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# Redetection of human papillomavirus type 16 infections of the cervix in mid-adult life

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ABSTRACT

*Purpose:* To assess whether HPV 16 originally detected in adolescent women can be redetected in adulthood.
*Methods:* A convenience sample of 27 adult women with known HPV 16 detection during adolescence was assessed for HPV 16 redetection. A comparison of the long control region (LCR) DNA sequences was performed on some of the original and redetected HPV 16 isolates.
*Results:* Median age at reenrollment was 27.5 years (interquartile range of 26.7–29.6). Reenrollment occurred six years on average after the original HPV 16 detection. Eleven of 27 women had HPV 16 redetected. Some of these HPV 16 infections had apparently cleared during adolescence. LCR sequencing was successful in paired isolates from 6 women; in 5 of 6 cases the redetected HPV 16 isolates were identical to those detected during adolescence,
*Conclusions:* HPV 16 may be episodically detected in young women, even over long time periods. HPV 16 redetection with identical LCR sequences suggests low-level persistent infection rather than true clearance, although newly acquired infection with an identical HPV 16 isolate cannot be excluded. However, this study suggests that a new HPV 16-positive test in a clinical setting may not indicate a new infection.

## 1. Introduction

Despite the high prevalence of HPV infection in women, only a small percentage of these infections lead to cervical dysplasia or cancer. Most become undetectable within 12 months of the initial HPV detection, a phenomenon commonly referred to as clearance [1,2]. However, in studies with longitudinal follow-up periods up to six years, episodic detection of oncogenic HPV (with long time periods of apparent clearance) has been regularly demonstrated in young women [3–6]. Although HPV 16 can be detected in the months or even years immediately prior to the diagnosis of cervical cancer, it is unclear is this represents episodic detection of a previously acquired infection (low-level persistence) or a new HPV 16 infection [8]. HPV, a DNA virus, uses the host cell machinery to replicate. The rate of mutation of HPV mirrors that of the human genome, and is stable over time with some estimating an evolutionary rate of only one magnitude greater than that of their human hosts [9]. Of the nine regions of the HPV 16 genome, the long control region is the most variable of all of the genome regions [10]. The sequence variability in this region may be as high as 5% among HPV 16 isolates and has been used to follow transmission of HPV 16 isolates among cohorts and to understand persistent HPV 16 infections [11,12].

Various models have been proposed to explain detection patterns of HPV after apparent clearance, but the clinical relevance of these patterns of detection are not well understood [13–15]. In certain developed countries, primary cervical cancer screening with HPV DNA followed by type determination will replace cytological screening in upcoming years. Episodically detected high-risk HPV (HR-HPV), including HPV 16, therefore has implications in this new method of screening. In addition, the attributable risks of episodically detected infections vs. incident infections acquired later in life are not known but may differ.

To test the hypothesis that some HPV redetection episodes may be due to reactivation of a previously acquired infection, we reenrolled 30 women from a longitudinal cohort study known as the Young Women's Project (YWP) [16]. During the YWP, these women were tested

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**Table 1**
Long Control Region (LCR) sequencing primers, nucleotide coordinates, and exact sequences.

| HPV 16 LCR segment | Nucleotide coordinates spanning the segment | Primer sequence |
|---|---|---|
| **Segment 1 Forward** | 7288-7626 | 5′-TGCTTGTGTAACTATTGTGTC-3′ |
| **Segment 1 Reverse** | | 5′-GTTGCACATAGTGCAGTGTAA-3′ |
| **Segment 2 Forward** | 7498-7788 | 5′-AGTTCTATGTCAGCAACTATGG-3′ |
| **Segment 2 Reverse** | | 5′-AACTAGGGTGACATTTAGTTGG-3′ |
| **Segment 3 Forward** | 7681-115 | 5′-CCTTACATACCGCTGTTAGG – 3′ |
| **Segment 3 Reverse** | | 5′-GTCCTGAAACATTGCAGTTCT – 3′ |

quarterly for HPV using self-collected vaginal swabs and annual cervical sampling. At reenrollment (the current study) data was gathered to assess whether 1) women with prior HPV 16 detection continued to have HPV 16 detected after a decade or longer, and 2) if the original and redetected HPV 16 isolates were identical or nearly identical (suggesting reactivation) or different (suggesting reinfection).

## 2. Material and methods

### 2.1. Study population and design

The current study was approved by the Institutional Review Board at Indiana University School of Medicine. Consent for re-contact of women enrolled in a prior study (the YWP) was already in place; however, all participants were consented again at enrollment for this study. We preferentially contacted women who had HPV 16 and or HPV 18 detected during their YWP observation [16–18]. Women in the YWP study (1998 through 2007) were contacted consecutively based on date of study enrollment, beginning with the earliest enrollment, and the first 30 to agree to participate verbally and present for their scheduled appointment constituted the study sample. A convenience sample of 30 women was re-enrolled. For 3 women who were reenrolled, no record of HPV 16 detection was found during their YWP observation, so these 3 women were excluded in this analysis that focused on HPV 16 re-detection.

