPV disease status in their female partner (11-15). In another study where HPV was assessed by DNA detection or penoscopy, 76% of male sex partners of women with HPV, were positive for HPV DNA, however type specific concordance was not assessed (46). Type specific concordance was also found to be higher than expected by chance in a recently published study of HPV in sexual partners, and support the sexual transmission of HPV between men and women (45). With an increased understanding of a man's role in HPV transmission to women, it is important to develop testing methods in men, in order to effectively manage HPV related diseases.

H. HPV Sampling and Detection Methods

In order to gain a better understanding of HPV infection in men and their role in transmission to women, sensitive and reliable sampling and HPV detection methods are necessary. At present, multiple types of sampling techniques for collection of exfoliated cells from the genital surface are used, these involve rubbing or rotating a swab or brush on the genital epithelium (6). Sampling techniques include the use of multiple types of swabs, including cotton tipped and Dacron swabs, cytobrushes, emery paper, or the combination of several of these methods (43).

HPV DNA detection in men has employed a variety of methods across studies. Most studies have used polymerase chain reaction (PCR) to detect HPV DNA (10, 13, 43, 47-57) with a variation in PCR primers by study. Of the studies using PCR to detect HPV DNA the majority use PGMY09/11 (10, 56), GP5+/6+ (47, 49, 51, 53, 57), or

MY09/11 (13, 43, 51, 52, 55) primer sets. Fewer studies of HPV in men have used DNA hybridization methods such as the Digene Hybrid Capture 2 assay and southern blotting techniques (46, 51, 58, 59). However, none of these studies assess the reliability of sampling or HPV detection methods in men.

I. Measures of Reliability

Reliability refers to the extent to which a measurement can be replicated if a test is repeated and is an index of agreement between repeated measurements (60, 61). Koepsell and Weiss (62) describe a good measurement tool as one that yields the same result repeatedly when underlying characteristics of the sample remain constant. Ideally, the only variability in a study should be that between study participants, however, this is not the case in real-life situations. Many factors can influence a measurement tool including variability due to the imprecision of the observer or method and variability within study participants (61). In order to quantify the reliability of a measurement tool various indices have been developed to assess continuous and categorical data (61, 63).

Selection of the appropriate index of reliability is dependent on the exposure measure (continuous or categorical) and whether it is an intermethod (more than one method where the measurement tool may differ) or intramethod (single method) reliability study (63). The primary distinction between the two is that intermethod studies involve two instruments and intramethod studies involve repeated measurements with the same instrument. Indices of reliability in intermethod studies are outlined elsewhere(63) as the current study is an intramethod study.

To assess continuous exposure measures in intramethod reliability studies the intraclass correlation coefficient (ICC) is commonly used (61, 63). The ICC is summarized by Shrout and Fleiss (64) and is based on the analysis of variance (65). The ICC measures homogeneity within groups as a ratio to the total variation among the groups. It reflects differences in mean values and measures the degree of correlation between two sets of measures (60).

For categorical measurements percent agreement, and Cohen's Kappa statistic are commonly used (60-62). Percent agreement, or concordance, is the proportion of all tested measures for which both measurements are the same. It is expressed as a percentage and is simple to calculate and understand (62). Concordance alone has a major limitation: it fails to account for agreement that could have resulted from chance. Due to this limitation, concordance is used to calculate another index of reliability, kappa. Kappa corrects for chance agreement and is widely used as a measure of reliability (62, 66, 67). Kappa represents the degree by which observed concordance exceeds that expected by chance and is expressed as a proportion of improvement beyond chance (62).

J. Significance

The goal of this study is to assess the repeatability of the sample collection methods used in the *HPV Detection in Asymptomatic Men* study. At present, there is limited knowledge on the reliability of HPV detection and sampling methods in men. This study is unique in that the repeatability of methods used to collect exfoliated skin cells from multiple anogenital sites are evaluated among asymptomatic, heterosexual men. Also, all

samples are tested for the presence of HPV DNA by PCR and are genotyped for 37 HPV types irrespective of HPV positivity or negativity by PCR. The reliability of HPV sampling methods in men has not been thoroughly investigated and the information gained from this analysis is an important step in the understanding of sampling in men. This may significantly contribute to the development of a sensitive testing protocol for detection of HPV in men.

