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RESEARCH ARTICLE

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Genotypic diversity of anogenital human papillomavirus in women attending cervical cancer screening in Harare, Zimbabwe

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Although anogenital cancers have been on a gradual rise in developing countries in the past few decades, they have been understudied. The objective was to investigate genotypic diversity of anogenital HPV amongst women reporting for routine cervical cancer screening in Harare in Zimbabwe. A cross-sectional study that enrolled 144 women ≥18 years from a cervical cancer-screening clinic was performed. Each woman provided a self-collected cervico-vaginal swab (VS) and a clinician-collected anal swab (CCAS). HIV testing was offered and cervical cytology was performed. Both VS and CCAS samples were HPV genotyped, using amplicon sequencing of the L1 gene region with Illumina technology. Mean age of the women was 39.9 (range 18-83 years, SD ± 11.0). HPV prevalence was 72% (104/144) in VS and 48% (69/144) in CCAS. The most common genotypes detected in both VS and CCAS were HPV18, HPV52, and HPV16. Sixty two percent of the subjects had multiple genotypic HPV infections. The odds of being HPV-positive among HIV-infected women were higher than in HIV-negative women in both the vagina and the anus (CCAS OR = 4.8; CI 2.4-9.8, $P < 0.001$) and (VS OR = 2.9; CI 1.3-6.4, $P = 0.005$). High HPV prevalence and diverse genotypes were detected in both the vagina and anus. Anal oncogenic HPV infection was common. HPV 52 was one of the most common oncogenic genotypes in both the vagina and anus. HIV co-infection played a significant role in the prevalence of HPV. These data have implications for design of primary and secondary programs for prevention of anogenital cancer in Zimbabwe.

KEYWORDS
anogenital, genotypes, human papillomavirus, sequencing, women, Zimbabwe

1 | INTRODUCTION

Human papillomaviruses (HPVs) are diverse DNA viruses that are known to infect mucosal surfaces and the skin.[1,2] They are common sexually transmitted agents that cause cancers of the cervix, vagina, vulvar, penis and anus, as well as genital warts.[3,4] The viruses are classified into two categories, the high-risk HPVs (HR-HPVs) and low-risk HPVs (LR-HPVs) genotypes, depending on their ability to cause cancer.[5] The most common HR-HPVs are HPV 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66, and 68 and infections with these types are associated with the majority of anogenital cancers.[3] Low-risk types such as HPV 6 and 11 are commonly associated with anogenital warts

Abbreviations: HPV, human papillomavirus; VIAC, visual inspection with acetic acid clinic; VS, cervico-vaginal self collected swabs; CCAS, clinician-collected anal swabs; HIV, human immuno-deficiency virus.

and lesions.[6] Normally, it is a persistent infection with HR-HPVs that may result in the development of anogenital cancers.[7] Globally, HPV 16 and 18 are the dominant HR-HPV genotypes and are responsible for causing most anogenital cancers such as cervical cancer in women.[8] In women with normal cervical cytology, HPV 16 is the dominant genotype worldwide, though there are exceptions in Eastern Africa, Japan, and Taiwan where HPV 52 is most common.[9] Other than HPV types 16 and 18, types 31 and 66 are also ranked as some of the most common in Europe.[10,11]

Cervical cancer is the most common anogenital cancer in Africa and is one of the major causes of mortality and morbidity in women.[12] In Zimbabwe, it accounts for one third of all cancers affecting African women.[13] Women who have a history of HPV-related cancers of the cervix, vagina, or vulva are known to be at increased risk of anal cancer.[14] In recent years, a notable increase in anal cancer cases has been observed.[15] In Zimbabwe, anorectal cancer accounts for 3% of all cancers, and the incidence is higher in women than in men.[13] There is evidence that HPV-associated cancers occur more frequently in HIV-infected individuals than in those who are HIV-uninfected.[16] Although anal cancers are rare, their incidence is higher in immunocompromised women than in immunocompetent subjects.[17] Data on the genotypic diversity of anogenital HPVs in Zimbabwean women is limited. Most studies have only concentrated research on genital HPV genotypes in women with cervical cancer.[18] Nothing is currently known about the epidemiology of anal HPVs in women in Zimbabwe. Also, no studies have previously investigated the genotypic diversity of both anal and genital HPVs in Zimbabwe. The aim of this study was to investigate the genotypic diversity of anogenital HPVs amongst women reporting for routine cervical cancer screening in Harare in Zimbabwe.

