Human papillomavirus in normal cervical smears from Cape Town

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Objective. To determine the prevalence of human papillomavirus (HPV) types in South African women with normal cervical cytology and to determine whether our results are comparable to what has been found elsewhere in the world.

Design. Cervical smears were collected from 262 women.

Setting. The Cape Town metropolitan area.

Participants. A total of 262 women, aged 19 - 85 years. Eighty-five women attended the family planning clinic of the Gynaecology Department at Groote Schuur Hospital and 177 women consulted private gynaecological practices.

Main outcome measures. The prevalence and types of HPV in 192 women with normal cervical cytology.

Results. The incidence of HPV DNA in cervical smears from Cape Town women with normal cytology was found to be 13% (25/192) using Southern blot hybridisation. The types of HPV found in normal cervical tissue from Cape Town did not differ significantly from those found elsewhere in the world. Nine per cent (17/192) were positive for 'high-risk' HPV types which are associated with premalignant and malignant cervical lesions. In the age group 20 - 39 years, 15 of 92 (16%) were positive for HPV DNA, while in the age group above 39 years, 3 of 53 (6%) women were positive for HPV DNA.

Conclusions. In this study, 25 of 192 (13%) women with normal cytology were positive for HPV.

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Specific types of human papillomavirus (HPV) have been shown to be strongly associated with cervical intra-epithelial neoplasia (CIN) and invasive cancer.[1-3] Analysis of cervical cytology (Papanicolaou smears) can identify lesions associated with HPV infection. These include koilocytes associated with HPV infection (low-grade lesions) as well as CIN and cervical cancer (high-grade lesions).[4] However, there is increasing evidence that HPV is present in a significant number of women with normal cervical cytology.[5-16] The incidence of HPV infection detected in women varies according to the methodology used to detect HPV DNA and the population being studied. De Villiers and co-workers[7] have performed the largest study — using filter *in situ* hybridisation — to detect HPV DNA and found that 9% (806/8 755) of women with normal cytology were positive for HPV DNA. The highest incidence of HPV infection was reported by Bauer *et al.*,[11] who used the polymerase chain reaction (PCR) to amplify HPV DNA from normal cervical smears from a university population. Thirty-one per cent (130/421) of the smears were HPV-positive.

Cervical cancer is the most common cancer among women in South Africa, comprising 18.6% of all cancers.[17] In 1989, the age-standardised incidence rate was 35.4/100 000 in black women.[17] HPV was detected in 81% (55/68) of cervical carcinoma biopsies and 66% (66/98) of CIN grade 3 biopsies from Cape Town.[18,19] HPV 16 was the predominant HPV type in both these studies.

At present there is no information on the prevalence and types of HPV DNA present in cervical smears from women with normal cytology in South Africa. HPV-infected women with normal cytology show elevated progression to CIN.[3] We therefore need to determine the prevalence of HPV types in South African women with normal cervical cytology and to determine whether our results are comparable to what has been found elsewhere in the world. This study examined cervical smears from women attending either family planning clinics or private gynaecology practices in Cape Town, South Africa. The cervical smears were collected from the women consecutively, as they presented to the family planning clinics or private gynaecology practices. HPV DNA related to high-, intermediate- and low-risk HPV types was detected by Southern blot hybridisation (SBH). Low-risk HPV types are associated with low-grade squamous lesions and rarely found in high-grade lesions, intermediate-risk HPV types are found more often in high-grade squamous intra-epithelial lesions (SILs) than in cancers, and high-risk HPVs are found in both high-grade SILs and invasive cancers.[20] Some of the HPV DNA samples that could not be typed by SBH were amplified by the PCR, cloned and sequenced.

Materials and methods

Clinical specimens

Cervical smears were collected from 85 women attending the family planning clinic of the Gynaecology Department at Groote Schuur Hospital, and 177 women consulting several private gynaecology practices in the Cape Town metropolitan area. Specimens from 56 women were eliminated from the study because smears yielded insufficient DNA. Of the 206 patients remaining in the study, cytology revealed that 192 had normal cytology, 9 had atypical cytology, 4 were classified as having koilocytotic HPV infection and 1 as CIN grade 1. Smears with minor nuclear abnormalities on the borderline between those of inflammation and CIN or HPV were classified as cytologically atypical.