MATERIALS AND METHODS

Parent Study: HPV Detection in Asymptomatic Men

A. Study Design

The parent study was a cross-sectional study of 463 men enrolled between 2003 and 2006 in Tucson, AZ, and Tampa, FL.

B. Population

Men were eligible if they were: 1.) between 18 and 40 years old, 2.) reported sexual intercourse with a woman within a year of enrollment, 3.) had no history of genital warts or penile or anal cancers, and 4.) no penile discharge, no pain during urination, or no current sexually transmitted infection at time of enrollment.

C. Recruitment

Men were recruited using advertisements in city and university newspapers (University of Arizona in Tucson and University of South Florida in Tampa), flyers disseminated on university campuses and throughout the communities, and through direct mailings. In-person recruiting was also conducted at the Davis Monthan Air Force Base clinic, the University of Arizona Student Health Center, and the Pima County Theresa Lee Health Center STD clinic.

D. Sample Collection

Sampling included the collection of anogenital swabs, semen, urine, and venous blood. A self administered demographic and sexual behavior questionnaire was also administered to each male participant

Current Study

A. Selection Criteria

Of the 463 men enrolled in the *HPV Detection in Asymptomatic Men* study, the last 139 were included in the current study. Men included in this study met the same eligibility criteria of those in the parent study and were drawn from the same populations of Tucson, AZ, and Tampa, FL, and were recruited using the same methods.

B. Sampling Methods

Exfoliated epithelial cells were independently collected from five different genital sites. The study clinician rubbed two separate saline-wetted Dacron swabs on the entire surface of the 1) glans penis/coronal sulcus, 2) penile shaft (including prepuce, if present), 3) scrotum, and 4) perianal area. Two saline-wetted Dacron swabs were also inserted into the anal canal up to the anal verge. Each swab was placed into separate tubes containing 320 µl of Specimen Transport Medium (STM, Digene Corporation, Gaithersburg, MD). All swabs had equal probability of sampling the entire surface area at each anatomic site. Swab tubes were labeled with the participant's study identification

number, date of collection, and specimen type. They were then stored at 4˚ C or –20˚ C, depending on clinic site, until further processing.

C. Sample Processing

Genital swabs were processed to maximize cellular material obtained from the collection. Following genital sampling, swabs were squeezed and rotated against the side of the collection tube to release as much liquid as possible. Approximately 0.5 cm of a 1000-µl pipette tip was sliced off using a sterile razor blade to make the opening larger. This tip was placed into the swab collection tube (Digene) and the swab was then placed inside the tip. The tube was spun at 3200 RPM for 3 minutes. This centrifugation released additional liquid that was trapped in the swab into the collection tube. The tip and swab were removed, and a 200 µl tip was used to draw any remaining liquid out of end of the 1000 µl pipette tip. Using an 800 µl pipette tip, the liquid remaining in the tube was mixed by drawing it into the tip several times. One aliquot of 200 µl was placed in a 1.5 ml pop-top conical tube for HPV DNA analysis. A second aliquot of the remaining liquid was archived in a 1.5-ml cryovial. This process carried out for each swab sample individually. Both aliquots were stored at –20˚ C until delivery to the analytical laboratory.

D. Laboratory Analysis

HPV testing of swabbed cellular material was conducted using polymerase chain reaction (PCR) for amplification of a fragment of the L1 gene. DNA extraction was

performed using the QIAamp Mini Kit (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Briefly, 200µl aliquots were digested with 20µl proteinase K for 1hr at 65˚ C, followed by 200µl of lysis buffer. DNA was eluted with 50µl of 10 mM Tris-EDTA buffer, pH 7.5 at 60˚ C. DNA was stored at –20˚ C until use.