Two study visits were required. At Visit 1, women were interviewed for intervening sexual histories and behaviors (from last date of YWP observation to current enrollment) as well as lifetime histories of previous and abnormal cervical cytological testing (Pap smear) and treatment for such abnormalities. After the interview, cervical swabs were collected for HPV testing and sequencing as described below, and a Pap smear was obtained. At Visit 2, participants provided a self-obtained vaginal swab for HPV testing. Pap smear and HPV results from Visit 1 were discussed with participants. The median time between Visit 1 and 2 for all participants was 33 days (IQR 22.5–54.5). Arrangements for follow-up with primary providers were made if the Pap smear was abnormal or if the participant's swab was positive for HPV types 16 or 18.

### 2.2. HPV testing

DNA was extracted from cervical or vaginal swabbed specimens as previously described [19]. The Linear Array HPV Genotyping test (Roche Molecular Diagnostics, Indianapolis, Indiana) (LA-HPV) was used for HPV detection and genotyping. This assay detects 37 HPV types using 5′ biotin-labeled primer pools for polymerase chain reaction (PCR) amplification within the L1 region of the HPV genome. Reactions were amplified in an Eppendorf Mastercycler® proS using the same parameters as previously described [20]. A positive control reaction (sample provided by Roche Molecular Diagnostics) and negative control reaction (no DNA) were performed with each assay. The GH20/PC04 human β-globin target was co-amplified to determine sample adequacy. Determination of specific HPV types was performed using the Roche Linear Assay (Roche Diagnostics, Indianapolis, Indiana) as previously described [19,21]. A semi-quantitative scoring system was used to estimate HPV viral load in samples from adolescent as previously described [22]. The low positive beta-globin band was assigned a value of 2, and the high positive band was assigned a value of 5. The intensity of HPV 16 bands on assay strips (“signal strength”) was compared to the low and high beta-globin bands and scored relative to these bands and given a score of 1–5.

### 2.3. Sequencing of the LCR region

The long control region (LCR) is the most variable region within the HPV genome with as much as 5% variability within variants, and has been used to characterize HPV isolates of various types [23–25]. When a participant tested positive for HPV 16 at one of the two study visits, a paired sampled was identified for comparison of LCR sequence. The paired sample consisted of the last HPV 16-positive swab during their YWP observation and an HPV 16-positive swab identified at re-enrollment. If amplification from the last positive swab during their YWP was unsuccessful, the next closest collected HPV 16-positive swab from that participant's longitudinal testing was used.

For sequencing, three overlapping primer pairs were developed such that the entire LCR was amplified (Table 1). The overlapping primer design was used to reduce error per sequencing reaction and increase sensitivity of the amplification [26]. The HPV 16 sequence used to determine the nucleotide number is located in GenBank under the accession number K02718.1. The percent homology between HPV 16 isolates was calculated by dividing the number of nucleotide non-identities by the total number of nucleotides sequenced, multiplied by 100. If sequencing for the identified pairs of HPV was more than 98% homologous, the pair was considered identical. As all of the participants were from a geographically limited area, determining the intertype variants may not have provided sufficient sequence variation to differentiate two isolates within the same type. de Villiers, et al. has noted a 2 to 5% variabilityin the LCR among HPV isolates of the same type [9]. Therefore, we selected this highly variable region for sequencing, and a cut-off of $\leq$2% difference in this region was used to differentiate HPV 16 isolates.

Five microliters of extracted DNA from each sample were used for amplification of the LCR region. PCR reactions were carried out using 5 μL of each primer (0.5 μM final concentration), 25 μL of Roche FastStart™ Master Mix (Roche Diagnostics), 10 μL of water on an Eppendorf Mastercycler® pro S for a final reaction volume of 50 μL. Reaction parameters were as follows: 94 °C for 9 min, 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s all for 40 cycles followed by 72 °C for 7 min for the final extension step.

Amplification products were visualized on an agarose gel and the products corresponding to the expected size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified LCR segments were sequenced using BIgDye terminator version 3.1 on an ABI 3730 XL instrument by ACGT, Inc. (Wheeling, IL). The sequences of the overlapping segments from the same sample were aligned using Basic Local Alignment Search Tool (BLAST) available on the NCBI website to create the full LCR sequence for that sample. The complete LCR sequence from one sample collected during the original YWP observation was then aligned with a sample from the re-enrollment period using BLAST.