

HPV L1 capsid protein detection in high-risk human papillomavirus-positive cervical smears

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Abstract

Background: The L1 capsid protein is a viral nuclear protein that encapsidates the human papillomavirus (HPV) DNA to build new infectious particles. Previous studies in immune-competent patients have shown that detectable L1 protein in low-grade squamous intraepithelial lesion (LSIL) smears is associated with remission in 60-75% of cases. Thus, the test can reduce interventions by approximately 75%.

Objective: This study was performed to evaluate the use of HPV L1 capsid protein detection on cytology samples in a population with relatively high human immunodeficiency virus (HIV) prevalence and with known high-risk HPV (hrHPV) DNA results.

Setting: Samples were obtained during a cervical cancer screening study at primary healthcare clinics in the Tshwane district, Gauteng. The HIV prevalence of the target group was estimated to be between 20% and 30%.

Method: Conventional cervical cytology smears of 575 women were microscopically assessed and diagnosed. In addition, women were tested for the presence of HPV DNA on a cervical or vaginal sample. Immunocytochemical analysis was performed on morphologically abnormal smears and on a 52 control group smears reported to be negative for HPV DNA and morphological abnormalities. The detection of L1 capsid protein was carried out with the Cytoactiv[®] HPV L1 screening set, an antibody-based immunocytochemical stain.

Results: A cytological diagnosis of LSIL was made in 19 women (3.3%), high-grade squamous intraepithelial lesions (HSILs) in 42 (7.5%) and malignancy in 1 (0.2%). Of the LSIL cases, 10 of 19 (52.6%) stained positive for the presence of the L1 protein, while only one of 42 HSIL (2.4%) cases stained positive. Three hundred and four cases (52.9%) tested positive for hrHPV DNA, including women with LSIL, HSIL and malignancy. All of the control cases stained negative for the L1 capsid protein.

Conclusion: hrHPV was not a useful triage test for women with abnormal cellular morphology in this population. Promising results were reported for Cytoactiv[®] immunostaining as a triage test for patients with LSIL, but it added no value to samples known to be HPV-negative or reported to be morphologically negative. The immunostaining of smears reported to be HSIL or worse were almost universally negative, supporting the diagnostic accuracy of cytology and the expectation of persistence or progression of these lesions.

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Introduction

The current prevalence of cervical precursor lesions in South Africa is not precisely known, but data from published studies suggest an increase in the incidence of abnormalities diagnosed on cytology.¹⁻³ Richter et al⁴ recently reported abnormal cytology results in 17% of 1 472 women screened

in an urban setting in South Africa, a prevalence that is higher than that described in the general population previously. This increase is attributed to a higher prevalence of infection with oncogenic human papillomavirus (HPV), a DNA virus that causes cervical carcinoma.⁵ HPV types are classified according to oncogenic potential into high-, intermediate- and low-risk categories.⁶

A higher prevalence of HPV infection, precursor lesions and cervical cancer in human immunodeficiency virus (HIV)-positive women has been demonstrated by numerous studies.⁷⁻⁹ In 2008, the national estimate of HIV prevalence in South Africans of all age groups and gender was 10.6%, and an average of 19.7% was reported for adult women aged 20 years and older. Peak prevalence of 32.7% was reported in women aged 25- 29 years.¹⁰

Screening by cytology contributes to a large number of patients in need of treatment and follow-up care, with healthcare and financial implications.^{11,12} While most high-grade squamous intraepithelial lesions (HSILs) persist or progress, a large number of low-grade squamous intraepithelial lesions (LSILs) regress, but cellular morphology cannot reliably differentiate between these groups.¹³ The search for new prognostic markers, to be used in addition to morphology, led to the development of immunocytology stains that showed great promise in initial studies that were mostly conducted in the developed world.¹⁴ An immunocytochemical stain for the L1 capsid protein of HPV DNA is one such marker. This nuclear protein encapsidates the HPV viral DNA to build new infectious particles, and is a major target of the human immune response.¹⁵

In 2003, Melsheimer et al showed that the majority of high-risk HPV (hrHPV)-associated LSILs express the HPV L1 capsid protein, but that the protein is absent in the majority of hrHPV-associated HSILs.¹⁵ They suggested that the loss of viral L1 capsid protein in HPV-infected HSILs may be used as a prognostic marker to predict the progression or regression of cervical lesions.^{15,16} Numerous studies have been conducted since then, which have confirmed a correlation with the progression of the lesions. In 2004, Griesser et al found that detectable L1 protein in LSIL smears was associated with remission rates of 76.4%, and in 2009, a follow-up study by the same group showed a remission rate of 66%.¹⁷ Sarmadi et al reported similar findings in 2011, with a slightly lower regression rate of 60.17% in L1-positive lesions.¹⁸