PCR amplification of HPV DNA

Specimens were tested for the presence of HPV by amplifying 5µl of the DNA extracts with the PGMY09/11 L1 consensus primer system and AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA). Each 50 µl amplification contained 1X PCR Buffer II; 2.5 mM MgCl2; 200µM (each) dCTP, dGTP, and dATP; 600µM dUTP; 7.5 U of AmplTaq Gold; 1µM of PGMY09; 1µM PGMY11; 2.5 nM of B_PC04; 2.5 nM of B_GH20; and 5µl of the template. For eventual inclusion of uracil-N-glycosylase to prevent product carryover, dTTP was replaced with dUTP. To determine specimen adequacy, the GH20/PC04 human β-globin target was co-amplified using the B_PCO4 and B_GH20 primers along with HPV consensus primer amplification. For every PCR plate a negative control (H_2O) and a positive control (CaSki Cells) were run to control for possible contamination and accuracy. The samples were amplified using Perkin-Elmer GeneAmp PCR System 9700. The following amplification profile was used: 95˚ C hotstart for 9 minutes, 95˚ C denaturation for 1 minute, 55˚ C annealing for 1 minute, and 72˚ C extension for 1 minute for 40 cycles; followed by a 5 minute terminal extension at 72˚ C; and a hold step at 4˚ C.

Genotyping

HPV genotyping was conducted using the reverse line blot method on all samples, regardless of HPV PCR result. This detection method utilizes the HPV L1 consensus PCR products labeled with biotin to detect 37 HPV types. The HPV genotype strip contained 39 probe lines, detecting 37 individual HPV genotypes and two concentrations—high and low—of the β-globin control probe (Roche Molecular Diagnostics, Alameda, CA). The following types are detected: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108. The PCR products labeled with biotin were denatured and added to the probe strip in a hybridization buffer. After strips were washed, a streptavidin-horseradish peroxidase conjugate was added to facilitate detection of the various HPV types. After the final wash, buffer was removed by vacuum aspiration, and strips were rinsed in 0.1 M sodium citrate. Color development was activated by incubation in a mixture of hydrogen peroxide in sodium citrate buffer and tetramethylbenzidine in dimethylformamide for 5 minutes on a rotating platform (70 RPM). Developed strips were interpreted and photographed for future reference. Strip interpretation was performed with a labeled overlay, with lines indicating the position of each probe relative to the reference mark.

E. Definition of Outcome Variables

A sample was considered positive for Any HPV if any HPV type was detected either by PCR or genotyping. A sample was considered positive for oncogenic types if it

was positive by genotyping for at least one of the following types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66. A sample was classified as positive for nononcogenic types if it was positive for at least one of the other 24 types not included in the oncogenic group, but not including unclassified types. A sample was considered positive for oncogenic and nononcogenic types if it was positive for at least one of the 13 oncogenic types and at least one of the 24 nononcogenic types. Unclassified types were defined as those positive by PCR but negative by genotyping for the 37 HPV types. Concordant samples were defined as those positive for HPV in both swabs, or negative for HPV in both swabs. Discordant samples were defined as those in which one of the two swabs was positive for HPV and the other was negative for HPV.

F. Statistical Analyses

Samples were included in the analysis if β-globin and/or HPV was detected on both swabs. Anatomic site specific HPV prevalence was determined by calculating proportion of men in whom one or both samples from that anatomic site were positive for HPV. Repeatability of detecting HPV between duplicate swabs positive for β-globin and/or HPV was determined by: 1.) estimating concordance of HPV positivity or negativity, and 2.) calculating the Kappa statistic and its 95% confidence interval. Concordance was estimated by calculating the proportion of concordant samples (positive and negative) for each anatomic site independently. Kappa statistics and corresponding 95% confidence intervals were calculated based on the percent concordance at each anatomic site.

Behavioral and demographic characteristics were also assessed as potential predictors of discordant samples for Any HPV. These included: demographic characteristics, clinic location, clinician reported circumcision status, age at first intercourse, number of new sexual partners in the past three months, frequency of sexual intercourse in the previous three months, frequency of sexual intercourse in the previous month, new partner at last intercourse, ever diagnosis with a STD, and smoking status. Pearson's chi squared test was used to compare differences in discordance between groups and simple logistic regression was used to calculate odds ratios to observe the magnitude of the association. All statistical analyses were conducted using Intercooled Stata, version 9.2 (StataCorp, College Station, TX).

RESULTS

Demographic Characteristics

Table 1 shows demographic characteristics of men from the parent study only (*HPV Detection in Asymptomatic Men* study) and the men included in the current study. Statistically significant differences were observed among categories of race and clinic site between excluded and included men. A marginally significant difference was detected between the excluded and included men for marital status. There were no significant differences observed for age and ethnicity.

