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Low-Level Persistence of Human Papillomavirus 16 DNA in a Cohort of Closely Followed Adolescent Women

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Most human papillomavirus (HPV) infections in young women become undetectable by standard assays after a few months. It is possible that many HPV infections do not actually clear, but persist at very low levels for years, becoming detected again later in life. The purpose of this study is to describe HPV 16 clearance, reappearance, and low-level persistence in a cohort of adolescent women. Adolescent women (N = 66), not vaccinated against HPV, were recruited from 1998 to 2008 into a longitudinal study. Self-collected vaginal samples were obtained quarterly and tested for HPV by Linear Array HPV Genotyping Test (LA-HPV). To explore low-level persistence, a type-specific nested PCR for HPV 16 (TSN-PCR-16) was developed. Women with HPV 16 detected by LA-HPV had their negative swabs retested with TSN-PCR-16. Forty-two participants with HPV 16, followed for a mean of 6.3 years, were analyzed. Using LA-HPV, the median duration of HPV 16 detection was 428 days (SD 852.5 days). TSN-PCR-16 detected HPV 16 during periods of LA-HPV non-detection in samples from many women. Using a combination of LA-HPV and TSN-PCR-16 results, the median duration of HPV 16 detection was 1,022.5 days (SD 943.7 days). The durations of detection differed significantly between the two methods ($P = 0.0042$) with a mean difference of 434.5 days. In adolescent females, duration of HPV 16 detection was significantly longer when TSN-PCR-16 was combined with LA-HPV. Some apparently cleared HPV 16 could be shown to persist at low levels using nested PCR. ***J. Med. Virol. 83:1362–1369, 2011.***

KEY WORDS: longitudinal; HPV; clearance; adolescence; persistence

INTRODUCTION

Human papillomavirus (HPV) is a sexually transmitted infection (STI) that peaks in prevalence among young women soon after initiation of sexual intercourse [Winer et al., 2003; Tarkowski et al., 2004; Manhart et al., 2006; Trottier and Franco, 2006]. In young women, infection of the genital tract with HPV may be asymptomatic or may cause several pathologic states including genital warts and cervical dysplasia [Schiffman, 1997]. While HPV infections in young women are extremely common, most infections are believed to "clear" within a few months, meaning that standard PCR assays do not detect the relevant type in two consecutive cervical or vaginal specimens after the initial detection [Evander et al., 1995; Ho et al., 1998; Moscicki et al., 1998; Sellors et al., 2003; Vinther and Norrild, 2003].

A late complication of oncogenic, or high risk (HR) HPV infection is cervical carcinoma, a malignancy that peaks in the 6th–7th decade of life [Gustafsson et al., 1997; Munoz et al., 2003]. Cervical cancer and

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other HPV-associated malignancies (vulva, vagina, anal) occur in a small percentage of infected women [Parkin and Bray, 2006]. Those women at highest risk appear to have "persistent" HR-HPV infection, meaning detection of the same oncogenic type over a one to several year period immediately prior to the diagnosis of cancer [Asato et al., 1994; Ho et al., 1995; zur Hausen, 1996; Koutsky, 1997; Walboomers et al., 1999; Bosch et al., 2002; Plummer et al., 2007].

While we have a good understanding of early events in the natural history of HPV and late event, such as cervical cancer, the long gap between early and late stages of HPV natural history is less well understood. The current longitudinal study utilized numerous specimens from young women followed for an extended time period to examine the possibility that incident HPV 16 infections (Family *Papillomaviridae*, genus *Alphapapillomavirus*, species *Human papillomavirus 16*), characterized by the Linear Array HPV Genotyping Test (LA-HPV; Roche Molecular Diagnostics, Indianapolis, IN), actually persist at low levels when assayed by type-specific nested PCR (TSN-PCR-16) and may therefore have a longer duration than previously believed. In addition, some young women's HPV 16 appeared to clear in standard PCR assays, and then reappear after several months to years. We therefore analyzed samples obtained during these periods of apparent clearance using TSN-PCR-16 to determine if low-level persistence (LLP) occurred.