The rate of regression in HPV-infected cases with cytological abnormalities is influenced by many factors, including the immune status of the individual, nutrition, the time of exposure to HPV and the viral subtype. Thus, the potential value of the L1 capsid protein immunocytochemical stain needs evaluation in settings where these factors, including HIV prevalence, are different from the countries where it was initially tested. Firstly, the prevalence of L1-positive stains in LSILs needs to be determined, and then the regression rate in those with positive staining studied.

Objective

This study was performed to evaluate the use of HPV L1 capsid protein detection on cervical cytology samples in a population with a relatively high HIV prevalence. In addition, observations were made to determine if the L1 capsid protein correlated with the results of HPV DNA analysis, in

order to evaluate the use of this immunostaining on patients screened with HPV tests.

Setting

Samples were obtained from a population-based cervical cancer screening study carried out in primary healthcare clinics in the Tshwane or Pretoria district, Gauteng. Women were recruited to this project at their local clinics, and also became part of the national screening programme. The target population of this screening study included women eligible for cervical screening according to government policy at the time. The method and main results of this study have previously been reported.^{4,19,20} Based on the wide age range, the estimated HIV prevalence of this group was between 19% and 33%.¹⁰

Method

After obtaining informed consent, cervical cytology was collected using an Aylesbury® wooden spatula. Material was smeared onto two glass slides and fixed with an aerosol fixative containing 96% ethanol. Clinical management during this study was based on cytology results as per national protocol. Women with LSIL are followed-up after one year. Those diagnosed with HSIL or worse are referred for further investigation, and are usually seen and treated at the same visit.

In addition to cytology, vaginal or cervical material was collected for DNA analysis, using either a dry swab or by tampon collection. Cervical swabs were taken at three clinics after specimens were collected for cytology. Two other clinics used self-collection with a tampon inserted by the participant at least one hour before the cytology specimens were taken.

The current study consisted of 575 participants, with an age range of 23-83 years. Cytology results, HPV DNA status (positive or negative) and HPV risk group assessment were obtained for all participants. Immunocytochemistry was carried out on 114 participants, selected according to the cytology results, for the detection of the L1 capsid protein. Sixty-two morphologically abnormal slides were selected, irrespective of age, HPV or HIV status, or any possible related symptoms. Fifty-two cases with normal cytology were included as a control group.

One glass slide per participant was stained routinely with regressive Papanicolaou stain. Screening was carried out using the national protocol, and reports formulated according to the Bethesda system of reporting. Cervical swabs were transported and stored dry, while tampons were placed in phosphate-buffered saline and 10% methanol solution directly after collection. These specimens were submitted for DNA extraction and HPV testing and typing, as previously reported.⁴

The HPV risk groups were defined as described by Munoz et al:

- *hrHPV*: 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 68 and 82.
- *Probable hrHPV*: 26, 53 and 66.
- *Low-risk HPV (lrHPV)*: 6, 11, 42, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81, 83 and 84.⁶

Participants were assigned to the highest risk group from which they tested positive for at least one viral type's DNA.

Detection of the L1 capsid protein was conducted on the additional native smear, with the Cytoactiv® HPV L1 screening set, an antibody-based immunocytochemical stain, according to the manufacturer's instructions. A positive control provided in the Cytoactiv® HPV L1 screening set showed nuclear positivity in each of the assays performed. Specimens were fixed for 20 minutes in 96% ethanol, dried at room temperature (17-27°C) and rehydrated using a degrading alcohol row (two minutes each in 96%, 70% and 50% alcohol, followed by two minutes in distilled water). The microwave method of antigen demasking was used as follows: microwaving in the citrate buffer for 3-4 minutes on maximum power, for 20 minutes on low power, cooling down for 10 minutes and washing. The washing step consisted of rinsing with buffer for one minute without a cover slip. One drop of Cytoactiv® antibody was added to each slide, enclosed with a cover slip to prevent drying out, and incubated for 30 minutes, followed by washing. One drop of detection agent was then added per slide, enclosed with a cover slip, and incubated for 10 minutes and washed. This was followed by one drop of chromogen, covering and incubation for five minutes. The same step was repeated before counterstaining with undiluted Mayer's haematoxylin for 10 seconds. Slides were immediately transferred to running tap water for 3-5 minutes; the blueing step.

