Original Research Article

Triaging women with prior ASCUS Liquid Based Cytology (LBC) results with Hr-HPV DNA testing in Harare, Zimbabwe.

ABSTRACT

Background: ASCUS is a borderline diagnostic category that leaves clinicians with a dilemma regarding how to manage the patient. Triaging women with prior ASCUS results with Hr-HPV testing helps to distinguish those with true neoplastic lesions from those with reactive epithelial lesions. This distinction helps to inform the best clinical management strategy.

Objectives: (1) To determine the proportion of LBC samples with prior ASCUS results that were positive for Hr-HPV. (2) To determine the Hr-HPV genotypes commonly detected in ASCUS LBC samples. (3) To determine an association between the detection of Hr-HPV and the presence of histology dysplasia in the follow up cervical biopsies.

Materials and Methods: This was a cross section descriptive study done at Cimas Medical Laboratories and KAVI molecular laboratory from June 2020 to March 2021. The study subjects were women with prior ASCUS cytology results. Hr-HPV testing was done using the HPV Genotypes 14 Real-TM Quant test kit. Follow up cervical biopsies were processed in a Citadel 2000 tissue processor and stained using the manual H&E staining protocol. The histology slides were reported by two independent pathologists. Discrepant findings were referred to a third pathologist who acted as a tie breaker. A Chi-square test was used to determine an association between the detection of Hr-HPV and the presence of histology dysplasia. A p-value <0.05 was regarded as statistically significant.

Results: The mean (SD) age of the study participants was 36.3 (8.5) years. Of the 109 tested LBC samples, 65.1% [71/109] were Hr-HPV positive and 34.9 [38/109] were Hr-HPV negative. The mean ages' of the HR-HPV+ and HR-HPV- patients were comparable (37.1 years vs. 35.9 years, respectively; p=0.526). Of the 71 Hr-HPV positive samples, 8.5 % [6/71] had multiple HPV genotypes detected. Therefore, a total of 78 Hr-HPV genotypes were detected from the 71 samples. HPV 52 was the most frequent; contributed 24.4% [19/78] of all the detected genotypes. Ten of the 17 (58.9%) cervical biopsies analyzed had a ≥LSIL diagnosis. The association between the detection of Hr-HPV and dysplasia on the cervical biopsy was statistically significant p=0.03.

Conclusions: The majority (65.1%) of women with prior ASCUS results were positive for Hr-HPV. HPV 52 is the most frequently detected Hr-HPV genotype. A strong association between the detection of Hr-

HPV and dysplasia on the cervical biopsy was noted in this study.

Recommendation: Triaging patients with prior ASCUS results with Hr-HPV DNA testing is useful in stratifying patients according to the risk of harboring more serious cervical lesions.

Keywords: *Human Papillomavirus, Liquid Based Cytology, Cervical Cancer, Atypical Squamous Cells of Undetermined Significance.*

Abbreviations: ***LBC:*** *Liquid Based Cytology*, ***HPV:*** *Human Papillomavirus*, ***ASCUS:*** *Atypical Squamous Cells of Undetermined Significance*, ***DNA:*** *Deoxyribonucleic Acid*, ***ASCCP:*** *American Society of Colposcopy and Cervical Pathology.*

1. INTRODUCTION

Human Papillomavirus is responsible for the majority (99.7%) of cervical cancer cases, the most common malignant neoplasm in Zimbabwe (1). The Pap smear has played a vast role in reducing the incidence of cervical cancer cases over the past six decades (2). Historically, several Pap smear reporting systems such as the dysplasia nomenclature, Pap Class nomenclature and the CIN nomenclature were used in different institutions and countries (3). This brought a barrier of communication and reproducibility of the results in the different institutions (3).

The Bethesda System for reporting cervical cytology was formulated in December 1988 to introduce uniform terminology in the reporting of cervical smears worldwide (3). This brought an end to multiple diagnostic systems previously used in different institutions and countries (3). This enhanced communication, reproducibility of results, and referral of patients between different countries and continents (4).

The Bethesda system has several diagnostic categories which can be summarized as Negative for Intraepithelial Lesion (NILM), Atypical Squamous Cells of undetermined Significance (ASCUS), Atypical Squamous cells-HSIL cannot be excluded (ASC-H), Atypical Glandular Cells (AGC), Squamous Intraepithelial Lesions (SIL's) and glandular lesions (3). SIL's can be further classified into Low Grade Squamous Intraepithelial Lesions (LSIL), High Grade Squamous Intraepithelial Lesions (HSIL) or Squamous cell carcinoma (3).Glandular lesions on the other hand, can be further classified into: Adenocarcinoma in Situ (AIS), endocervical adenocarcinoma and endometrial adenocarcinoma (5).

