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Diagnostic value of human papillomavirus (HPV) 16 and HPV18 viral loads for the detection of high-grade cervical intraepithelial neoplasia (CIN2+) in a cohort of African women living with HIV

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Highlights

- HPV16 and HPV18 E6 DNA loads increase with the grade of cervical lesions
- HPV18 viral load is less preformant than HPV16 viral load for idenfying CIN2+
- HPV16 viral load may be considered as a triage test in HPV16/HIV-coinfected women

ABSTRACT

Background: African women living with HIV (WLHIV) are at high risk of cervical cancer but rarely adequately screened. Better strategies enabling identification of WLHIV with high-grade cervical intraepithelial lesions (CIN2+) are required.

Objectives: To investigate the diagnostic value of HPV16 and HPV18 viral loads in a cohort of African WLHIV.

Design: HPV16 and HPV18 viral loads were determined by quantitation of the E6 gene DNA by real-time PCR in cervical specimens collected at baseline and endline (16 months) from 245 African WLHIV positive for HPV16 or/and HPV18. Cervical biopsies were graded using the histopathological CIN classification.

Results: Women with CIN2+ had higher viral load for HPV16 (p<0.0001) or HPV18 (p=0.03) than those without CIN2+. HPV16 viral load ≥3.59 log copies/1,000 cells detected CIN2+ with sensitivity and specificity of 93.5% (95%CI: 81.7 to 98.3%) and 74.1% (95%CI: 66.3 to 80.6%), respectively, whereas HPV18 viral load ≥1.63 log copies/1,000 cells detected CIN2+ with sensitivity and specificity of 59.1% (95%CI: 38.7 to 76.7%) and 66.9% (95%CI: 58.8 to 74.1%), respectively. A high baseline HPV16 viral load was significantly associated with

persistence of, or progression to CIN2+ at endline; these findings were not observed for HPV18.

Conclusions: HPV16 viral load is a powerful marker of CIN2+ in African WLHIV. HPV18 viral load is of lower diagnostic value in this population.

Keywords: HPV16; HPV18; viral load; cervical intraepithelial neoplasia

1. Background

Cervical cancer is the fourth most common cancer in women worldwide and the most common cancer and main cause of cancer mortality in women living in low income and developing countries, particularly in sub-Saharan Africa [1-2]. Virtually all cases of cervical cancer result from a persistent infection with high-risk human papillomaviruses (HR-HPV), HPV16 and HPV18 being associated with over 70% invasive cervical cancer cases [3].

Women living with HIV (WLHIV) have an increased risk of persistent HR-HPV infection and development of precancerous and cancerous lesions [4], and preventative strategies targeting this highly exposed population are required. In women currently not eligible for HPV vaccination, prevention of cervical cancer relies on the detection and treatment of precancerous lesions. In most sub-Saharan African countries, access to cervical cancer screening by cytological examination of cervical cells (pap test) is limited by a lack of resources [5]. Visual inspection with acetic acid and Lugol's iodine (VIA/VILI) performed by trained physicians or nurses is more widely used in sub-Saharan African countries, but the diagnostic performance of this simple cost-effective screening method varies across studies and is very dependent on the level of training of the observer [6-7]. Detection of HR-HPV DNA in cervical specimens has proven high sensitivity for identifying women with cervical precancerous/cancerous lesions [8]. Simple and affordable HPV DNA tests may be used for cervical cancer screening in resource-poor settings [9-10]. We have shown that this approach was effective for detecting high-grade cervical intraepithelial neoplasia (CIN) among WLHIV in Burkina Faso and South Africa [11]. However, in this highly-infected

population, the high sensitivity of HR-HPV DNA testing for detection of high-grade cervical lesions is counterbalanced by a relative low specificity [11-12], and a triage test would be required to determine which women may benefit from the more expensive and less easily accessible colposcopy referral. It has been reported that HPV16 DNA levels in cervical samples increase with lesion severity and thus, measurement of HPV16 viral load might be considered as a triage tool for identifying women at risk of high-grade CIN [13-20]. Data on HPV18 viral load are more sparse, and the association with cervical lesion severity is less clear [18, 20, 21-24].

2. Objectives

The objective of the present study was to investigate the diagnostic value of HPV16 and HPV18 viral loads in a cohort of WLHIV enrolled in a prospective evaluation of cervical cancer screening approaches in sub-Saharan Africa.