Ages were available for 145 of the patients with normal cytology. The age of the patients ranged from 19 to 85 years, with a mean of 38 years.

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Cytology

The cytology smears were examined either at the Groote Schuur Cytopathology Laboratory or at private cytopathology laboratories in Cape Town where the doctors were University of Cape Town-trained pathologists. The quality control measures in these laboratories included random 10% rescreening of smears and direct rescreening of all high-risk cases. The smears were examined blind by the cytotechnologists and cytopathologists in these laboratories and subjected to routine quality control. A smear was reported normal if all cellular modalities including squamous, endocervical and metaplastic cells showed no significant alteration in nuclear/cytoplasmic ratio and showed no irregularity of nuclear chromatin.

Atypical cellular changes are recognised cytologically when there is an increase in the nuclear/cytoplasmic ratio. If there is a corresponding hyperchromasia, nuclear membrane folding and chromatic clumping, the diagnosis of cervical intra-epithelial neoplasm is made. The degree of nuclear/cytoplasmic ratio disturbance and the presence or absence of syncytial cell groups influence the grading of CIN. HPV infection is diagnosed when three particular cell types are found on cervical smear. These include the koilocyte, which undergoes only a slight increase in nuclear/cytoplasmic ratio, with a crumpled hyperchromic nucleus. Classically the perinuclear cytoplasm is cleared giving a perinuclear halo. Dyskeratotic squamous and metaplastic cells are recognised and are seen as aggregates of orangeophilic cells with dense irregular hyperchromatic nuclei. Atypical immature metaplastic cells are the third cell type seen with HPV infections. These may be accompanied by bi- or multinucleate squamous cells.

Two specimens were taken with a spatula from the endo- and ectocervix. The first one was submitted for cytology and the exfoliated cells from the second specimen were resuspended in 10 ml of sterile phosphate-buffered saline (PBS) and stored at –20°C until needed for DNA extraction. No review of either methods was performed once the results were known.

DNA extraction

Half of each stored specimen was used for DNA extraction. The cervical cells in 5 ml PBS were thawed. Sodium dodecyl sulphate (SDS) and proteinase K (Boehringer Mannheim, Germany) were added to give final concentrations of 0.5% and 100 µg/ml respectively. The cell suspension was incubated at 55°C for at least 3 hours. After two extractions with phenol and one extraction with chloroform/isoamyl alcohol (24:1), the DNA was precipitated and resuspended in 50 µl TE buffer per specimen.

Preparation of HPV-specific DNA probes

Plasmids containing cloned HPV 6, HPV 11, HPV 16 and HPV 18 were provided by Dr E. M. de Villiers (German Cancer Research Centre, Heidelberg, Germany). Plasmids containing HPV 31 and HPV 35 were provided by Dr A. T. Lorincz (Bethesda Research Laboratories, Gaitersberg, Md, USA) and the plasmid containing HPV 33 was provided by Dr G. Orth (Pasteur Institute, Paris, France). HPV DNA was separated and purified from the flanking vector sequences by agarose gel electrophoresis and electro-elution with a Biotrap apparatus (Schleicher and Schuell, Germany) according to the manufacturer's instructions. The HPV DNA fragments were labelled with ^{32}P-dCTP (Amersham PLC, UK) by the random primer technique (Multipriming Kit; Amersham PLC, UK) for use in hybridisation experiments.

Southern blot hybridisation

For SBHs, 5 - 10 µg of DNA from each biopsy were digested with 20 units of *Pst* I (Boehringer Mannheim, Germany), and followed by electrophoresis through a 1% agarose gel. The agarose gels were immersed in 0.25M HCl, denatured and neutralised.[21] The DNA was transferred onto Hybond N nylon membrane (Amersham PLC, UK) and UV crosslinked. The membranes were prehybridised at 42°C overnight in a solution containing 30% (v/v) formamide, 6 X SSC (0.9M NaCl, 0.9M sodium citrate), 0.5% (w/v) fat-free milk powder (Carnation), 1% SDS and 0.2% salmon sperm DNA (Sigma, USA). The hybridisations were performed in the prehybridisation buffer at 42°C (Tm –35°C) for 18 - 24 hours. The membranes were then washed three times in 2 X SSC 0.1% SDS for 1 hour and twice in 0.5 X SSC, 0.1% SDS for 1 hour at 42°C before being subjected to autoradiography.