The Atypical Squamous Cells of Undetermined Significance (ASCUS) is a diagnostic category of uncertainty (3). ASCUS is a borderline lesion whose differential diagnosis includes an exuberant reactive lesion and a true neoplastic lesion (6). An ASCUS diagnosis gives clinicians a dilemma regarding the nature of the lesion and the appropriate clinical management strategy (5). Knowing the nature of the lesion helps clinicians to decide on the appropriate management strategy (3).

An ASCUS interpretation is reported in about 4% of all Pap smears (7).The American Society for Colposcopy and Cervical Pathology (ASCCP) recommends follow up by either repeat testing with a Pap smear after 12months or triaging with high HPV testing to determine the nature of the lesion (non-neoplastic or neoplastic)(5). The HPV testing is the preferred follow up approach as it can inform whether the lesion is non-neoplastic or neoplastic (3). There are several high-risk HPV (Hr-HPV) genotypes; however, HPV16 and HPV18 genotypes are the most detected HPV genotypes in CIN3 or invasive cervical cancer biopsies (8). Hr-HPV DNA is detected in half of all ASCUS lesions (8) and this helps to identify patients with a higher risk of having neoplastic lesions so that they can be followed up closely (7).

The aim of this study was to perform Hr-HPV DNA testing on LBC samples with prior ASCUS results to determine their nature (neoplastic or non-neoplastic). The Hr-HPV testing helped the laboratory to issue conclusive results. This helped clinicians to decide on the most appropriate management strategy. Samples with ASCUS/HPV+ results were classified as neoplastic lesions and those with ASCUS/HPV- were classified as non-neoplastic lesions.

2. MATERIAL AND METHODS

2.1. Study design: This study was a cross-sectional descriptive study conducted from June 2020 to March 2021.

2.2. Sampling method: Consecutive sampling method.

2.3. Subjects: Women with prior ASCUS results.

2.3.1. Inclusion criteria: *Women who have never been diagnosed of cervical cancer.*

2.3.2. Exclusion criteria: *Samples with invalid Hr-HPV results.*

2.4. Study sites: Cimas Medical Laboratories and KAVI molecular laboratory.

2.5. Sample size: A total of 109 LBC samples with a prior ASCUS cytology result were evaluated for the presence of Hr-HPV DNA.

2.6. Study objectives

1. To determine the proportion of LBC samples with prior ASCUS results that were positive for Hr-HPV.
2. To determine the Hr-HPV genotypes commonly detected in ASCUS LBC samples.
3. To determine an association between the detection of Hr-HPV and the presence of histology dysplasia in the follow up cervical biopsies.

2.7. HR-HPV DNA testing

Hr-HPV DNA testing was done using the HPV Genotypes 14 Real-TM Quant test kit (Sacace Biotechnologies - 44 – 22100, Como, Italy) according to manufacturer's specifications. This is a qualitative test that detects 14 high risk HPV types : HPV 16,18, 31,33,35,39,45, 51,52,56,58,59,66 and 68.

2.8. Histology sample processing

The formalin fixed tissues were processed in a Citadel 2000 tissue processor (Thermo Scientific- Leicestershire, LE115RG, UK). The tissue samples were then embedded using a TBS embedding machine (Cole-Parmer Scientific Experts - Vernon Hills, IL60061, USA) and sectioned using a ME+ Finesse Microtome (Thermo Scientific- Leicestershire, LE115RG, UK) . During sample processing, SOPs were adhered to and machines used had current service records. The slides were stained using the manual Haematoxylin and Eosin staining protocol. The slides were reported by two pathologists. Discrepant findings were referred to a third pathologist who acted as a tie breaker.

2.9. Data management

The data was analyzed using SPSS version 25. All statistical tests were performed at 5% level of significance. The mean ages' of the HR-HPV+ and HR-HPV- patients were compared using the independent t test. A Chi-square test was used to determine an association between the detection of Hr-HPV and the presence of histology dysplasia. Descriptive statistics were presented as proportions, percentages, tables and charts.

2.10. Ethical approval

Ethical approval was obtained from the Joint Research Ethical Committee of University of Zimbabwe and Parirenyatwa Hospital (JREC), certificate number: JREC 124/2020. Permission was also granted by Cimas Medical Laboratories.

3. RESULTS AND DISCUSSION

During the study period from June 2020 to March 2021, a total of 3571 women were screened for cervical cancer using LBC at Cimas Medical Laboratories. One hundred and nine (n=109, 3.1%) of the women had ASCUS results. Hr-HPV DNA testing was performed serially on the residual LBC samples.