3. Study design

3.1. Study population

The HARP (HPV in Africa Research partnership) study enrolled women with HIV-1 infection aged 15-49 in Ouagadougou, Burkina Faso, and Johannesburg, South Africa, for a prospective study over 18 months. The HARP study methodology has been described in detail elsewhere [11, 25]. In brief, at enrolment (baseline) and at the follow-up visit scheduled at month (M) 18 (endline) and that actually occurred at a median (IQR) 16 (15.5-16.8) months later, women benefited from cervical screening using cytology, VIA/VILI, HR-HPV DNA screening using digene HC2 test (at baseline) or *care*HPV tests (at endline) (both Qiagen, Gaithersburg, MD) and colposcopic examination. Systematic 4-quadrant cervical biopsies, including directed biopsy of any suspicious lesions, were obtained for participants who had abnormalities detected by cytology, VIA/VILI or colposcopy, or who had a positive HR-HPV DNA test. Local histological findings using the CIN classification were reviewed by the HARP Endpoint Committee consisting of five pathologists for consensus classification of

the cervical lesions [26]. In addition, HPV DNA detection and typing using the INNO-LiPA HPV genotyping Extra assay (Fujirebio, Courtaboeuf, France) was performed as previously described [27] for all women enrolled in the HARP study at baseline and endline. CD4+ cell counts were measured by flow cytometry using the FACScount system (Beckton Dickinson, Franklin Lakes, NJ, USA). The present study was conducted among the sub-group of HARP participants who were positive for HPV16 or/and HPV18 DNA at enrolment and/or at endline. We intended to treat all women with CIN2+ lesions, but this was not possible for all women during the follow-up period for a variety of reasons, mostly women not attending their gynaecological clinic appointments in South Africa. Written informed consent was obtained from all participants. Ethical clearance was obtained from the Ministry of Health of Burkina Faso, the University of Witwatersrand in Johannesburg, and the London School of Hygiene & Tropical Medicine.

3.2. HPV 16 and HPV18 viral loads

Determination of HPV16 and HPV18 viral loads were performed as previously described [16, 24] on the DNA extracts used for the genotyping assay. In brief, viral loads were measured by quantitation of HPV16 and HPV18 E6 gene DNA by real-time PCR. Standard curves were obtained by serial dilution of pBR322-HPV16 and pBR322-HPV18 plasmids. Whole cell DNA was measured by quantitation of the GAPDH gene using a human DNA standard curve. SiHa (1-2 copies of HPV16 genome per cell), Caski (>500 copies of HPV16 genomes per cell) and HeLa (multiple copies of HPV18 genome) cell lines were used as controls. The lower limit of quantitation for E6 DNA for both HPV types was calculated to be 0.05 copies/1,000 cells.

3.3. Data analysis

Women without CIN or with CIN1 were categorized as <CIN2, and women with CIN2, CIN3 and higher grade lesions were categorized as CIN2+.

Results were expressed as the log number of copies of E6 DNA per 1,000 cells. Values of E6 DNA loads under the lower limit of quantitation (-1.30 log copies/1,000 cells) were set at this value.

For each genotype, viral loads were compared among women with no CIN, CIN1, CIN2 and CIN3 using the Kruskal-Wallis test and between <CIN2 and CIN2+ using the Mann-Whitney U test. Receiver operating characteristic (ROC) curve analyses with calculation of area under the curve (AUC) were used to evaluate the ability of HPV16 and HPV18 E6 viral loads to separate women with CIN2+ from <CIN2. For association of persistence or progression of lesions between baseline and endline with E6 DNA levels, logistic regression was used to calculate odds ratios (OR) with 95% confidence intervals (95%CI). Data were analysed using the XLSTAT2016 software (Addinsoft, Paris, France).

4. Results

Among the 1238 WLHIV enrolled in the HARP study who were tested for HPV detection and genotyping, 245 (19.8%) were positive for HPV16 or HPV18 at baseline and/or endline. These women had a median (IQR) age of 34 (30-41) years, had a median (IQR) CD4+ T-cell count of 417 (324-557) cells/µL, and 158 of them (64.5%) were on antiretroviral therapy (ART).