MATERIALS AND METHODS

Participants

A longitudinal study of STI and sexual behaviors in a cohort of adolescent women was recently described [Brown et al., 2005; Batteiger et al., 2010]. Participants were recruited from primary care clinics in Indianapolis, IN, under the main study protocol, which was approved by the local institutional review board [Brown et al., 2005; Batteiger et al., 2010]. Participant and parental informed consent were obtained at enrollment. All participants received financial compensation for their time and effort. From the larger cohort of participants enrolled, 120 women with the longest duration of observation comprised the sample of women that defined the HPV study. This analysis uses the first 66 women with completed HPV testing.

Sample Collection and HPV Genotyping

A self-collected vaginal sample for STI testing was obtained every 3 months from each subject. Samples were analyzed for specific HPV types using LA-HPV as previously described [Gravitt et al., 2000; Brown et al., 2005, 2009; Shew et al., 2006; Fife et al., 2009]. A participant was considered HPV 16-positive if at least one of their samples contained HPV 16 DNA when tested with LA-HPV. For some participants during the entire duration of follow-up, HPV 16 was detected in multiple samples. Each of these samples was considered to be part of the same HPV 16 detection period, rather than counting each positive sample as a separate HPV 16 event. In other words, after a period of non-detection of HPV 16, a re-detection of HPV 16 in subsequent samples was considered to be part of the original detection period, rather than a new event. Forty-two of 66 participants were HPV 16-positive by LA-HPV testing and are included in the current analysis.

The Linear Array HPV Genotyping Test (LA-HPV)

DNA was extracted from participant samples using the QIAamp MinElute Media Kit as directed by the manufacturer (Qiagen, Germantown, MD). The LA-HPV assay involves two steps: DNA amplification by PCR followed by DNA detection using a reverse blot strip assay with type specific probes for individual HPV types [Gravitt et al., 1998, 2000]. The PCR for the LA-HPV assay utilizes pooled, non-degenerate 5′ biotin-labeled primers designed to amplify 37 individual genital HPV types indicated in Figure 1 [Gravitt et al., 1998, 2000]. The portion of the L1 open reading frame amplified in LA-HPV spans approximately 450 bp between nucleotides 6500 and 7000, with some variability depending on the specific HPV type.

To determine specimen adequacy, the GH20/PC04 human β-globin target was co-amplified with HPV sequences. Reactions were performed using a Perkin Elmer TC9600 Thermal Cycler (Perkin Elmer, Foster City, CA) as described previously [Brown et al., 2009]. Known HPV-positive and negative (no DNA) specimens were included in each assay as controls. The PCR product was then denatured and hybridized under high stringency conditions to Roche paper strips containing 39 BSA-conjugated probes, representing the 37 HPV genotypes and two concentrations of the β-globin control probe. After hybridization, streptavidin-alkaline phosphatase conjugate was added to permit identification of hybridized HPV sequences to the strip, which was performed by visual comparison to the HPV Type Reference Guide provided in the Roche kit.

Type-Specific Nested PCR

A type-specific nested PCR test for HPV 16 (TSN-PCR-16) was developed. The nested PCR uses two sets of primers to specifically amplify a portion of the HPV 16 L1 gene as shown (Fig. 1) [Seedorf et al., 1985; Kennedy et al., 1991]. DNA was extracted from samples using the QIAamp MinElute Media Kit (Qiagen, Inc.-USA, Valencia, CA), using the protocol provided by the manufacturer.

Two separate rounds of PCR were performed for each sample tested. The process of amplification was a two-step process where the larger (396 bp) region of L1 was amplified for 30 cycles from the sample. One microliter of this product was then used for the subsequent nested PCR reaction. The nested PCR was performed on a separate thermocycler in a separate