Smears were enclosed with aqueous mounting medium and a large cover slip, and evaluated under a light microscope. L1 is found predominantly in the nucleus, and thus requires a nuclear stain. Staining intensity varies and depends on the amount of L1 capsid protein produced within the cell. A single-stained nucleus on a cytology slide is regarded as positive (Figure 1). Cytoplasmic staining is regarded as non-specific, as shown in Figure 2.

Results

Cellular morphology revealed no abnormality or less than a LSIL in 513 participants (89.2%), which were then considered to be negative. A cytological diagnosis of LSIL was made in 19 women (3.3%), HSIL in 42 (7.5%), and malignancy in 1 (0.2%). Valid HPV DNA test results were available for 575 participants. Of these, 304 cases tested positive for hrHPV types, 12 for probable hrPVP types, and 207 for lrHPV DNA. A small group of 52 patients tested negative for any HPV. Importantly, the prevalence of hrHPV was 52.9%, and all specimens reported to have LSIL, HSIL and malignancy were

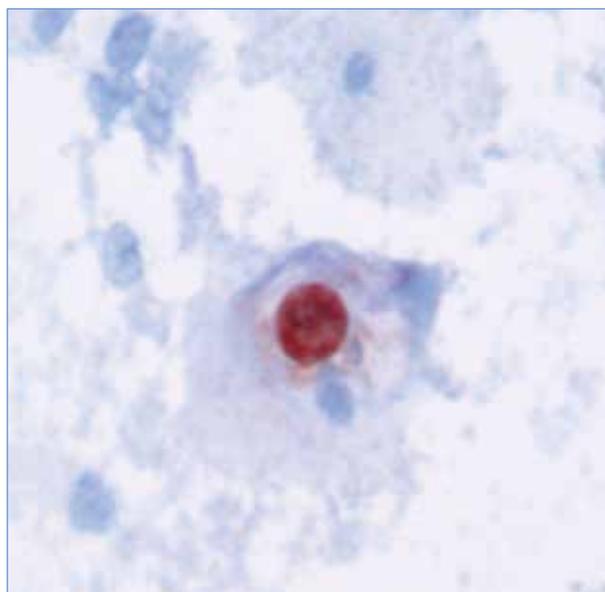


Figure 1: L1-positive low-grade squamous intraepithelial lesion (40 x)

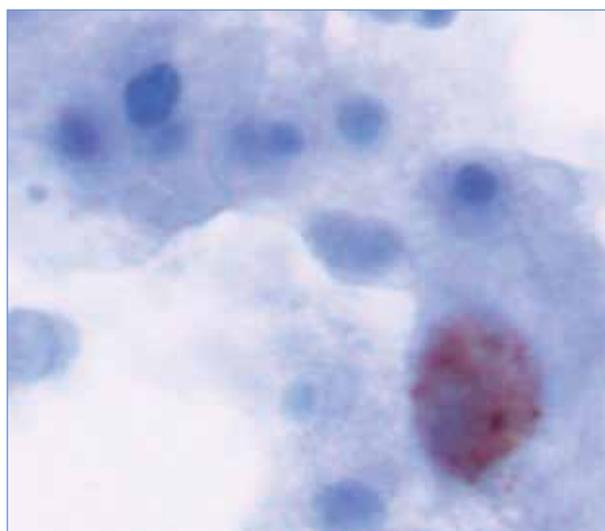


Figure 2: Inconclusive cytoplasmic staining (40 x)

also positive for hrHPV. The prevalence of current cytological abnormalities in hrHPV-positive women was 20%, while 6.3% had LSIL and 13.8% had HSIL or worse on a single round of cervical cytology. The results of cytology and HPV testing are shown in Table I.

Of the LSIL cases, 10 of 19 (52.6%) stained positive for the presence of the L1 protein, while only one of 42 HSIL (2.4%) cases stained positive, and the one case reported as malignancy did not stain for L1. All control cases stained negative for the L1 capsid protein.

Eleven specimens with hrHPV DNA were positive for L1, reflecting a good prognosis or a 60% chance of regression. Ten of these patients only had a LSIL on cytology. None of the HPV-negative specimens that were tested were positive for the L1 capsid protein. The immunocytochemistry results are highlighted in Tables II and III.