Table 1: Demographic Characteristics of Men in Both Studies

	Parent Study[a] N = 324	Current Study[b] N = 139	χ^2 p-value
Age (years)	N (%)	N (%)	
18-19	38 (12)	12 (9)	
20-24	131 (40)	50 (36)	
25-29	57 (18)	33 (24)	
30-34	48 (15)	15 (11)	
35-40	38 (12)	21 (15)	p=0.285
Ethnicity			
Hispanic	56 (17)	23 (17)	
Non-Hispanic	262 (81)	114 (82)	p=0.931
Race			
American Indian/Alaska Native	7 (2)	2 (1)	
Asian	11 (3)	8 (6)	
Black/African American	13 (4)	20 (14)	
White	236 (73)	88 (63)	
Other/unknown	57 (18)	21 (15)	p=0.001
Marital status			
Single	219 (68)	108 (78)	
Married	45 (14)	14 (10)	
Cohabiting	25 (8)	4 (3)	
Divorced/separated/widowed	28 (9)	7 (4)	p=0.051
Clinic site			
Tucson	267 (82)	92 (66)	
Tampa	57 (18)	47 (34)	p<0.001

[a] *HPV Detection in Asymptomatic Men* study only; [b] Current Study only

Proportion of HPV positive samples

Site-specific prevalence was highest at the penile shaft for all but the unclassified types. For unclassified types prevalence was observed to be the highest on the scrotum. The lowest prevalence was observed at the perianal area and anal canal, with all but the oncogenic type prevalence lowest at the anal canal (Table 2).

Table 2: Proportion of HPV Positive Samples by Type

	HPV Prevalence[b] (%)				
Site	**Any[a]**	**Oncogenic[a]**	**Oncogenic and Nononcogenic[a]**	**Nononcogenic[a]**	**Unclassified[a]**
Coronal Sulcus/ Glans Penis	44.8	9.7	11.2	18.7	6.7
Penile Shaft	62.5	14.0	16.8	26.5	11.8
Scrotum	50.0	10.9	8.6	21.7	13.0
Perianal Area	22.1	4.7	4.7	9.4	5.4
Anal Canal	21.8	5.3	3.0	9.0	5.3

[a] Adequate samples: β-globin and/or HPV detected in both swabs
[b] Positive for HPV in at least one of the two swabs

Concordance and Kappa Estimates for Any HPV

Table 3 shows concordance and Kappa statistics for Any HPV type. Site specific concordance for Any HPV type between duplicate swabs was highest at the perianal area, followed by anal canal, penile shaft, coronal sulcus/glans penis, and scrotum. Overall concordance calculated from all samples was 86.2%.

The kappa statistic for Any HPV type was highest for the penile shaft and coronal sulcus/glans penis, followed by the perianal area (all substantial agreement), and scrotum and anal canal (moderate agreement). Substantial agreement was observed for the overall kappa estimate calculated from all samples.

Table 3: Concordance and Kappa Estimates for Any HPV

Site	**N[a]**	**Concordance (%)**	**Kappa (95% CI)**	**Interpretation[b]**
Coronal Sulcus/ Glans Penis	134	85.8	0.70 (0.57-0.82)	Substantial
Penile Shaft	136	87.5	0.75 (0.63-0.86)	Substantial
Scrotum	138	78.3	0.54 (0.40-0.69)	Moderate
Perianal Area	128	91.4	0.68 (0.51-0.86)	Substantial
Anal Canal	133	88.0	0.55 (0.35-0.74)	Moderate
All Samples	669	86.2	0.69 (0.63-0.75)	Substantial

[a] Adequate samples: β-globin and/or HPV detected in both swabs
[b] Guidelines by Landis and Koch (*Biometrics* 1977; 33:159-74)

Concordance and Kappa Estimates by Oncogenic and Nononcogenic Types

For the oncogenic only type infection category, concordance was the highest at the perianal area and anal canal while the kappa statistic was highest at the penile shaft (substantial agreement). Lowest concordance was observed at the scrotum which also had the lowest kappa. However, moderate agreement was still observed at this site and all sites exhibited substantial agreement for oncogenic types (Table 4).

