High HPV 16 Viral Load is Associated with Increased Cervical Dysplasia in Honduran Women

Nelba Táborá, Annabelle Ferrera, Judith M. J. E. Bakkers, Leon F. A. G. Massuger, and Willem J. G. Melchers*
Department of Microbiology, Universidad Nacional Autónoma de Honduras (UNAH), Tegucigalpa, Honduras; Department of Medical Microbiology, Radboud University Nijmegen Medical Centre Nijmegen, Nijmegen University Centre for Infectious Diseases, Nijmegen, The Netherlands; Department of Obstetrics and Gynaecology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Abstract. Cervical cancer is believed to have a co-factorial etiology in which high-risk human papillomavirus (HPV) infections are considered an essential factor and other elements play an ancillary role. Besides the importance of specific HPV genotypes, other viral cofactors as viral load may influence the progression likelihood. In this study the relationship between HPV 16 viral load with respect to the grade of cervical disease in Honduran women was investigated. A real-time PCR allowing quantification of both HPV 16 genome and β-globin gene to normalize the measuring HPV 16 load in cervical cells was used. The data in 87 women with cervical dysplasia or cervical cancer and in 23 women with a negative Pap smear were evaluated. The highest average of HPV 16 viral load was detected in women with High Squamous Intraepithelial Lesions (HSIL). An increasing amount of HPV in higher cervical lesions was found, which could indicate a dose-response association between viral load and precancerous lesion grade.

INTRODUCTION

Cervical cancer is a significant public health problem in Latin America and the Caribbean. By the year 2000, some 76,000 cervical cancer and almost 30,000 deaths were estimated for the whole region, which represents 16% and 13% of the world burden, respectively.1 The vast majority of cervical cancer cases and deaths occur in developing countries where women have little or no access to effective disease prevention services. According to WHO statistics, Central America has one of the highest rates of death from cervical cancer in the world. Honduras is no exception with an age-standardized rate of 30.6 per 100,000 inhabitants.2 Most women who die of cervical cancer—particularly in developing countries—are in the prime of their lives. They may be raising children, caring for their families, and contributing to the social or economic life of their towns. Their deaths are both personal tragedies and sad, unnecessary losses to their families and communities. Their deaths are unnecessary because there is compelling evidence that cervical cancer is one of the most preventable and treatable forms of cancer, as long as it is detected early and managed effectively. Unfortunately, the majority of women in developing countries still do not have access to cervical cancer prevention programs. The consequence is that cervical cancer is not detected until it is too late to be cured.

The effect of high-risk types of HPV DNA infection on CIN development is highly influenced by viral number copies. In this context, measuring HPV load could improve the positive predictive value of HPV testing based on the assumption that higher load values are more strongly associated with severe disease. Several studies have suggested that a high HPV-DNA viral load may be a candidate marker that could help identify women at greater risk of CIN progression.3–6 In fact, some studies have pointed out that high viral load in cytological normal epithelium could also be a risk factor for neoplastic progression.3,4

To increase our knowledge of HPV-induced carcinogenesis, we evaluated the relationship between HPV 16 copy numbers with respect to the grade of cervical disease to identify the existence of a HPV type-dependent viral load effect in Honduran women.

MATERIALS AND METHODS

Study population. The samples analyzed are part of a study that was conducted in Tegucigalpa, Honduras from January 2003 to December 2005 in which 760 women were enrolled for the analysis of HPV. Cervical samples were obtained from 281 women aged 18 to 65 years with cervical dysplasia or cervical cancer attending San Felipe General Hospital, a referral center for cancer patients. The histological diagnosis based on biopsy results determined the classification for each case in the study. At the same time, cervical samples were also taken from 479 women with normal cervix and a negative Pap smear; 2 per women with cervical dysplasia or cervical cancer whenever possible, matched by age to a ± 5-year interval and randomly selected from cervical screening clinics.

After formal written consent, a thorough physical examination was performed, and a structured epidemiological questionnaire was administered to each woman. The study was reviewed and approved by the correspondent Ethical Committee. A total of 118 samples from the women with cervical dysplasia were positive for HPV 16 (42%) and 44 of the women with normal cytology (9%).

From these 162 samples positive for HPV 16 there was not enough DNA to do the viral load from 52 samples, so 110 frozen cervical DNA specimens were available for this study, 23 from women with normal cytology, 25 samples from women with low squamous intraepithelial lesions (LSIL), and 62 from women with high squamous intraepithelial lesions (HSIL).