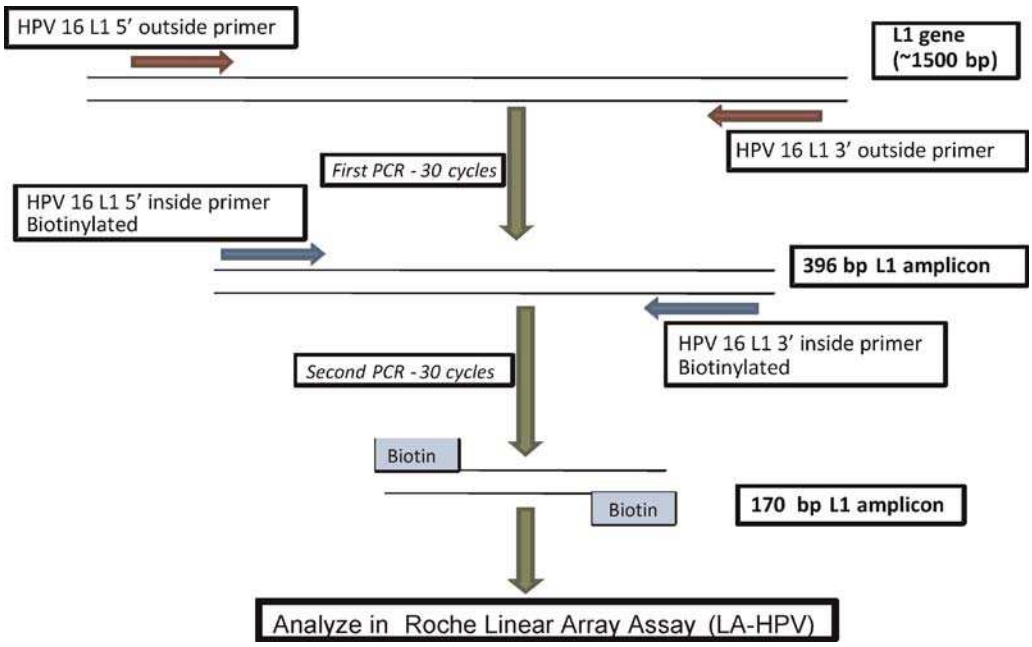

Fig. 1. General schema of nested PCR process. The "outside" primers were designed to amplify a 396 bp region of the L1 gene. The product of this reaction was then used as the template for the subsequent "nested" PCR. The 170 bp product was then analyzed in the Roche Linear Array Assay (LA-HPV).

room. Thus, the two reactions were strictly isolated to reduce contamination. This portion of the amplification was carried out for an additional 30 cycles.

All pipettes, microcentrifuge tubes, and reagents for both processes were kept separately in their respective rooms. Each was used only for one step of the process. The reactions were performed as two separate processes with two sets of primers, and used two different templates. The first PCR ("outside") amplified a 396 bp portion of L1 using extracted DNA as template. The "outside" primer sequences were as follows:

5′AGGCTCTGGGTCTACTGCAA 3′ (nucleotides 6471–6491) and 5′ GGGGGGGGTTGTAGACCAAA 3′ (nucleotides 6866–6847). The reaction conditions for the first PCR were: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, 72° for 1 min, followed by an elongation step of 72°C for 5 min.

The second PCR ("inside") amplified a 170 bp nested portion of L1 using 1 μl of the first 50 μl as template. The "inside" primer sequences were as follows:

5′ TTACAACGAGCACAGGGCCA 3′ (nucleotides 6573-6592) and 5′TCCTCCCCATGTCGTAGGT 3′ (nucleotides 6742–6724). Reaction conditions for the second PCR were: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 60.5°C for 30 sec, and 72°C for 45 sec, followed by elongation at 72°C for 5 min. The "inside" primers were biotin-labeled by the manufacturer, Invitrogen (Carlsbad, CA), to allow for DNA detection using the Roche Linear Array Assay HPV Genotyping Test using the same protocol as LA-HPV [Gravitt et al., 2000; Brown et al., 2005].

Sample Testing With TSN-PCR-16

To identify samples to be tested with TSN-PCR-16, the LA-HPV results for each HPV 16-positive participant were grouped chronologically. For each subject, all individual samples testing positive for HPV 16 with LA-HPV were not retested with TSN-PCR-16. One LA-HPV positive sample from each participant was used as a positive control for that participant's TSN-PCR-16. In total, 36 LA-HPV positive samples from the same participant were retested with TSN-PCR-16, and in six cases in which adequate specimen was not available, an LA-HPV positive sample from a different participant was utilized. All 42 of these specimens tested exclusively positive for HPV 16, in spite of the detection of numerous additional types by LA-HPV (data not shown).