For re-use of blots, the probes were stripped from the membranes by boiling in 0.1% SDS according to the manufacturer's instructions (Amersham PLC, UK). Before rehybridisation to other probes, the membranes were exposed to X-ray film to confirm that the probe had been removed.

PCR amplification and cloning of PCR products

Nested primer PCR amplification of a 335 bp fragment of the HPV L1 gene was performed as described by Williamson and Rybicki.[22] The PCR-amplified fragments were cloned into pUC 18.

DNA sequencing and sequence analysis

Reagents for the DNA sequencing procedures were obtained in kit form (Sequenase, USB., USA) and used according to the manufacturer's specifications. To ensure accuracy, the sequence was verified by independent sequencing of more than one clone of the HPV DNA and sequencing of both strands of all PCR products. The sequences obtained were compared with other HPV sequences at the National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) network service.[23] Further alignments were done using Genepro (Riverside Scientific Enterprises).

Results

Detection of HPV by SBH

Southern blots of *Pst* 1-digested DNA from each of the 206 cervical smears were hybridised with DNA from HPV types 16, 18, 31, 33, and 35 as a pooled probe to detect high-risk and intermediate-risk HPV types. The membranes were then stripped and hybridised with a mixed HPV 6 and HPV 11 probe under moderate stringency conditions to detect low-risk HPV types. There was no cross-hybridisation between the two probes.

Cervical smears from 25 of the 192 (13%) patients who had normal cytology were positive for HPV DNA; 2 of the 4 patients who had koilocytotic HPV, and 2 of the 9 (22%) patients with atypical cytology were positive for HPV DNA (Table I). HPV DNA was not detected in the patient with CIN 1. The probe designed to detect high- and intermediate-risk HPV types detected HPV DNA in 17 of the 25 (68%) SBH-positive cytologically normal cervical smears and the low-risk HPV probe detected HPV DNA in 8 of the 25 (32%) SBH-positive cytologically normal cervical smears (Table I).

Table I. Results of detection of HPV DNA in cervical scrapes by Southern blot hybridisation

	No. of HPV-positive specimens/total		
Cytology	Low-risk HPV types	High-risk HPV types	HPV-positive/ total
Normal	8/192 (4%)	17/192 (9%)	25/192 (13%)
Atypical	0	2/9	2/9
HPV	1/4	1/4	2/4

HPV typing by SBH and sequencing of cloned PCR amplification products

In this study we were able to type the HPV in 17 of the 29 SBH HPV-positive specimens. Eleven were typed by SBH and a further 6 were typed by sequencing of cloned PCR amplification products. The typing of 4 of the HPVs by SBH was also confirmed by sequencing of the cloned PCR amplification products. The results are given in Table II. In 1 patient, HPV 18 was detected by PCR sequencing, but Southern blot analysis revealed band sizes of 6 400 bp, 4 050 bp and 2 550 bp which indicate that this patient probably had a dual infection HPV 18 and another unclassified HPV type. Twelve of the HPV-positive samples were not typed by either SBH or sequencing of cloned PCR amplification products and these were scored as unclassified HPVs.

Table II. Summary of HPV types present in cervical scrapes determined by Southern blot hybridisation and sequencing of cloned PCR products

	No. positive for HPV DNA		
HPV type	Normal cytology	Atypical cytology	CIN or HPV on cytology
6	3		
11	2		
16	2	1	
18	7*	1	
31	0		
33	0		1
35	0		
Unknown	12*		1
HPV positive/total	25/192 (13%)	2/9	2/5

* One patient had a dual infection with HPV 18 and an unclassified HPV type.

Age distribution of patients

In the age group 20 - 39 years, 15 of 92 women (16%) were positive for HPV DNA while in the age group aged 40 years and above, 3 of 53 women (6%) were positive. The relative risk (RR) of the younger group of women with detectable HPV DNA compared with the older group of women is 2.88.

Private patients v. Groote Schuur patients

The incidence in the private patients (higher socio-economic group) where 19 of 115 (16.5%) showed evidence of HPV infection was similar to that in the Groote Schuur patients (low socio-economic group), where 6 of 44 (11.4%) showed evidence of HPV infection.