3.1. Age characteristics of the study participants

The women from whom the LBC samples were collected had age characteristics that are illustrated in Table 1 below:

Table 1: Age characteristics of the study participants

Age variable	Years
Mean age	36.3
Age SD	8.5
Age range	28-83
Age mode	41

3.2. Hr-HPV DNA findings in ASCUS women

Of the 109 tested LBC samples, 65.1% [71/109] were Hr-HPV positive and 34.9 [38/109] were Hr-HPV negative. The mean ages' of the HR-HPV+ and HR-HPV- patients were comparable (37.1 years vs. 35.9 years, respectively; *p*=0.526). The other results are summarized in figure 1 below:

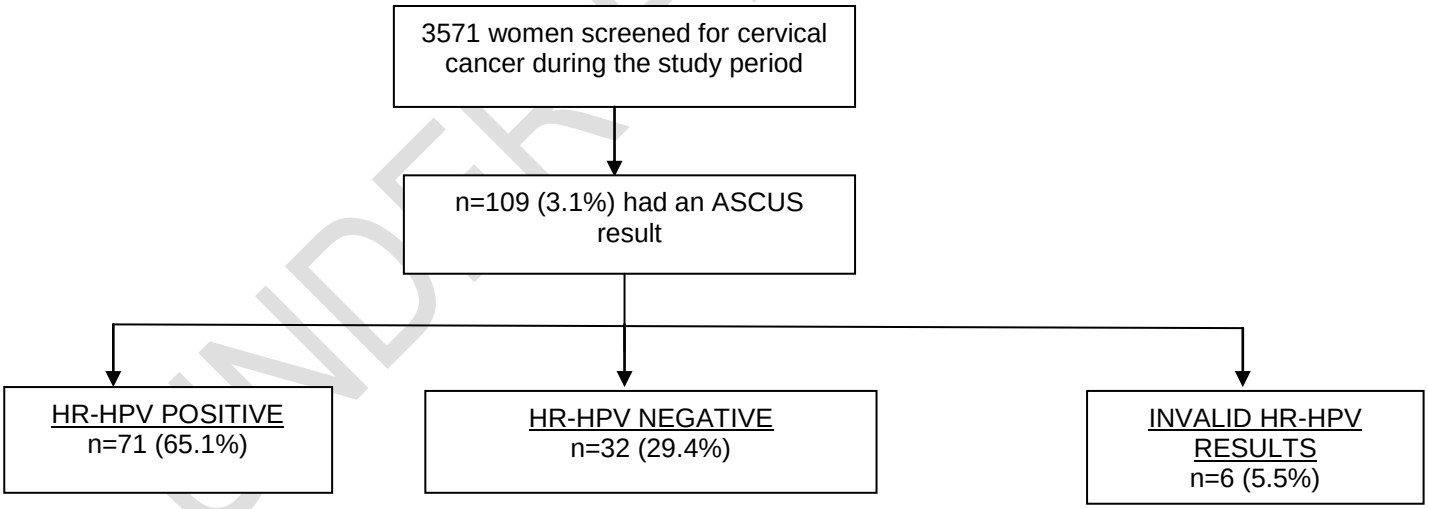

Figure 1: HR-HPV DNA findings in ASCUS women

3.3. Number of HPV genotypes detected per sample.

Of the 71 Hr-HPV positive samples, 8.5 % [6/71] had multiple HPV genotypes detected from their samples. Therefore, a total of 78 Hr-HPV genotypes were detected from the 71 samples. The number of HPV subtypes detected per sample are summarized the table 2 below.

Table 2: Number of HPV subtypes detected per sample

Number of subtypes	Number of samples	Relative frequency (%) (n=71)
1	65	91.5
2	5	7.1
3	1	1.4
Total	71	100.0

3.4. Hr-HPV genotypes detected from ASCUS LBC samples.

A total of 78 HR-HPV genotypes were detected from the 71 samples. HPV 52 was the most frequent; contributed 24.4% [19/78] of all the detected genotypes. The other detected genotypes are summarized in Table 3 below.

Table 3: Frequencies of Hr- HPV subtypes detected from samples with ASCUS results

HR-HPV genotype	Number of subtypes	Relative frequency (%) (n=78)
16	7	9.0
18	9	11.5
31	2	2.6
33	1	1.3
35	11	14.1
39	1	1.3
45	4	5.1
51	2	2.6
52	19	24.4
56	3	3.8
58	7	9.0
59	1	1.3
66	5	6.4
68	6	7.7

3.5. Histology findings from biopsies taken

Of the 109 women with ASCUS results, 17 (15.6%) had cervical biopsies taken. Of the 17; 10 had prior Hr-HPV positive results, 6 had Hr-HPV negative results and 1 had an invalid results (this had a LSIL histology result on biopsy). The cervical biopsy results are illustrated in Table 4 below:

Table 4: Cervical biopsy findings

Histology diagnosis	Number	Relative frequency (%)
Normal	3	18.8
Chronic cervicitis	4	6.3
LSIL	9	68.8
HSIL	1	6.3

3.6. Association between the detection of Hr-HPV and dysplasia on cervical biopsy (≥LSIL).

Sixteen cases with known histology and Hr-HPV results were used to evaluate the presence of an association between the detection of Hr-HPV and dysplasia on the cervical biopsy. The association was statistically significant *p*=0.03. The results are summarized in table 5 below.