From HPV 16-positive participants (by LA-HPV), all samples that were negative for HPV 16 by LA-HPV testing were re-tested with TSN-PCR-16 (Fig. 2). This included samples collected both before and after the period of HPV 16-positivity in the LA-HPV assay.

Statistical Analysis

Descriptive and summary statistics were reported for test results of LA-HPV and TSN-PCR-16. For each

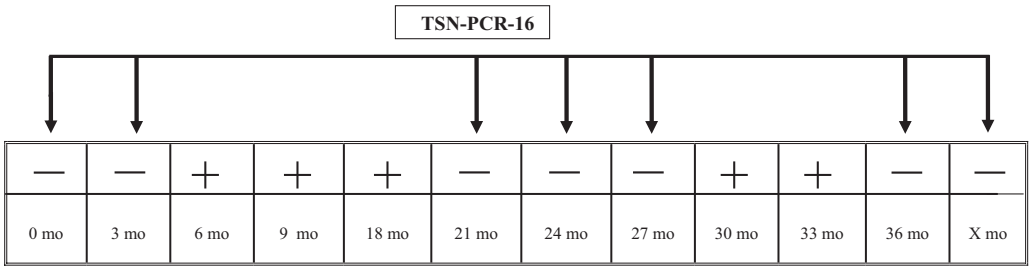

Fig. 2. Serial samples from a hypothetical subject analyzed first by the Roche Linear Array Assay HPV Genotyping Test (LA-HPV). Each sample is represented by a +/− symbol representing a sample that is either positive or negative for HPV 16, respectively, by LA-HPV. The time of acquisition is represented in the lower row, where "mo" is the time in months. The arrows represent samples that would be tested in the current study by type-specific nested PCR for HPV 16 (TSN-PCR-16) for the presence of HPV 16.

individual subject, durations of HPV 16 detection was calculated as the time difference between first and last detection by LA-HPV, as well as by a combination of LA-HPV or TSN-PCR-16 testing. The end of an HPV detection period was defined as the final chronologic positive test followed by one negative test, and no subsequent positive tests. If the last sample in study period was positive for HPV16 by a particular testing method the precise duration of detection cannot be determined. In these situations, the duration of the detection period is considered censored for the purposes of statistical analysis. Durations of the same detection period defined by the two testing methods were presented as Kaplan–Meier curves, which take into account censoring, and compared using a Cox proportional hazard model with random subject effect. The random subject effect was introduced to accommodate the potential correlation between the two different duration measurements within the same study subject.

RESULTS

Participants

Of the 66 women with completed longitudinal HPV testing for the entire study period, 42 had a HPV 16 detected by LA-HPV. Mean duration of follow-up was 6.3 years (range 3.9–9.1; SD 1.4). The mean age of the 42 participants with HPV 16 detection was 15.1 years at enrollment (range 14–17; SD 0.93), and the majority of the participants (88.1%) were African-American. At enrollment, 28 of the 42 HPV 16-positive participants (66.7%) reported prior sexual activity (vaginal intercourse); all reported sexual activity during follow-up. Mean age at first vaginal intercourse was 14.5 years (range 11–18; SD 1.8). None of the participants received HPV vaccination because it was not yet available in the clinics from which participants were recruited.

Sample Testing With TSN-PCR-16

A comparison of relative sensitivity of TSN-PCR-16 and LA-HPV was performed using serial 10-fold dilutions of a clinical sample known to contain HPV 16. In serial dilution experiments using a known HPV 16-positive clinical specimen, TSN-PCR-16 was more sensitive than LA-HPV for HPV 16 by at least 10-fold (Fig. 3).

A total of 968 samples were collected from 42 participants with HPV 16 detected by LA-HPV. As above, samples testing HPV 16-positive by LA-HPV ($n = 206$) were not retested with TSN-PCR-16. Testing by TSN-PCR-16 was therefore attempted for 762 samples. However, 35 samples did not have sufficient sample to complete the TSN-PCR-16, so this assay was performed on 727 samples. Using TSN-PCR-16, HPV 16 was detected in 89 of 727 samples (12.2%).