Discussion

The incidence of HPV DNA in cervical smears from Cape Town women with normal cytology was found to be 13% using SBH. This result is high compared with most studies based on direct detection of HPV DNA by hybridisation-based techniques. Most of the reports give an HPV prevalence rate in the range of 1 - 9%, with only Fuchs *et al.*[8] reporting a prevalence of 15% using SBH in normal smears from a random population of American women.

Because SBH analysis underestimates HPV prevalence, the true prevalence of HPV in normal cervical smears from Cape Town women is probably much higher than reported in the present study. Studies using PCR as their detection assay report rates that are similar to ours; 16% reported by Kallio *et al.*,[10] 16.4% reported by Vandenvelde *et al.*[13] and 19.2% reported by Evander *et al.*[14] Bauer *et al.*[11] detected HPV in 31% of female students (mean age 22.9 years) with normal cytology. However, when the same group was subjected to analysis by Virapap hybridisation which would be expected to have similar sensitivity to SBH, the apparent prevalence was substantially lower at 7%, indicating that the Virapap method underestimated the real prevalence by a factor of 4.

Detection rates are also affected by the specificities of the probes used. In the present study only a small panel of probes (HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 6 and HPV 11) was used, and women who were infected with HPV types not detected by this panel of probes may have been missed. Another factor to consider was that only 2 of the 4 smears that were classified as having HPV infection were positive on SBH. This is further evidence for the lack of sensitivity of the direct hybridisation technique that was used. If all these factors are taken into account, the HPV prevalence in this population may be considerably higher, at least in the range of 40%.

The types of HPV found in normal cervical tissue from Cape Town did not differ significantly from those found elsewhere in the world. However, we detected more high-risk and intermediate-risk HPV types than low-risk HPV types in this study. The most prevalent type detected was HPV 18. Because many previous studies screened mainly for the presence of HPV 16,[9,12] there is a lack of data on the prevalence of the low-risk types 6 and 11 in women with normal cytology. However, we found that one-third (8/25) of the HPVs detected with normal cytology were related to low-risk HPV types.

Of great interest is the fact that HPV 18 was the most prevalent type detected in the local population. This result was rather surprising, as HPV 18 had a low incidence in CIN 3 and cervical cancer biopsy studies carried out in Cape Town previously.[18,19] In another South African study conducted by Cooper *et al.*[24] in Durban, the prevalence of HPV 18 was also low in CIN 3 biopsies. The high incidence of

HPV 18 cannot be attributed to contamination with plasmid DNA as all the HPV 18 sequences detected by PCR had a distinct point mutation. In addition, HPV typing by sequencing of cloned PCR products correlates with fragments observed on SBH analysis. HPV 18 has previously been shown to be associated with a greater risk of progression or more rapid transition to malignancy,[25] and HPV 18-associated cervical cancers may be more aggressive than those associated with other HPV types.[26] Given that South Africa has a very high incidence of cancer of the cervix,[17] but a low incidence of HPV 18 in premalignant and malignant lesions, we need to conduct prospective studies to determine whether the prevalence of HPV 18 in South African women with normal cytology is predictive of progression to malignancy. Interestingly, the patient with atypical cytology and HPV 18 later went on to develop CIN.

In our study 16% of women under the age of 39 years and 6% of women over 39 years were infected with HPV. The trend therefore was that detection of HPV was more likely in women below the age of 40 years (RR = 2.88). There have been previous reports that the prevalence rates of HPV infection are related to age. Melkert *et al.*[27] reported that significant differences ($P = < 0.001$) in HPV prevalence were found between groups of women aged 15 - 34 years and those aged 35 - 55 years. They also observed that a gradual decrease in HPV prevalence was found after the age of 30 years. The decrease in HPV infection in older women may reflect a decrease in virus shedding with age and may be related to post-menopausal hormonal changes that result in less replication of the virus. The decrease may also be due to low exposure to reinfection. Alternatively, it is possible that in young women HPV infections are transient and that many HPV infections can be cleared; this would explain why not all HPV-infected women develop cervical cancer.

This study has confirmed that a significant number of women with normal cytology are infected with HPV. However, because high-risk HPVs are implicated in the development of CIN and cervical cancer it is important to identify individuals with an increased risk of progression to cervical cancer.

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