2 | MATERIALS AND METHODS

2.1 | Study subjects, ethical approvals, and sample collection

This was a cross-sectional epidemiological study at a cervical cancer-screening clinic at Parirenyatwa Hospital, a tertiary referral hospital in Harare, Zimbabwe. The study subjects were women visiting the VIAC clinic situated within the hospital. The ethical approvals to conduct the study were obtained from the Joint Parirenyatwa Hospital and College of Health Sciences Research Ethics Committee (reference number: JREC210/14) and the Medical Research Council of Zimbabwe (reference number: MRCZ/A/1911). Written informed consent, in English or vernacular language was obtained from the women who were ≥18 years, sexually active and had no history of a total abdominal hysterectomy. Enrolment of study subjects was from February to April 2015. A data collection tool was administered by a research nurse to capture demographic data and questions addressing risk factors such as parity, sexual debut, number of sexual partners, and HIV status. Women who did not have documented HIV results were offered counseling and testing using Alere Determine™ HIV-1/2 rapid test for screening and First Response HIV 1-2.0 rapid test, using finger prick blood, for confirming positive results. All HIV test results were documented. Two specimens were requested from each woman, one self-collected vaginal swab (VS) and one clinician (nurse)-collected anal swab (CCAS). The recruiting nurse collected the CCAS by gently inserting the swab until the shaft could not move further. The swab was rotated for 10-30 s. The nurse then explained the procedure of vaginal self-collection. All swabs were immediately broken into cryotubes soon after collection and were stored in 500 µL lysis buffer from bioMérieux (containing guanadine thiocyanate) at −80°C until laboratory processing. After both swabs were collected, the nurse inserted a speculum and a cytobrush was used to collect cells from the transformation zone of the cervix. A monolayer smear was made on a frosted glass slide and cytospray was used immediately to fix the slide. The slides were transported to the laboratory for conventional Papanicolaou staining.[19] Cytology was reported using the Bethesda method.[20]

2.2 | Laboratory processing of specimens

DNA was extracted from anal and vaginal swabs as previously described.[21] The extracted DNA was stored at −80°C until HPV DNA amplification.

2.3 | Amplification of HPV DNA and sequencing

The detection and genotyping of HPV was done by amplification of a 450 bp L1 gene fragment using the PGMY09/11 primers and sequencing using the MiSeq platform.[22] PGMY amplicons with Illumina-tailed adapters were generated in 20 µL volumes using Phusion Master Mix (Thermo Fischer Scientific, MA), 0.1 µM of each primer (PGMY09 and PGMY11) and 5 µL sample DNA template under the following thermocycling conditions: 1 cycle of 98°C for 30 s, 40 cycles of 98°C for 10 s, 56°C for 30 s, and 72°C for 15 s, before a final extension at 72°C for 10 min. Amplicons were cleaned up using modified PERFORMA® DTR V3 96-Well Short Plates and Quick-StepTM2 96-Well PCR Purification Kit (Edge Biosystems, MD) protocols. Amplicons were diluted 1:100 before being used as templates in indexing PCR in 20 µL volumes using Phusion Master Mix, 0.5 µM each index and 1 µL template under the following conditions: 1 cycle of 98°C for 2 min, 12 cycles of 98°C for 20 s, 65°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 5 min. The resulting amplicon libraries were pooled using 5 µL from each library and cleaned up using 0.8× AMPure XP (Agencourt Beckman Coulter, CA) and 1 µL was run on the Bioanalyzer (Agilent Technologies, CA) for quality control and quantitation using the High Sensitivity DNA Analysis kit. The libraries containing PGMY amplicons were sequenced on the MiSeq platform (Illumina, CA) using V3 chemistry and 2 × 300 bp reads.