Table I: Cytology and human papillomavirus results

	Cytology-negative	LSIL	HSIL	Malignancy	Total n (%)
HPV-negative	52	0	0	0	52 (9)
IrHPV	207	0	0	0	207 (36)
Probable hrHPV	12	0	0	0	12 (2)
hrHPV or worse (%)	242 (79.6%)	19 (6.25%)	42 (13.8%)	1	304 (53)
Total (%)	513 (89.2%)	19 (3.3%)	42 (7.5%)	1 (0.2%)	575

HPV: human papillomavirus, hrHPV: high-risk human papillomavirus, HSIL: high-grade squamous intraepithelial lesion, IrHPV: low-risk human papillomavirus, LSIL: low-grade squamous intraepithelial lesion

Table II: L1 staining versus human papillomavirus risk group results

	L1-negative	L1-positive	L1 not evaluated	Total
HPV-negative	52	0	0	52
IrHPV-positive	0	0	207	207
Probable hrHPV-positive	0	0	12	12
hrHPV-positive	51	11	242	304
Total	103	11	461	575

HPV: human papillomavirus, hrHPV: high-risk human papillomavirus, HSIL: high-grade squamous intraepithelial lesion, IrHPV: low-risk human papillomavirus, LSIL: low-grade squamous intraepithelial lesion

Table III: L1 staining versus the cytology screening results

	L1-negative	L1-positive	L1 not evaluated	Total
Cytology (normal)	52	0	461	513
LSIL	9	10	0	19
HSIL	41	1	0	42
Cytology malignancy	1	0	0	1
Total	103	11	461	575

HSIL: high-grade squamous intraepithelial lesion, LSIL: low-grade squamous intraepithelial lesion

Discussion

Cytological abnormalities (LSIL and HSIL) occurred in more than 10% of women in this study. The prevalence was higher than that usually reported for the general population, but slightly less than that pertaining to the results of a larger screening study from which these participants were selected.⁴ The cytology findings are explained by the very high prevalence of HPV infection, specifically infection with high-risk HPV types. High hrHPV prevalence is described in settings with a very high background prevalence of HIV infection,⁴ assumed to be between 19% and 33% in this study population.¹⁰ It is clear that women with HSILs within this environment need treatment.

Even though HSILs are more common, LSILs still contribute to a large number of women with abnormal screen results. These numbers are expected to increase when the entry age for screening is lowered in HIV-positive women. The current protocol is to follow up these women and repeat testing, but this approach is not cost-effective, and large numbers of patients are lost during the process. Treating these women in a system that is overloaded with women

with HSILs would be difficult, would have financial implications and would lead to significant overtreatment.

Previous studies demonstrated that Cytoactiv[®] testing can reduce further testing and unneeded interventions by an estimated 60-75% in women with cytological abnormalities. Previously reported results of HPV L1 staining on specimens with cytological abnormalities are as follows: Approximately 80% slides were positive for LSIL (mild to moderate dysplasia),¹⁵ approximately 25% specimens with HSIL were positive (severe dysplasia),¹⁵ and less than 5% of slides were diagnosed as malignant.²¹

Of the women with HSIL in this trial, the prevalence of a positive L1 stain was 2.3%, in comparison to the expected 25%. These results suggest that in our population, almost all of these lesions have the potential to progress, as L1 protein expression is lost, and thus the HPV has evaded the immune system. In the current study, immunocytochemistry added no value to the prognostic evaluation and management of women with HSIL as almost all of these specimens stained negative. Similarly, L1 testing was not useful in patients who were either known to be HPV-negative, or known to have no cytological abnormality.

The L1 capsid protein staining results obtained during this study on LSIL specimens were also less favourable than results from similar studies. In the current study, 52% of LSIL smears stained positive for the L1 capsid protein, compared to the expected 80%, as reported by Griesser et al.^{17,22} Thus, it was expected that the progression rate for these lesions in our patients would be higher. Potentially, this could be a consequence of the estimated high rate of HIV infection in the target population. However, it is essential to determine the progression and regression rates by a longitudinal follow-up study to confirm these assumptions, and before large-scale implementation is contemplated.

Conclusion

All of the findings in this study support a higher prevalence of squamous intraepithelial lesions and poorer prognosis, with less regression in patients in our population. In spite of these findings, our results show some potential for the L1 capsid protein as a prognostic marker when used selectively for specimens with LSILs.

Prognostic biomarkers may be especially useful if no further specimen collection is needed and reflex testing with a triage test is available. The current pilot study was limited to the initial evaluation of this marker in a small group of specimens. The results suggest potential usefulness as a triage test for a selected group of patients to allow safe and cost-effective follow-up versus immediate treatment. This approach should be tested in a larger study, with a longitudinal design and stratified according to HIV status and age.