HPV 16 was detected by TSN-PCR-16 before some participant's first LA-HPV positive sample, and after some participant's last LA-HPV positive sample. HPV 16 was detected by TSN-PCR-16 before 14 of the participant's first LA-HPV positive test. For these 14 participants, the mean time from TSN-PCR-16-positivity to the first LA-HPV positive was 478.6 days (median 224, range 85–1,537, SD 512.2 days). Fifteen participants had positive TSN-PCR-16 tests after their last positive LA-HPV test. For these 15 participants, the mean time from the last LA-HPV positive to last TSN-PCR-16 positive sample was 771.7 days (median 449, range 85–2,616, SD 820.3 days). It is possible that HPV 16 could have been present at a point in time less than 85 days before or after a positive LA-HPV test. However, due to the timing of the sample collection (approximately every 3 months), there were not specimens collected during that time period.

Duration of HPV 16 Detection

Duration of HPV 16 detection was defined as time from first positive HPV 16 test to last positive test using either assay. The median duration of HPV 16 detection calculated using only LA-HPV results was 428 days, or 1.2 years (SD 852.5 days); mean duration was 718.9 days or 1.9 years (SD 852.5). When duration was calculated using a combination of both the TSN-PCR-16 and the LA-HPV results, the median

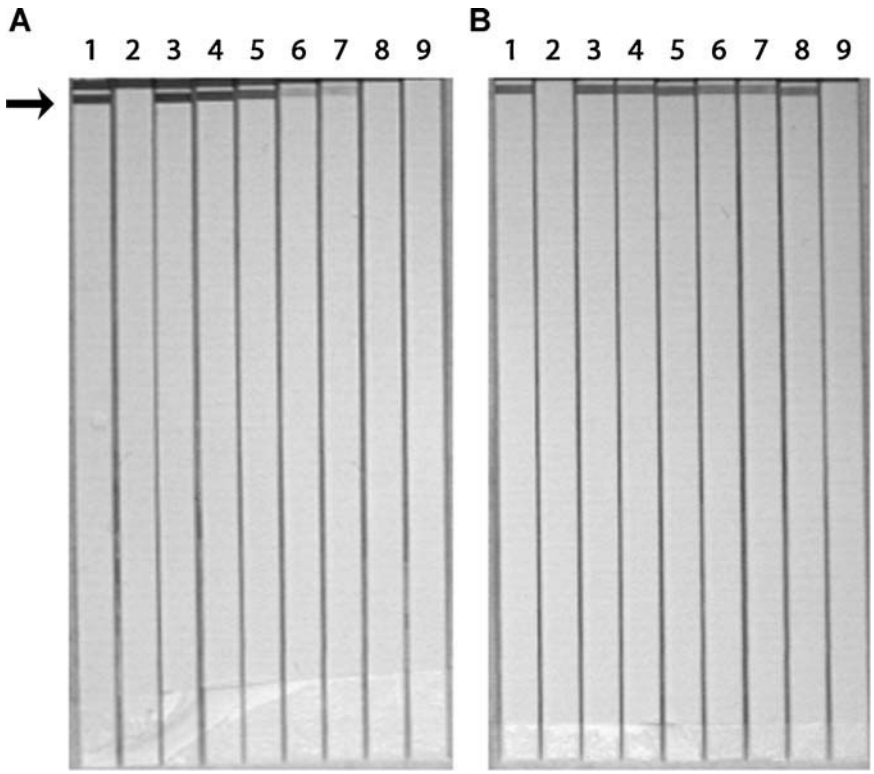

Fig. 3. Comparison of sensitivity of HPV 16 detection using LA-HPV (**Panel A**) or TSN-PCR-16 (**Panel B**). The readout assay in both systems utilizes the Roche Linear Array strips than include type-specific oligonucleotide probes for 37 HPV types, including HPV 16 (positive signal shown by the arrow). **Lane 1**: PCR using pBR322-HPV 16 whole genomic clone as positive control. **Lane 2**: PCR with no added DNA template. **Lane 3**: PCR using 2×10^2 copies of pBR322-HPV 16 whole genomic clone per reaction, **Lane 4**: 2×10^4, copies per reaction, **Lane 5**: 2×10^3 copies per reaction, **Lane 6**: 2×10^2 per reaction, **Lane 7**: 20 copies per reaction, **Lane 8**: 2 copies per reaction, Lane 9: 0.2 copies per reaction.

duration increased to 1,022.5 days, or 2.8 years (SD 943.7 days); mean duration increased to 1,153.3 days or 3.2 years (SD 1,943.7) (Table I). The duration of detection based on the combination of TSN-PCR-16 and LA-HPV results was significantly longer than the duration based on LA-HPV testing alone ($P = 0.0042$). Kaplan–Meier curve illustrating duration of detection is shown in Figure 4.