2.4 | Data analysis

Adaptors, primers, and low quality bases were removed from the raw sequence reads by Cutadapt Version 1.8.3.[23] Sequences longer than 50 bp after trimming were aligned to 175 HPV reference type genomes using Bowtie 2 Version 2.2.5.[24] Aligned reads were counted using

FeatureCounts from the Subread package Version 1.4.6.[25] A minimum of 100 sequence pairs mapped to a HPV genotype was used as a HPV positivity cutoff. All data were entered and analyzed on STATA 13.0 for calculations of prevalence, frequencies, and associations. A Chi-square Fishers test was used to test for association between two categorical variables where necessary to do so. Odds ratios were also calculated to compare HPV-positive patients with and without HIV.

3 | RESULTS

3.1 | Demographic data of study subjects

A total of 144 women were enrolled into the study. The age of the women ranged from 18 to 83 years with a mean and SD of 39.9 ± 11.0 years. Demographic data and clinical history of the participants are summarized in Table 1.

3.2 | Genotypic diversity of HPV

In this study, diverse genotypes of anogenital HPV were detected. Thirty-four and 30 different genotypes were detected in CCAS and VS, respectively. In all the HPV-positive swabs, the most common genotypes were 18 (21%), 52 (21%), and 16 (19%). The prevalence of HPV genotypes in vaginal swabs was 72% (104/144) of which the highest proportion (36%) was observed in women aged 26-35 years. The most common vaginal HPV genotypes were 18 (24%), 52 (23%), and 16 (16%) (Fig. 1). The prevalence of anal HPV genotypes was 48% (69/144), of which the highest proportion (38%) was observed in women aged 26-35 years (Table 2). The most common anal HPV genotypes were 52 (19%), 18 (17%), and 16 (16%) (Fig. 1). Women aged 26-35 and 36-45 years had the highest HR-HPV genotypes on both vaginal and anal sites (Table 2). In this age group, the 9-valent vaccine genotypes 16, 18, 52, and 58 are the most detected on both vaginal and anal sites. HPV 6 is also ranked among the top four most common genotypes on the vaginal site. Amongst the HPV-positive samples, 60% of VS and 62% of CCAS had at least two HPV genotypes (Table 3). The highest number in one person was eight genotypes in a VS sample and 10 genotypes in one CCAS sample. All 9-valent HPV genotypes were detected, with HPV types 31 and 33 giving the lowest prevalence (Table 4). Only HPV genotypes 45 and 62 showed significant association in their detection in both anal and vaginal samples ($P = 0.002$ and $P = 0.004$, respectively).

3.3 | HPV genotypes and HIV infection

The prevalence of HIV infection in the study subjects was 49% (70/144). HPV and HIV infections were associated. The odds of being HPV-positive among the HIV-infected were higher than in their HIV-negative counterparts; (CCAS OR = 4.8; CI 2.4-9.8, $P < 0.001$) and (VS OR = 2.9; CI 1.3-6.4, $P = 0.005$). Vaginal HPVs 53 ($P = 0.004$), 62 ($P = 0.036$), 89 ($P = 0.028$), and anal HPV 69 (0.029) had significant association with HIV status.