For the oncogenic and nononcogenic category, concordance was highest at the anal canal and the highest kappa statistic was observed for the penile shaft. Agreement ranged from fair to almost perfect, with agreement the lowest (fair) observed in the perianal area and anal canal (Table 4).

Concordance was the highest at the perianal area and anal canal in the among nononcogenic type only category. Kappas were the highest for the coronal sulcus/glans penis and the penile shaft. Substantial agreement was observed across all sites for nononcogenic types.

Table 4: Grouped Oncogenic and Nononcogenic Types

Site	N[a]	Concordance (%)	Kappa (95% CI)	Interpretation[b]
Only Oncogenic Types				
Coronal Sulcus/ Glans Penis	134	96.3	0.74 (0.53-0.96)	Substantial
Penile Shaft	136	95.6	0.79 (0.62-0.95)	Substantial
Scrotum	138	93.5	0.54 (0.29-0.80)	Moderate
Perianal Area	128	98.5	0.74 (0.40-1.0)	Substantial
Anal Canal	133	97.7	0.72 (0.41-1.0)	Substantial
Oncogenic and Nononcogenic Types				
Coronal Sulcus/ Glans Penis	134	97.0	0.83 (0.69-0.99)	Almost Perfect
Penile Shaft	136	97.1	0.88 (0.77-1.0)	Almost Perfect
Scrotum	138	97.1	0.76 (0.54-0.99)	Substantial
Perianal Area	128	96.9	0.32 (-0.17-0.81)	Fair
Anal Canal	133	97.7	0.39 (-0.16-0.94)	Fair
Only Nononcogenic Types				
Coronal Sulcus/ Glans Penis	134	93.3	0.74 (0.58-0.90)	Substantial
Penile Shaft	136	91.2	0.74 (0.61-0.88)	Substantial
Scrotum	138	89.1	0.60 (0.42-0.78)	Substantial
Perianal Area	128	96.1	0.72 (0.48-0.95)	Substantial
Anal Canal	133	95.5	0.64 (0.38-0.91)	Substantial

[a] Adequate samples: β-globin and/or HPV detected in both swabs
[b] Guidelines by Landis and Koch (*Biometrics* 1977; 33:159-74)

Concordance and Kappa Estimates of Unclassified Types

Table 5 shows that agreement was not at the same levels for unclassified types as was observed in any HPV and the oncogenic and nononcogenic types. Agreement ranged from fair to substantial among unclassified types, with the lowest agreement at the anal canal (fair) and the highest agreement at the coronal sulcus/glans penis (substantial). All other sites showed moderate agreement.

Table 5: Analysis of Unclassified Types

Site	**N[a]**	**Concordance (%)**	**Kappa (95% CI)**	**Interpretation[b]**
Unclassified Types				
Coronal Sulcus/ Glans Penis	134	96.3	0.60 (0.27-0.92)	Substantial
Penile Shaft	136	91.9	0.44 (0.16-0.71)	Moderate
Scrotum	138	91.3	0.45 (0.20-0.71)	Moderate
Perianal Area	128	96.9	0.48 (0.05-0.92)	Moderate
Anal Canal	133	95.5	0.23 (-0.16-0.63)	Fair

[a] Adequate samples: β-globin and/or HPV detected in both swabs
[b] Guidelines by Landis and Koch (*Biometrics* 1977; 33:159-74)

Potential Factors Associated with Discordant Samples

Of the behavioral and demographic characteristics assessed to determine their association with discordant samples for Any HPV, there were no consistent predictors of discordant samples. However, clinic location showed variation by anatomic site in the measure of effect observed

Table six shows the variation in the odds ratios for the level of discordance among the clinic locations, by anatomic site. The only anatomic site that showed statistical significance in the odds ratio was the penile shaft, indicating that there was a higher likelihood of a discordant sample at the penile shaft for any HPV at the Tampa clinic location. For the other anatomic sites we see both a lower and higher likelihood of discordance for the remaining anatomic sites, however none are statistically significant.