Intermittent Detection of HPV 16

In 12 participants, "intermittent detection" of HPV 16 occurred in the LA-HPV assay, as defined by positive LA-HPV tests for HPV 16, followed by at least two consecutive negative LA-HPV tests, followed later by additional LA-HPV positive tests for HPV 16. Among these 12 participants, 18 periods of non-detection of HPV 16 lasting 6 months or longer were found. A total of 103 samples LA-HPV negative samples were collected during these periods of non-detection. Testing of these 103 samples using TSN-PCR-16 yielded an additional 19 HPV 16-positive samples, thereby reducing the number of HPV negative samples by 18.4%.

DISCUSSION

This study was performed to gain new insights into the natural history of HPV 16 genital infections in adolescent women. This cohort was closely followed for about 6 years on average, some for up to 9 years. Multiple, sequential samples from each participant were analyzed for HPV 16 using two different methods. In participants with apparent clearance of HPV 16 detected by the LA-HPV assay, TSN-PCR-16 testing resulted in continued detection of HPV 16 in many cases. Utilizing TSN-PCR-16 in addition to LA-HPV testing revealed that HPV 16 was detected

TABLE I. Duration of HPV Infections Using Roche Linear Array Assay HPV Genotyping Test (LA-HPV) and Type Specific Nested PCR 16 (TSN-PCR 16); Where "N" Equals the Number of Participants

Variable	N	Median (days)	Minimum (days)	Maximum (days)	Standard deviation
LA-HPV	42	428	1	3,270	852.5
LA-HPV plus TSN-PCR 16	42	1,022.5	1	3,270	937.9

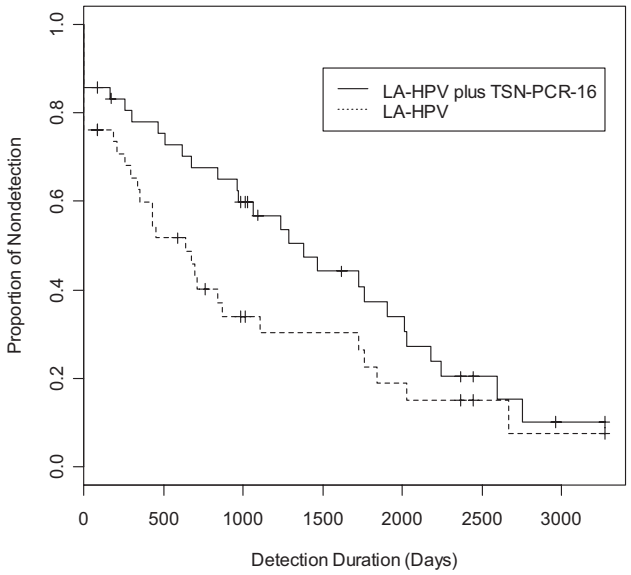

Fig. 4. Kaplan–Meier curve comparing the Roche Linear Array Assay HPV Genotyping Test (LA-HPV) alone versus LA-HPV and Type Specific Nested PCR for HPV 16 (TSN-PCR-16) combined testing results.

for 1.6 years longer on average than when LA-HPV was used alone.

Overall, the prolonged duration of participant follow-up combined with an HPV 16 detection method with enhanced sensitivity resulted in an expanded characterization of the natural history of HPV 16 in adolescent women. Application of the TSN-PCR-16 allowed for this further characterization of HPV 16 in adolescent women. Many samples from the adolescent women would have been deemed HPV 16-negative if LA-HPV alone was utilized for testing, thus affecting the overall infection characteristics, including duration. Using TSN-PCR-16, HPV 16 was detected at time points when the infection was not detected by LA-HPV, including before first positive LA-HPV test, during periods of non-detection between positive LA-HPV tests, and after infection was thought to have "cleared."