TABLE 1 Demographic data, social, and clinical history of participants

Variables	Outcome
Age in years, mean (SD)	39.9 (11.0)
Sexual debut, mean (SD)	20.1 (3.6)
Parity, median (Interquartile range (IQR))	3 (0-7)
Education level, *n* (%)	
Primary	45 (31)
Secondary	71 (49)
Tertiary	28 (19)
Marital status	
Co-habiting	2 (1)
Divorced	15 (10)
Married	94 (65)
Single	6 (4)
Widowed	27 (19)
Residence, *n* (%)	
Rural	26 (19)
Urban	113 (81)
Clinical and social history variables	Outcome
Use of vaginal herbs, *n* (%)	3 (2)
With multiple sex partners at the time of recruitment, *n* (%)	9 (6)
Spouse has multiple sex partners at the time of recruitment, *n* (%)	18 (13)
Partner circumcised, *n* (%)	
Yes	27 (19)
No	82 (57)
Did not know or did not answer	35 (24)
History of STI, *n* (%)	48 (34)
Use of contraception, *n* (%)	
Yes	81 (57)
Contraception method, *n* (%)	
Condom	17 (21)
Oral tablets	15 (18)
Intra muscular injection (Depo provera)	11 (14)
Implant (Jadelle)	17 (21)
Other	21 (26)
HIV positive	70 (49)
On Anti Retroviral Therapy (ART), *n* (%)	65 (94)
CD4 count (cells/mL), median (IQR)	194 (10-700)
Duration on ART (months), median (IQR)	39 (18-72)
Pap smear results	*n* (%)
Negative for Intraepithelial Lesion or Malignancy (NILM)	101 (70)
Not satisfactory for evaluation	9 (6)
Atypical squamous cells of undetermined significance (ASCUS)	6 (4)
Atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H)	4 (3)

(Continues)

TABLE 1 (Continued)

Variables	Outcome
Low-grade squamous intraepithelial lesion (LSIL)	6 (4)
High-grade squamous intraepithelial lesion (HSIL)	14 (10)
HSIL with invasion	4 (3)

4 | DISCUSSION

Human papillomaviruses are very diverse viruses that can infect the anogenital compartments of humans. In this study, the genotypic diversity of HPVs was investigated from two anatomical sites (vaginal and anal) in women reporting for routine cervical cancer screening at Parirenyatwa Hospital in Harare in Zimbabwe. Seventy percent of these women had normal cytology and 49% were HIV-positive. Cervico-vaginal HPV prevalence in this cohort was high (72%) in comparison to a previous study carried out in HIV-negative women from Zimbabwe with a prevalence of 25%.[26] In 2005, a Zimbabwean study reported an HPV prevalence of 43% in women with normal and abnormal cytology outcomes.[27] Literature shows that prevalence of HPV in women with normal cytology is higher in resource limited settings than in more developed parts of the world, averaging 24% in Africa.[10] This is largely associated with the high prevalence of HIV in developing countries.[28] Some studies have detected HPV DNA prevalence as high as 83% and 90% (cervical and anal, respectively).[29] The high prevalence of HPV DNA in the current study is largely attributed to HIV co-infection, because half the study population was HIV infected.

In this study, HPV genotypic frequencies were stratified into five age groups. Women who were 26-35 years had the most HPV infections in both vaginal and anal sites. Most studies record the highest prevalence of HPV infections among 18-25 years age group. The high proportion of HPV positive women shown in mid-adulthood is tempered by the sample size and age distribution of this group of women. Most women were aged 26-45 and there were only 9/144 (6%) women aged 18-25 years.

This study is the first to detect diverse HPV genotypes from different anatomical regions in women from Zimbabwe, reporting HPVs 18 and 52 as the most common genotypes in the vaginal and anal samples, respectively. The three most common HPV genotypes in both anatomical sites were the same (HPVs 18, 52, and 16), differing in proportion only. Previous studies in Zimbabwe have looked at cervical HPV only. In 2008, Hill and colleagues reported 16/94 (17%) women (with HIV/HPV co-infection) infected with HPV 16 and 18.[30] In 2010, another study in Zimbabwe reported HPV 58, 16, 70, and 18 as the most common genotypes.[26] In a broad worldwide meta-analysis of HPV genotypes in women with normal cytology, HPV types 16, 18, 31, 52, and 58 were the most commonly detected. Inasmuch as the methods used in these studies were not next-generation-sequencing, molecular based assays such as