Table 6: Association of Discordant Samples with Clinic Location by Anatomic Site

	Glans Penis/ Coronal Sulcus	**Penile shaft**	**Scrotum**	**Perianal area**	**Anal Canal**
Clinic					
Tucson					
OR (95% CI)	1.00	1.00	1.00	1.00	1.00
Tampa					
OR (95% CI)	0.62 (0.21-1.85)	3.17 (1.12-8.97)	0.52 (0.20-1.31)	2.22 (0.64-7.73)	1.20 (0.41-3.54)

DISCUSSION

The results from this study indicate a high level of repeatability in the sample collection methods used for the detection of HPV among a subsample of men participating in the *HPV Detection in Asymptomatic Men* study. These results also suggest that the sampling methods are robust in their ability to sample an adequate amount of cells to detect a wide range of HPV types across several male anogenital anatomic sites. Here, we evaluate the repeatability of HPV sample collection methods for five anogenital sites in men.

We observed a high degree of adequate samples collected to detect HPV, based on the presence of β-globin and/or HPV in duplicate swabs. The scrotum yielded the highest percentage of adequate samples collected (99.3%), while the perianal area yielded the lowest (92.1%). The penile shaft, coronal sulcus/glans penis, and the anal canal all had comparable levels of adequacy in the samples collected (97.8%, 96.3%, and 95.7%, respectively). In previous studies where β-globin was used to evaluate sample adequacy the prepuce, shaft, glans, corona, and scrotum were most likely to yield adequate DNA, with β-globin positivity ranging from 70%-98.5% (43, 53, 68-72). The adequacy of samples in this study was observed to be at the higher end of this range.

Prevalence of HPV positive samples in this study across sites, were comparable to those observed in other studies that assess the presence HPV DNA. We observed the highest prevalence at the penile shaft at 62.5% which was higher than that observed in previously published literature that showed the prevalence at this anatomic site ranging from 5.6%-51.5% (43, 68, 70). Prevalence at the scrotum was also higher than that

previously reported. We observed a prevalence of 50.0%, whereas it has been previously reported ranging from 7.1%-46.2% (43, 46, 68, 70, 72).

For all other anatomic sites evaluated the observed prevalence fell within the range previously reported; 6.5%-50% for the corona and/or glans, and 0%-32.8% for the perianal area, anus, or rectum (6). However, they were also observed to be on the upper end of the previously reported ranges. Prevalence was observed at 44.8% for the coronal sulcus/glans penis, and 22.1% and 21.8% for the perianal area and anal canal, respectively. Across the HPV types evaluated (any HPV, oncogenic types, nononcogenic types, oncogenic and nononcogenic combined, and unclassified types) the penile shaft was consistently observed to have the highest prevalence, with the exception of unclassified types where the prevalence was highest at the scrotum.

When evaluating agreement between duplicate swabs by anatomic site, we consistently see the penile shaft to have the highest agreement among detectable types of HPV, where substantial to almost perfect agreement is observed. The coronal sulcus/glans penis also has a high degree of agreement among detectable types of HPV, with agreement in the same range as the penile shaft. These two anatomic sites, along with the prepuce are noted as the best anatomic sites for sampling in men because they consistently yield adequate samples, and sample collection is simple and painless (6). Not only was high agreement observed in these two anatomic sites, but a high degree of sample adequacy was also present in the current study.

Among detectable types of HPV, agreement between duplicate swabs at the scrotum ranged from moderate to substantial, with moderate agreement observed for the

any HPV type and oncogenic types only categories, and substantial agreement was observed for the nononcogenic types only category and oncogenic and nononcogenic types category. In the perianal area and anal canal agreement ranged from fair to substantial across detectable types of HPV. The lowest agreement at these two sites was observed for the oncogenic and nononcogenic types combined (both with fair agreement), however no specific pattern was observed as to which anatomic site yielded the lowest agreement and it varied by HPV types.

When comparing agreement between oncogenic only and nononcogenic only HPV categories, we do not see major differences in the levels of agreement. Across all anatomic sites, moderate to substantial agreement is observed, indicating a high degree of repeatability for oncogenic only and nononcogenic only type infection. This suggests that our ability to accurately detect HPV does not differ by oncogenic versus nononcogenic HPV types. However, when looking at the group of men with both oncogenic and nononcogenic type infection, we see a wider range of agreement (fair to almost perfect). This may be resulting from the fact that in this category we are detecting at least two HPV types compared to the oncogenic only or nononcogenic only categories where a sample could be positive with only one HPV type infection.