At baseline, 122 and 103 of these women were positive for HPV16 and HPV18, respectively, 25 of them being positive for both HPV16 and HPV18. At endline, 78 and 66 women were positive for HPV16 and HPV18, respectively, 11 of them being positive for both HPV16 and HPV18. Among the 122 women positive for HPV16 at baseline, 50 remained positive (persistence) and 72 became negative (clearance) at endline, whereas among the 123 women negative for HPV16 at baseline, 25 acquired an incident HPV16 infection. Similarly, among the 103 women positive for HPV18 at baseline, 41 showed persistence and 62 showed clearance, whereas among the 142 women negative for HPV18 at baseline, 25 acquired an incident HPV18 infection.

Overall, 68% of participants at baseline and 65.4% at endline were co-infected with one or more HR-HPV types other than HPV16 or HPV18, the most prevalent types being HPV52 (25.0%), HPV51 (17.0%), and HPV35 (16.5%)

E6 viral loads for each HPV type according to the histological grades are shown in Table 1. E6 DNA levels increased with histological grade and women with CIN2+ had significantly higher levels of E6 than those without CIN2 (<CIN2). Within each CIN category, absolute values of E6 DNA loads were higher for HPV16 than for HPV18.

The ROC curve analysis indicated a better performance of HPV16 E6 DNA load (AUC=0.877) compared to the HPV18 E6 DNA load (AUC=0.640) for the diagnosis of CIN2+ (Fig. 1). The best performance in terms of sensitivity and specificity was observed for a cutoff HPV16 E6 DNA load of 3.59 log DNA copies/1,000 cells and a cutoff HPV18 E6 DNA load of 1.63 log DNA copies/1,000 cells (Table 2)**.** Therefore, E6 DNA levels equal or higher than these values for each genotype were considered as 'high' viral loads in subsequent analyses. For these cutoff values, sensitivity, specificity, positive and negative predictive values were higher for HPV16 viral load than for HPV18 viral load (Table 2).

As shown in Table 3, there was no significant difference between HPV16 or HPV18 viral loads and CD4+ cell count levels. Women treated with ART had lower HPV16 but not HPV18 viral loads than untreated women. HPV16 and HPV18 viral loads were higher in single infections than in multiple HR-HPV infections.

As shown in Table 4, a high HPV16 viral load at baseline was associated with persistence of untreated CIN2+ or progression from <CIN2 to CIN2+ ; these findings were not observed for HPV18. Overall, among women infected with HPV16, those who remained <CIN2 between baseline and endline had a median (IQR) baseline viral load of 1.55 (-1.30 to 3.50) log DNA copies/1,000 cells, whereas those showing persistence of CIN2+ or progression from <CIN2 to CIN2+ had a higher viral load of 5.13 (4.49 to 5.67) log DNA copies/1,000 cells (p=0.001). Notably, 4 women had a histological diagnosis of <CIN2 at baseline (1 CIN-negative, 3 CIN1)

associated to a high viral load (4.25 to 8.21 log DNA copies/1,000 cells) and all had a diagnosis of incident CIN2+ at endline. Among women infected with HPV18, those who remained <CIN2 between baseline and endline had a median (IQR) baseline viral load of 1.48 (-1.30 to 2.83) log DNA copies/1,000 cells, whereas those showing persistence of CIN2+ or progression from <CIN2 to CIN2+ had a viral load of 0.37 (-1.30 to 3.08) log DNA copies/1,000 cells (p=0.87).

Between baseline and endline, HPV16 and HPV 18 viral loads increased in women with progression to CIN2+ and decreased in women with CIN2+ regression following treatment, with DNA level changes >1 log DNA copies/1,000 cells. Viral loads were more stable (<1 log DNA copies/1,000 cells) in women with no progression to CIN2+ or with CIN2+ persistence (Table 5).