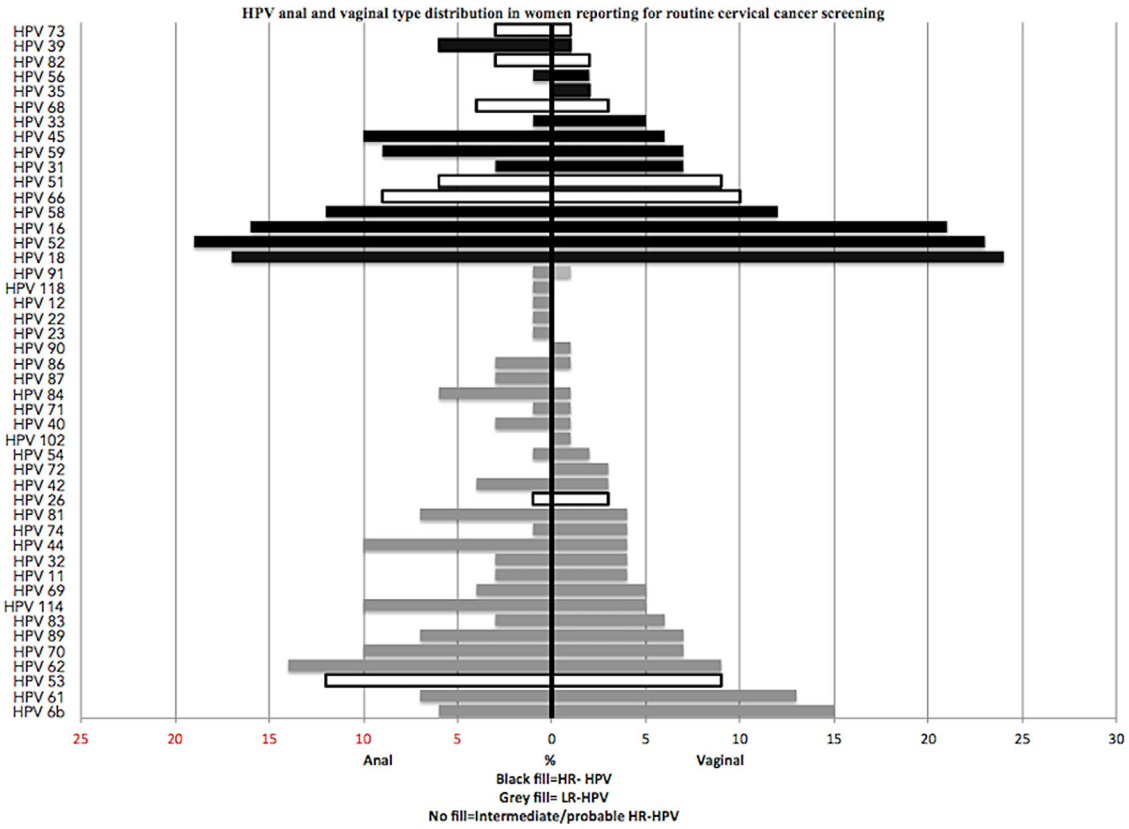

FIGURE 1 Prevalence of HPV DNA detected in Cervico-vaginal and anal samples from women reporting for routine cancer screening. Black bars represent HR-HPV, grey bars represent LR-HPV and the clear bars represent the intermediate (probable HR-HPV) genotypes as described in literature.[39,40] The left side is prevalence for the anal samples (CCAS), while the right-hand side is for vaginal samples (VS).

TABLE 2 Proportions of HPV and HR-HPV positive samples stratified on age for both sample types

	HPV positive		HR-HPV positive	
Age category (years)	VS (*n* = 104) (%)	CCAS (*n* = 69) (%)	VS (*n* = 44) (%)	CCAS (*n* = 28) (%)
18-25	8	6	5	11
26-35	36	38	41	43
36-45	35	33	41	36
46-65	19	23	14	11
>65	3	0	0	0

VS, self-collected vaginal swabs; CCAS, clinician-collected anal swabs.

polymerase chain reaction and Hybrid Capture were used.[11] The top three HPV genotypes detected in the current study (16, 18, and 52) agree with those detected in the meta-analysis. Other studies have reported HPV 16 and 18 to be lower in women with normal cytology and higher in pre-neoplastic and in cancer cases.[10] A study carried out in Uganda reported HPV 16 as the most frequent genotype, followed by HPV 33, 35, 45, and 58 in descending order.[31] HPV 52 is not commonly detected in America and Europe. However HPV 52 and 58 have been the most prevalent in HIV-positive women with high-grade squamous intraepithelial lesions in Zambia.[32] HPV 58 is also mostly reported from Asia.[33,34] Knowing the genotype-specific HPV prevalence is crucial for the choice of HPV vaccine. The nanovalent vaccine covers the most common high-risk genotypes detected in this study. Although substantially high coverage is still achieved by a vaccine based on 6, 11, 16, and 18 (the quadrivalent vaccine), it is important to note how much more a 9-valent vaccine may be able to offer for this group of women.