In the oncogenic and nononcogenic type infection category a sample was considered positive if it was positive for at least one of the 13 oncogenic types *and* one of the 24 nononcogenic types. In the oncogenic only category a sample could be positive if it was positive with at least one of the 13 oncogenic types and none of the 24 nononcogenic types. Similarly, in the nononcogenic only category a sample could be

positive if it was positive for at least one of the 24 nononcogenic types and none of the 13 oncogenic types. To assess whether the wider range of agreement observed in the oncogenic and nononcogenic category was a result of multiple infections, further analysis can be done assessing the effect of multiple infections on concordance.

As previously noted we consistently observed the highest levels of agreement at the penile shaft and coronal sulcus/glans penis across all detectable types of HPV, and in unclassified types, the highest agreement was observed at the coronal sulcus/glans penis. In addition, we consistently observed the highest prevalence at the penile shaft, coronal sulcus/glans penis, and scrotum. These anatomic sites have been previously indicated as the best anatomic sites for sampling (6). The results presented here of a high degree of agreement at these anatomic sites also support the findings for the optimal site for sampling in the *HPV Detection in Asymptomatic Men* study (data not currently published).

Although some of the demographic characteristics were shown to be significantly different between men from the parent study only and those in the current study no consistent predictors of discordance were identified. In this analysis a large number of predictors were assessed against five outcomes (by anatomic sites), which increased the risk for type I error. However, since there were no consistent predictors identified the effect is negligible. In addition, the effect measures associated with most of the behavioral and demographic characteristics were small and not statistically significant.

The observation that there were variations in the effect measures for the clinic location, by anatomic site, is not surprising. In studies of this nature where more than

one clinic location is involved, variations in the sampling methods used may exist. This may be contributing to the differences we observed in the degree of discordance between anatomic sites and can be noted as a potential limitation to this study. However, considerable attention was given to training of clinicians in order to keep sampling methods consistent between clinic locations. When conducting future research on the reliability of HPV sampling methods in men, this needs to be taken into consideration to limit the variability that may exist between sampling at different clinic locations.

Another potential limitation to this study could be the generalizability of these findings. In this study, men were recruited from a population that was sexually active (by selection criteria) and the majority of the men recruited were younger. In this study 45% of the men were between the ages of 18 and 24 years, and among men only in the parent study 52% were in the same age range. The fact that we have recruited from a sexually active population could be contributing to the higher prevalence of HPV in this sample of men.

Here we demonstrate the ability to reliably detect HPV from samples collected at five anogenital sites in a group of asymptomatic men. Several methods employed in this study allowed for the greatest opportunity to detect HPV in the samples. First, this study uses an extensive sampling method strategy with a wide range of anatomic sites sampled in men. Second, samples collected from each anatomic site were processed and analyzed independently as opposed to pooling samples collected from each anatomic site. Since the PCR reaction that occurs during testing for HPV in each sample can be inhibited by proteins in the sample (73), individual testing of anatomic sites allows the greatest chance

to detect HPV if it is present in one sample but absent in others. Third, HPV genotyping was conducted on all samples regardless of HPV positivity or negativity by PCR. This practice is not common in studies that use similar strategies to detect HPV DNA; however it yielded more sensitive detection of HPV. All are strengths of this study because they allow us to extensively study a wide range of anatomic sites, and use a more sensitive approach for the detection of HPV by genotyping all samples irrespective of PCR results.

This study is important in that it will contribute to our understanding of the HPV detection methods in asymptomatic men. At present, little is known about the reliability of HPV sampling and detection methods in men. Understanding the reliability of HPV sampling methods is an important step in the development of a sensitive HPV screening method that can be used for the prevention of HPV infection in men, and more importantly prevention of transmission to women. The next step necessary is to assess the reliability of the HPV detection methods used in the *HPV Detection in Asymptomatic Men* study. This will be an important piece in the evaluation of this method as a reliable HPV sampling and detection procedure for detection in men.

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