5. Discussion

Results obtained in this study indicate that HPV16 viral load, as determined by quantitation of E6 DNA, could be a reliable marker of high-grade CIN (CIN2+). HPV16 viral load significantly increased with histological grades, and a threshold of 3.59 log DNA copies/1,000 cells detected CIN2+ with a sensitivity and specificity of 93.5% and 74.1%, respectively, and a high negative predictive value (97.2%). Moreover, among the women initially <CIN2, a high HPV16 viral load at baseline was associated with incident CIN2+. The threshold of 3.59 log DNA copies/1,000 cells to predict prevalent or incident CIN2+ is in line with the results from Saunier et al. [17] who reported that a HPV16 viral load of 22,000 (4.34 log) DNA copies/1,000 cells allowed the identification of prevalent CIN2+ with a high specificity, and with the results reported by Carcopino et al. [28] who determined that a value of $3.0x10^6$ DNA copies/10^6cells (3.48 log DNA copies/1,000 cells) was a threshold for CIN2+ identification.

Among women infected with HPV18, an increase in viral load with histological grade was also observed, and a HPV18 viral load of 1.63 log DNA copies/1,000 cells identified CIN2+ with a sensitivity and specificity of 59.1% and 66.9%, respectively, and a negative predictive value of 91.3%. These results indicate that absolute HPV18 viral loads are lower than HPV16

viral loads, and that HPV18 viral load is less performant than HPV16 viral load for identifying CIN2+, which is supported by the difference in AUC values in the ROC curve analysis. These observations are in agreement with previous reports suggesting that HPV18 viral load is low in precancerous lesions [20] and has a poor diagnostic performance for detecting CIN2+ [21-22].

This study was conducted among WLHIV and, as expected, high prevalences of HR-HPV infection and CIN were observed. However, the majority of these women were not profoundly immunosuppressed and we did not observe a significant difference in HPV16 or HPV18 viral loads between women with CD4+ T-cell count greater or lower than 350 cells/µL, which was the ART eligibility cutoff used in these African countries at the time of the study. We have previously reported that ART has an impact on the control of HPV infection and development of cervical neoplasia [25], and the finding of lower HPV16 viral loads in women on ART is in line with this previous finding. Interestingly, we observed that HPV16 and HPV18 viral loads were higher in single HPV16 or HPV18 infections than in multiple HR-HPV infections, suggesting an involvement of coinfecting HR-HPV types other than HPV16 and HPV18 in the development of CIN. Indeed, we have reported that HR-HPV types other than HPV16/18, notably HPV58, were associated to CIN2+ in these women [29]. The high frequency of multiple HR-HPV infection in this population may have limited the performance of viral load for identifying CIN2+ in the present study. In particular, the lower performance of HPV18 viral load could be due in part to the fact that other HR-HPV types might be involved in most CIN2+ associated with a low HPV18 viral load.

In summary, our findings in a population of WLHIV indicate that, as previously reported for HIV-negative women, a high HPV16 viral load represents a useful marker of high-grade cervical lesion and might be considered as a triage test for women identified as HPV16-positive. A standardized commercially available test for HPV16 quantitation, affordable to low income countries, should allow to more precisely identify women at risk of cervical

carcinoma. On the other hand, HPV18 viral load appears to be of lower diagnostic value in this population.

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Contributors

MND, PM and MS conceived and planned the study, JN performed the experiments, MS and HK analysed the data, TO, OGL and SD performed histological diagnosis and slide reviews, MS drafted the manuscript, all authors revised and approved the manuscript.

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Competing interest

The authors have no conflicts of interest to disclose

Ethical approval

Ethical clearance was obtained from the Ministry of Health of Burkina Faso, the University of Witwatersrand in Johannesburg, South Africa, and the London School of Hygiene & Tropical Medicine. Written informed consent was obtained at the screening visit and all women provided a second written informed consent at the enrolment visit for enrolment and follow-up over 18 months.

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Figure legends

Fig.1. Receiver operator characteristic (ROC) curve analysis of HPV16 (A) and HPV18 (B) E6 DNA load for identifying women with high-grade lesions (CIN2+).

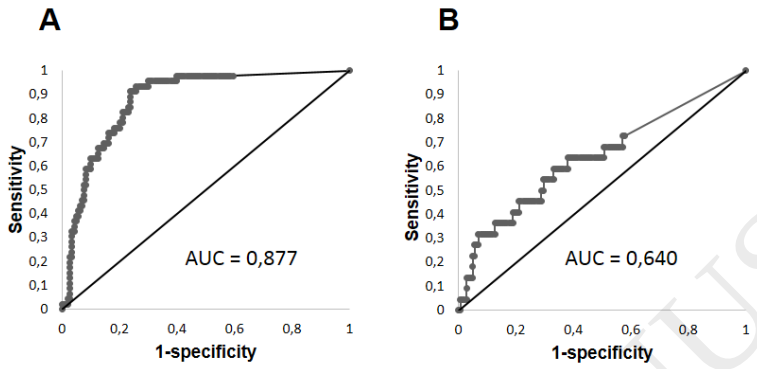

Table 1. HPV16 and HPV18 E6 DNA loads according to histologic grades of cervical lesions.