Table 5: Association between the presence of HR-HPV and dysplasia on cervical biopsies.

	Histology results		df =1 Pearson's value = 4.82	*p* value = 0.03
	Non neoplastic	Neoplastic (≥LSIL)		
HR-HPV -	5	1		
HR-HPV +	2	8		

3.4. Discussion

Atypical Squamous Cells of Undetermined Significance (ASCUS) account for about 5-7% of all cervical cancer reports (10). ASCUS is a diagnosis of uncertainty that leaves clinicians with a dilemma of how to manage the patient (7). Triaging such cases with Hr-HPV helps to distinguish those with true neoplastic lesions from those with reactive epithelial lesions (4). This study tested residual LBC samples with prior ASCUS results for the presence or absence of Hr-HPV in order to issue conclusive laboratory reports to clinicians. The majority (65.1%) of the LBC samples were positive for Hr-HPV.

The Hr-HPV positivity rate in this study (65.1%) was higher than the 48.4% reported by Wong et al and the 41% reported by Khunamornpong et al (7,11). This may have been attributed by a higher threshold of diagnosing ASCUS in this study. This resulted in the correct cytological classification of most of the lesion as abnormal. The HPV positivity rate was however, comparable to the 59% reported by Evans et al (9). The 29.4% who were Hr-HPV negative in this study were able to avoid unnecessary colposcopy. The figure was slightly lower than the 44% reported by Kim et al (12).

In a study by Castle et al, the baseline prevalence of HPV 16 in women with ASCUS was 14.9% (13). This was considerably higher than the 9% we discovered in this study. In addition, in a study by Wong et al, HPV 16 and 18 contributed 38.4% of all Hr-HPV genotypes (7). This was significantly higher than the 20.5% we realized in this study. This may partly be explained by the different Hr-HPV genotypes that cause cervical neoplasia in different countries (14). In a study done by Fitzpatrick et al in Zimbabwean women, HPV 52 was reported to be the most frequent high risk HPV genotype (14). These findings are consistent with findings of this study where HPV 52 was the most detected Hr-HPV genotype. However, it is noteworthy to state that when we characterised the Hr-HPV persistence patterns in Zimbabwean women in another study, HPV 16 was the most persistent Hr-HPV genotype (15).

In this study, 32 (29.4%) specimens had ASCUS/ Hr-HPV negative result combinations. This can be caused by exuberant reactive lesions which were incorrectly classified as ASCUS lesions (4). However, this may have been caused by a false negative HPV result (4). Catteau et al demonstrated that such false negative results may be due to low volumes of the Preserv Cyt solution (16). Quiroga –Garza et al also reported that the ASCUS/HPV- discrepant finding could be due to rare HPV subtypes such as HPV 90 which are not available on current commercial kits (17)**.** A few (n=6, 5.5%) of the samples had invalid results. This can be caused by improper samples preservation (4). One common similarity on all the samples that had invalid results was a bloody macroscopic appearance. This may have contributed to the invalid result. However, more studies are needed to validate this claim.

Of the 17 samples that had cervical biopsies taken, 10 (58.8%) had neoplastic changes (≥LSIL). This is slightly higher than the 41.4% recorded by Khunamornpong et al (11). The higher figure in our study can be attributed to the inability to classify the ASCUS lesions into either LSIL or HSIL on the Pap smear with certainty. This may be due to sampling errors that may result in a few cells showing definite features of LSIL or HSIL causing definite classification difficult (4).

4. CONCLUSIONS: The majority (65.1%) of women with prior ASCUS results was positive for Hr-HPV. HPV 52 was the most frequently detected Hr-HPV genotype. A strong association between the detection of Hr-HPV and dysplasia on the cervical biopsy was noted in this study.

5. RECOMMENDATION: Triaging patients with ASCUS results with Hr-HPV DNA testing is useful in stratifying patients according to the risk of harboring more serious cervical lesions.

ETHICAL APPROVAL

Ethical approval was obtained from the Joint Research Ethical Committee of University of Zimbabwe and Parirenyatwa Hospital (JREC), certificate number: JREC 124/2020. Permission was also granted by Cimas Medical Laboratories. During the study, strict patient confidentiality was observed. Cervical sample collection is a safe procedure. However, minor complications such as mild bleeding may be encountered in patients with cervicitis. Such spotting is usually self limiting and usually ends on its own in a few hours.

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