This is the first study to document anal HPV DNA prevalence among women in Zimbabwe. This has been a neglected anatomical region because of social and cultural factors in Zimbabwe. One limitation however, is that the association of anal HPV DNA prevalence to practice of anal intercourse could not be demonstrated because these women were not asked questions directly linked to practicing anal intercourse. Nevertheless, literature supports that with vaginal intercourse only, anatomic spread to the peri-anal canal is possible. Globally, most studies on anal HPV focus on men-having-sex-with-men (MSM). Although anal cancers are rare, 90-93% of the cases are attributed to HPV infection.[35] Our study reported anal HPV genotypic prevalence of 48%. An American study detected HPV DNA in 76% and 42% of HIV-positive and HIV-negative women, respectively.[17]

Multiple HPV genotypic infections were commonly found in the study population, especially for HIV-infected women. Multiple HPV infections are associated with a higher rate of persistent HPV infection than single infections.[36,37] Also, persons infected with multiple HPV genotypes types are more likely to have larger tumors and a poorer response to cancer treatment when compared with their counterparts with single HPV genotype infections.[38] HIV-infected immunocompromised individuals commonly have a higher prevalence of HPV infection and tend to have a higher frequency of persistent HPV infections that progress to cancer faster than in HIV-negative women.[17] Cancer prevention and treatment programs may need to accommodate the issue of multiple HPV infections and HIV status, as this population may need more frequent screening schedules than those with a low HIV prevalence.

Primer competition in multiple HPV genotype infected samples may potentially lower the sensitivity of detecting mixed infections. This may occur specifically when one genotype has substantially lower titer than the other. One hundred sequence pair cutoff was chosen as a trade-off between reducing false positive genotyping results and detection of low titer HPV that are likely not clinically relevant.

In general, the sample size limits the extent of stratification of these results. More information on concordance of HPV genotypes in relation to anatomical site, cervical cytology outcome, and HIV status would have been valuable. Although, the uniqueness of this study is largely on having HPV DNA tested on two anatomical sites, very few individuals with paired genotypes were observed. Concordance of HPV 45 and 62 is probably an under representation of the larger population. Prospective studies with larger sample size are recommended.

TABLE 3 Multiple HPV infection among vaginal and anal samples

	VS (*n* = 104)	CCAS (*n* = 69)
Num of HPV genotypes	Freq. *n* (%)	Freq. *n* (%)
1	41 (39%)	26 (38%)
2	30 (29%)	20 (29%)
3	14 (13%)	10 (14%)
≥4	19 (18%)	13 (19%)

VS, self-collected vaginal swabs; CCAS, clinician-collected anal swabs.

TABLE 4 Prevalence of nonavalent vaccine HPV genotypes detected in the study population

HPV genotype	Positive VS (*n* = 104) (%)	Positive CCAS (*n* = 69) (%)
6	15	6
11	4	3
16	21	16
18	24	17
31	7	3
33	5	2
45	6	10
52	23	19
58	12	12

VS, self-collected vaginal swabs; CCAS, clinician-collected anal swabs.

5 | CONCLUSION

HPV genotypes were diverse in this group of women in both the cervico-vaginal tract and the anus, with significantly higher prevalence in the HIV-infected compared with HIV-uninfected women. These data will assist the Zimbabwe Ministry of Health and Child Care to formulate a policy for HPV vaccination and screening of anogenital cancers. We recommend more studies to be carried out on anogenital HPVs other than in the cervix in both women and men, particularly those who are HIV infected.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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