HPV DNA detection. Sample collection. For the analysis of HPV, cervical samples were taken from the transformation zone with an Ayre wooden spatula. Cells were eluted from the spatula in 5 mL of PBS (Phosphate Buffered Saline), pH 7.6 containing 0.05% thimerosal. Cells were vortexed, centrifuged for 10 min at 4500 rpm, then resuspended in 1 mL of PBS and centrifuged again for 10 min at 4500 rpm, resuspended in 0.5 mL of PBS and stored at −20°C for further analysis. DNA was extracted from thawed cells according to the standard Boom method as previously described.7 In brief, 200 μL of material was pipetted into a reaction vessel containing 30 μL of celite and 1 mL of a guanidium thiocyanate (GuSCN)-containing lysis buffer, released nucleic acid (NA) was bound to the celite, forming complexes that could be rapidly sedimented by centrifugation. These complexes were

* Address correspondence to Willem Melchers, Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, The Netherlands. E-mail: W.Melchers@mmb.umcn.nl
then washed twice with 1 mL of a GuSCN-containing washing buffer, twice with 1 mL of 70% ethanol, and once with 1 mL of acetone; the complexes were dried, and NA was subsequently eluted in a final volume of 100 μL nuclease-free water; 10 μL were used for each of the various PCR analyses. Every 5 samples, a negative extraction control was processed as well.

To avoid contamination by PCR products, sample preparation and the amplification reaction were all performed in separate rooms.

**Qualitative β-globin detection.** From the start, all samples were prescreened to assess DNA sample integrity with the β-globin primers PCO3 (5′-ACA CAA CTA CGT TGT TCA CTA GC 3′) and PCO5 (5′-GAA ACC CAA GAG TCT TCT CT 3′).

**PCR amplification of HPV DNA.** Broad-spectrum HPV DNA amplification was performed using a short PCR fragment assay (HPV SPF10 Line Blot 25, Labo Bio-medical products B.V. Rijswijk, The Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame, and allows detection of at least 43 different HPV types.8,9

Appropriate negative and positive controls were used to monitor the performance of the PCR method in each experiment.

**HPV detection by DEIA.** The presence of HPV DNA was determined by hybridization of SPF10 amplimers to a mixture of general HPV probes recognizing a broad range of high-risk, low-risk, and possible high-risk HPV genotypes in a microtiter plate format, as described previously.9,10 All HPV DNA positive samples were further genotyped.

**HPV genotyping by reverse hybridization using the HPV SPF10 Line Blot 25 genotyping system.** The 28 oligonucleotide probes that recognize 25 different types were immobilized as parallel lines to membrane strips (Labo Bio-medical products B.V. Rijswijk, The Netherlands). The HPV genotyping assay was performed as described previously.9,10 Each experiment was performed with separate positive and negative PCR controls.

The LiPA strips were manually interpreted using the provided reference guide. The samples that tested positive using the DNA Enzyme Immuno Assay but showed no results on the LiPA strip were considered to be HPV X-type (i.e., genotypes not available on the LiPA strip).

**Quantitative HPV 16 viral load determination.** The amount of HPV 16 DNA in cervical samples was determined by real-time PCR using the LightCycler System (Roche). The amplification was performed in a 20 μL volume containing 1× Hybridization Probe mix (Roche); 3.5 mM MgCl2, 20 μM concentration of each probe (HPV16-Fi: 5′-ACA AAA GGT TAC AAT ATT GTA ATG GGC TCT labeled with fluorescein and HPV16: 5′-CGG GTT CTG CTT GTC CAG CTG G –PH labeled with LC Red 640), 50 μM concentration of each primer (HPV16-1: 5′-GAG GAG GAG GAT GAA ATA GAT GGG T3′ and HPV16-2: 5′-GCC CAT TAA CAG GTC TTC CAA T3′), and 5 μL of DNA. The amplification ramp included an initial hold step of 10 min followed by a denaturation cycle of 3 sec at 95°C, an annealing cycle of 15 sec at 56°C, and an elongation cycle of 9 sec at 72°C for 45 cycles.11

For quantification of HPV 16, standard curves were generated using 10-fold dilutions of HPV 16 plasmid (ranging from 100 to 10,000,000 fg per reaction). The β-globin gene was quantified with the LightCycler Control Kit DNA, using a standard curve of 10-fold dilutions of human genomic DNA ranging from 3 pg to 3 ng per reaction.