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References

1. Allan BR, Marais DJ, Denny L, et al. The agreement between cervical abnormalities identified by cytology and detection of high risk types of human papillomavirus. *S Afr Med J*. 2006;96(11):1186-1190.
2. Fonn S, Bloch B, Mabina M, et al. Prevalence of pre-cancerous lesions and cervical cancer in South-Africa: a multi-centre study. *S Afr Med J*. 2002;92(2):148-156.
3. Taylor S, Kuhn L, Dupree W, et al. Direct comparison of liquid-based and conventional cytology in a South African screening trial. *Int J Cancer*. 2006;118(4):957-962.
4. Richter KL, Becker PJ, Horton A, Dreyer G. Age-specific prevalence of cervical human papillomavirus infection and cytological abnormalities in women in Gauteng Province, South Africa. *S Afr Med J*. 2013;103(5):313-317.
5. Zur Hauzen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer*. 2002;2(5):342-350.
6. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 2003;348(6):518-527.
7. Ahdieh L, Munoz A, Vlahov D, et al. Cervical neoplasia and repeated positivity of human papillomavirus infection in human immunodeficiency virus-seropositive and seronegative women. *Am J Epidemiol*. 2000;151(12):1148-1157.
8. De Vuyst H, Lillo F, Broutet N, Smith JS. HIV, human papillomavirus, and cervical neoplasia and cancer in the era of highly active antiretroviral therapy. *Eur J Cancer Prev*. 2008;17(6):545-554.
9. Palefsky J. Human papillomavirus-related disease in people with HIV. *Curr Opin HIV AIDS*. 2009;4(1):52-56.
10. Shisana O, Rehle T, Simbayi LC, et al. South African national HIV prevalence, incidence, behaviour and communication survey 2008: a turning tide among teenagers? Cape Town: HSRC Press; 2009.
11. Parkin DM, Bray FL, Devesa SS. Cancer burden in the year 2000. The global picture. *Eur J Cancer*. 2001;37(Suppl 8):S4-S66.
12. Kurman RJ, Henson DE, Herst AL, et al. Interim guidelines for management of abnormal cervical cytology. *JAMA*. 1994;271(23):1866-1869.
13. Cantor SB, Atkinson EN, Cardenas-Turanzas M, et al. Natural history of cervical intraepithelial neoplasia. *Acta Cytol*. 2005;49(4):405-415.
14. Nucci MR, Castrillon DH, Bai H, et al. Biomarkers in diagnostic obstetrics and gynaecological pathology: a review. *Adv Anatom Pathol*. 2003;10(2):55-68.
15. Melsheimer P, Kaul S, Dobeck S, Bastert G. Immunocytochemical detection of human papillomavirus high risk type L1 capsid proteins in LSIL and HSIL as compared with detection of HPV L1 DNA. *Acta Cytol*. 2003;47(2):124-128.
16. Stanley M. Immune response to human papillomavirus. *Vaccine* 2006;24 Suppl 1:S16-S22.
17. Griesser H, Sander H, Walczak C, Hilfrich R. HPV vaccine protein L1 predicts disease outcome of high-risk HPV+ early squamous dysplastic lesions. *Am J Clin Pathol*. 2009;132(6):840-845.
18. Sarmadi S, Izadi-Mood N, Pournashkari M, et al. HPV L1 capsid protein expression in squamous intraepithelial lesions of cervix uteri and its relevance to disease outcome. *Arch Gynecol Obstet*. 2012;285(3):779-784.
19. Dreyer G, Mnisi E, Maphalala A. Challenges in preventative care and research in primary healthcare facilities: information obtained during implementation of a cervical cancer screening project in the Tshwane Health District. *South Afr J Gynaecol Oncol*. 2013;5(2):S10-S14.
20. Richter KL. Alternative sampling methods for cervical cancer screening: practical perspectives from the laboratory. *South Afr J Gynaecol Oncol*. 2013;5(2):S5-S9.
21. Birner P, Bachtiry B, Dreier B, et al. Signal-amplified colorimetric in situ hybridization for assessment of human papillomavirus infection in cervical lesions. *Mod Pathol* 2001;14(7):702-709.
22. Griesser H, Sander H, Hilfrich R, et al. Correlation of immunocytochemical detection of HPV L1 capsid protein in Pap smears with regression of high risk HPV positive mild/moderate dysplasia. *Anal Quant Cytol Histol*. 2004;26(5):241-245.