HPV 16 has been shown to persist longer than other HR types; median duration of HPV 16 infections reported in prior studies ranged 7.3–11 months [Ho et al., 1998; Woodman et al., 2001; Trottier et al., 2008]. Using LA-HPV tests alone to define duration, periods of HPV 16 detection in the current study had a median duration of 1.2 years, which is somewhat longer than reported in previous longitudinal studies. The inclusion of TSN-PCR-16 tests in the determination of duration of HPV 16 detection increased the median duration to 2.8 years, suggesting that HPV 16 is detectable for longer than widely accepted. As illustrated in Figure 4, the majority of HPV infections still became undetectable during follow-up. In this research, the end of an infection is defined as "at least one negative test that is not followed by positive test results before the end of follow-up." Such a definition leads to a more conservative estimate of duration because one negative test before the end the observation does not necessarily signify a true "clearance," which is usually defined by at least two negative tests after a positive test.

These finding suggest that low-level persistence of HPV 16 does occur in some cases. Low-level persistence is defined here as HPV 16 detection by TSN-PCR-16 in samples that were negative for HPV 16 by LA-HPV. These data suggest a possible explanation of why, in a previous longitudinal cohort study by Strickler et al. [2005] HPV previously not detected appeared in follow-up testing in women who reported no sexual activity. In the referenced study, new HPV types were detected at follow up testing in a cohort of HIV-positive women despite no sexual activity in the interim.

The clinical relevance and implications of low-level persistence of HPV 16 are not known, nor is the cause of low level persistence. In a previous study by Collins et al. [2009] integration of HPV 16 resulted in a markedly lower viral copy number per cell. It is possible that this phenomena occurred in our study. Integration is an important event in the series of events leading to the development of cervical cancer [Li et al., 2008; Collins et al., 2009]. Alternatively, low-level persistence may signify containment of HPV-infected cells by cellular immunity resulting in a small lesion that may be difficult to adequately sample by standard methods.

In this study, some periods of HPV 16 detection using LA-HPV were characterized by periods of non-detection followed later by redetection. In some of these cases, HPV 16 could be detected by TSN-PCR-16 during the periods of non-detection by LA-HPV. In light of these findings of low-level persistence, we hypothesize that redetection after "apparent clearance" is probably due in most instances to reactivation rather than HPV 16 reinfection. Our findings of redetection are supported by Winer et al. [2010] in which redetection occurred in 19.4% of the original infections and in most cases, was associated with the same HPV variant as the original infection. Understanding factors associated with redetection (or reactivation) need to be more fully understood, and future studies in our laboratory will explore this important issue. For these future studies, in cases of HPV 16 detection, non-detection, then redetection, PCR and sequencing will be performed on highly variable regions, such as the long control region (LCR).

This study has some limitations. While the overall number of participants was small, participants were followed closely for many years. There is little similar data to compare these to, and additional testing of more participants will expand what is presented here. There was little racial diversity among the participants, and findings cannot be directly extrapolated to other ages or populations. The potential risk factors, such as sexual behaviors, numbers of sex partners, hormonal contraceptive use, and smoking, potentially

associated with persistent HPV were not analyzed in this cohort. Additionally, in five participants, HPV 16 was detected by LA-HPV in only one sample during their entire participation. None of these participants had additional TSN-PCR-16 positive samples when testing was completed. It is possible, therefore, that these five cases of one time HPV 16 detection represented transient vaginal deposition of HPV 16 (e.g., following unprotected penile-vaginal intercourse) rather than established infection. The inclusion of these five "one time detections" may have led to an underestimation of the duration of HPV 16 detection. The TSN-PCR-16 assay has some limitations as well, including the need for highly controlled laboratory conditions to prevent false positive results, and the higher amount of labor involved compared to standard PCR assays. Proof of HPV infection includes detection of viral transcripts. This analysis focused on detection, and admittedly, a number of these detections could represent deposition during sexual intercourse. However, repeated detection of HPV 16 provides better evidence of infection.

Multiple unknowns about the natural history of HPV still exist. The data presented here support reconsideration of the word "clearance" in relationship to HPV infections, as periods of non-detection by LA-HPV, often longer than 6 months, followed by redetection of HPV 16 were present among this cohort. Data from this study suggest that, at least for HPV 16, low-level persistence exists.

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