**Analysis of results and statistical methods.** Normalized viral load was calculated dividing the HPV copy number by the β-globin copy number (based on 2 β-globin copies per cell) and was expressed as copies of virus per cell equivalent according to the formula described by Carcopino and others (2006)12.

**Viral load (HPV copies/cell) = Number of HPV copies/Number of β-globin copies.** Calculations and statistical analysis were done using Epi Info 2000 v 3.3.2 (CDC, Atlanta, GA, USA), Excel X (Microsoft, USA), and MATLAB Statistics Toolbox 6 (The Mathworks, USA) programs. Statistical significance was determined by Kruskal Wallis test. Any P value ≤ 0.05 was considered significant. Lilliefors and Jarque-Bera tests were used as evidence for normality of the log-transformed data.

**RESULTS**

We investigated whether the amount of HPV 16 DNA correlates with cervical disease severity by applying a real-time PCR-based assay to HPV 16 positive samples. The results were stratified by clinical group and the underlying distribution was examined. Figure 1 shows log-normal cumulative distribution for HPV 16 viral load populations obtained for the 3 clinical groups studied. Analytical cumulative log-normal distributions determined using observed mean and standard deviation are also plotted for clarity. The hypothesis for log-normality in the observed cumulative distributions was not rejected with alpha 0.05 using Lilliefors and Jarque-Bera tests for normality (P = 0.46/0.5 NORMAL, P = 0.44/0.13 LISR, and P = 0.43/0.49 HSIL). Log normal distributions were assumed in all population analysis for determination of confidence intervals shown in Table 1; however, for determination of significance of mean changes among groups, the non-parametric Kruskal-Wallis was used.

The overall results are shown in Table 1 and have been stratified by condition and subsequently by age group and infection type within the corresponding clinical group. The main stratification by clinical type exhibited statistically significant differences in the populations. In this stratification, the highest average viral loads of HPV 16 were detected in women with HSIL (75 HPV DNA copies/cell). In LSIL samples an average viral load of 9 HPV DNA copies/cell was found, whereas an average HPV 16 viral load of 0.7 was found in women with a normal Pap smear result.

The amount of HPV DNA/specimen varied significantly within a single group and between the different groups. The

**FIGURE 1.** Cumulative frequency of the viral loads of the clinical groups evaluated. The lines were obtained from the log-normal curves using the observed mean and standard deviations.
Mean HPV 16 viral load in cytological normal women and in women with cervical lesions stratified by clinical group, age, and infection type

<table>
<thead>
<tr>
<th>Overall stratification</th>
<th>N</th>
<th>Mean</th>
<th>min</th>
<th>max</th>
<th>95% CI</th>
<th>Kruskal Wallis significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normal</td>
<td>23</td>
<td>0.72</td>
<td>0.20</td>
<td>2.6</td>
<td>5.6E-01</td>
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<tr>
<td>LSIL</td>
<td>25</td>
<td>8.8</td>
<td>2.9</td>
<td>27</td>
<td></td>
<td></td>
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<tr>
<td>HSIL</td>
<td>62</td>
<td>75</td>
<td>37</td>
<td>150</td>
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Subgroup stratification by age

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<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>min</th>
<th>max</th>
<th>95% CI</th>
<th>Kruskal Wallis significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal &lt; 35</td>
<td>10</td>
<td>0.48</td>
<td>0.13</td>
<td>1.7</td>
<td>9.6E-01</td>
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</tr>
<tr>
<td>35–45</td>
<td>6</td>
<td>1.1</td>
<td>0.013</td>
<td>95</td>
<td></td>
<td></td>
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<tr>
<td>&gt; 45</td>
<td>7</td>
<td>0.92</td>
<td>0.044</td>
<td>19</td>
<td></td>
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</tr>
<tr>
<td>LSIL &lt; 35</td>
<td>9</td>
<td>4.6</td>
<td>0.34</td>
<td>64</td>
<td>8.6E-01</td>
<td></td>
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<tr>
<td>35–45</td>
<td>11</td>
<td>13.7</td>
<td>2.8</td>
<td>67</td>
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<td>&gt; 45</td>
<td>5</td>
<td>10.3</td>
<td>0.68</td>
<td>160</td>
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<tr>
<td>HSIL &lt; 35</td>
<td>18</td>
<td>51.2</td>
<td>13</td>
<td>210</td>
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Subgroup stratification by infection type