	Median (IQR) viral load (log E6 DNA copies/1,000 cells)							
HPV type	**CIN-negative**	**CIN1**	**CIN2**	**CIN3**	***P* ***	**<CIN2**	**CIN2+**	***P* ****
HPV16	n = 104 0.35 (-1.30 – 3.65)	n = 45 2.88 (0.29 – 4.53)	n = 28 5.14 (4.25 – 6.18)	n = 19 5.71 (4.39 – 6.37)	<0.0001	n = 149 0.86 (-1.30 – 3.67)	n = 47 5.44 (4.29 – 6.35)	<0.0001
HPV18	n = 93 -0.92 (-1.30 – 2.20)	n = 48 1.03 (-1.30 – 2.67)	n = 17 1.90 (-1.18 – 3.55)	n = 5 4.60 (-1.30 – 5.93)	0.03	n = 141 -0.27 (-1.30 – 2.42)	n = 22 1.96 (-1.27 – 4.23)	0.03

Table 2. Performance of HPV16 and HPV18 E6 DNA load threshold values for the diagnosis of CIN2+ among 245 African WLHIV.

Performance indicator	HPV16 E6 DNA 3.59 log copies/1,000 cells % (95% CI)	HPV18 E6 DNA 1.63 log copies/1,000 cells % (95% CI)
Sensitivity	93.5 (81.7 - 98.3)	59.1 (38.7 - 76.7)
Specificity	74.1 (66.3 - 80.6)	66.9 (58.8 - 74.1)
PPV	53.8 (46.6 - 60.8)	21.7 (15.9 - 30.6)
NPV	97.2 (92.2 - 99.1)	91.3 (86.3 - 94.6)

CI, confidence interval ; PPV, positive predictive value ; NPV, negative predictive value

Table 3. HPV16 and HPV18 E6 viral load according to CD4+ cell count, antiretroviral therapy (ART) and HR-HPV coinfection.

Parameter	HPV16 E6 viral load log DNA copies/1,000 cells	*P* *	HPV18 E6 viral load log DNA copies/1,000 cells	*P* *
CD4+ cell count				
<350 cells/µL	3.24		0.47	
≥350 cells/µL	2.37	0.81	0.006	0.78
ART				
No	4.12		-0.07	
Yes	1.90	0.01	0.38	0.68
HPV coinfection				
No	3.62		1.88	
Yes	2.00	0.08	-0.35	0.02

* Mann Whitney U test

Table 4. Associations of E6 DNA levels of HPV16 and HPV18 measured at baseline with evolution of cervical histology over 16 months among WLHIV with absence of CIN2+ lesions or untreated CIN2+ lesions at baseline.

HPV type	Baseline E6 DNA level*	No progression**	Persistence or progression***	OR (95%CI)	*P*
HPV16	<3.59	18	0		
	≥3.59	6	11	65.46 (3.36-1274.90)	0.006
HPV18	<1.63	14	4		
	≥1.63	13	3	0.81 (0.15-4.32)	1.0

CI, confidence interval ; OR, odds ratio

*3.59 log DNA copies per 1,000 cells and 1.63 log DNA copies per 1,000 cells are the thresholds determining high viral loads for HPV16 and HPV18, respectively.

**<CIN2 at baseline and at endline.

***Persistence : untreated CIN2+ at baseline and found again at endline ;

Progression : <CIN2 at baseline and CIN2+ at endline.

Table 5. E6 DNA level changes between baseline and endline (16 months later) according to the evolution of cervical lesions.

	E6 DNA level change (Δ log DNA copies/1,000 cells)	
Lesion evolution	**HPV16**	**HPV18**
No progression	-0.51	-0.16
Persistence	+0.74	+0.34
Progression	+1.50	+3.35
Regression*	-3.43	-1.34

* CIN2+ at baseline and <CIN2 at endline, mostly post-treatment