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<tr>
<th>Clinical Type</th>
<th>N</th>
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<th>max</th>
<th>95% CI</th>
<th>Kruskal Wallis significance</th>
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<td>Single-HPV16</td>
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<td>0.83</td>
<td>0.16</td>
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<tr>
<td>LSIL Single-HPV16</td>
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<td>10.0</td>
<td>3.3</td>
<td>31</td>
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<tr>
<td>Multiple</td>
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<td>6.1</td>
<td>0.19</td>
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<tr>
<td>HSIL Single-HPV16</td>
<td>41</td>
<td>79</td>
<td>36</td>
<td>176</td>
<td>8.6E-01</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>21</td>
<td>67</td>
<td>16</td>
<td>286</td>
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</tbody>
</table>

CI = confidence interval; LSIL = low squamous intraepithelial lesions; HSIL = high squamous intraepithelial lesions.

Discussion

In this study, the highest viral loads of HPV 16 were detected in women with HSIL, whereas lower viral loads were detected in women with negative Pap smears. These differences were statistically significant and suggest that the increasing amount of HPV in higher CIN stages indicates a dose-response association between virus viral load and lesion grade. The analysis of the results revealed that there was a broad distribution among the HPV 16 viral loads and no clusters were observed in any of the groups analyzed and the presence of dispersed values was more evident with the progression of cervical lesions. Previous studies have also shown that average HPV DNA copy number increases significantly with the grade of CIN, mainly for HPV 16, but not for other high-risk types.4,13,14

Real-time PCR techniques have been developed to quantify HPV in clinical samples. Moreover, the Hybrid Capture II provides a semi-quantitative measurement of HPV-DNA and some studies have demonstrated that the estimated hc2 load correlates well with the precise load generated by real-time PCR.15–19 However, real-time PCR assays more accurately measure HPV16 viral load by adjusting the signal obtained for HPV-16 DNA with the amount of cellular DNA calculated for amplification of a human gene, therefore providing a more accurate HPV viral load.4,13–16,20–23 Such correction is essential if bias in viral load measurements is to be avoided because more cellular material can be obtained from sampling high-grade cervical lesions, thus potentially generating artificially higher HPV viral loads.4 However, due to low multiplicity for different hr-HPV types, real-time PCR methods are not (yet) suitable as a high-throughput “screening tool.”

In the present study, the method used allowed for normalizing the numbers of copies of viral DNA against the quantity of host DNA, permitting calculation of true viral load in terms of the number of DNA copies/cell, thus eliminating the fluctuation due to variation in cell content among specimens from different subjects.

Although in general the amount of HPV 16 found in patients with normal cervix was low, 2 patients had a high HPV 16 viral load (>100 HPV copies/cell), close to viral load levels observed in patients with high-grade squamous intraepithelial lesion. It would be helpful to follow-up these patients to differentiate transient infection from possible progression to high-grade squamous intraepithelial lesion. However, financial and logistic restraints partially impaired follow-up. Considering that Honduras does not have a well-organized National Cervical Cancer Program, it was difficult for the women positive for HPV 16 to comply with the follow-up visits and was the main limitation of this study.

In our survey, age strata analysis revealed a correlation between high viral load and severity of cervical lesion in all the age groups studied; even though results of this study were obtained with a single measurement of exposure, it was not possible to know whether an ordinal measurement of HPV DNA could be a predictor of progression. However, results were consistent with some findings observed in follow-up study designs that studied viral load of HPV and development of cervical cancer that indicated that measurement of viral load, as a surrogate for HPV persistence, may identify those women at increased risk of developing cervical lesions.3–6

Our results also showed higher HPV 16 viral loads in single infections as compared with multiple infections. Some studies have shown, besides viral load, an association between persistent HPV infection and the presence of multiple types.24 On the contrary, other studies have shown that clearance of a type-specific HPV infection seem to be independent of the presence of a coinfection with other types.25

The study findings have emphasized the significance of HPV 16 viral load in the risk determinant of cervical SIL and invasive cancer using a method that allows adjusting for differences in the number of cells collected for viral quantitation.
In the context of this study and given the need for additional biomarkers that could distinguish transient infections from those that impose a risk of progressing into cervical cancer, it appears appropriate to evaluate quantitative PCR assays in larger prospective studies of cytological normal women, which will help to determine the natural history and biology of latent infection.

Taken together, the data presented here could advance our understanding of the relationship of HPV 16 viral loads with respect to the grade of cervical disease in Honduran women.

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