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Research Article

Molecular detection of HPV 16/18 E6 genes from cervical cells

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ABSTRACT

Background: Epidemiological, clinical and molecular studies have established the link between genital infection with high risk human papillomavirus (HPV) and cervical cancer but there is great challenge in establishing early infection by both clinicians and the laboratories. The virus cannot be grown in conventional cell cultures and serology cannot different between active and past infection. Molecular studies remain the goal standard as it detects viral nucleic acid or cellular antigens indicative of oncogenic potential in cytology or biopsy specimen. The study was aimed to molecularly determine the presence of HPV 16/18 and expression of E6 gene in squamous intraepithelial lesions.
Methods: Cervical cells were collected from 18 women with positive cytology test results and 32 controls in gynaecology clinic of Murtala Muhammad Specialist Hospital, Kano Nigeria and HPV 16/18 were detected with E6 gene specific PCR primers.
Results: Overall, HPV E6 gene was found in 76% of the women, 88.3% of positive cytology specimens and 71.2% controls.
Conclusions: There is very high prevalence of HPV infection. The presence of HPV 16/18 E6 genes in cervical intraepithelial lesions may serve as a useful predictor of diagnosis and possible clinical outcome of the disease.

Keywords: Cervical cancer, HPV, E6 gene, Molecular detection

INTRODUCTION

Human papillomaviruses (especially types 16 and 18) are the causative agents of cervical cancer,[1] the second leading female cancer in the world; the most frequently reported cancer in most developing countries and the principal cause of mortality from cancer in general. The virus encodes for 2 oncoproteins E6 and E7 which directly contribute towards initiation and maintenance of tumour development.[2] Protein products of these genes interfere with the normal function of tumour suppressor genes. E7 binds and inactivate retinoblastoma protein RB while E6 binds P^{53} protein and directs its degradation leading to resistance to apoptosis, uncensored cell growth and malignant transformation.[3] E6 is of particular interest because of its multiple roles in the cell and in interacting with many other proteins. It reacts with E6-AP and form an ubiquitin-protein ligase a process leading to degradation of the P^{53} gene.[4] It also targets NFX1-9 cellular protein which normally represses reduction in telomerase (a protein that make cells to divide unlimited times) and consequently keep cell growth uncheck.[5] E6 acts as a translational co-factor, specifically transcription activator when interacting with cellular transcription factor E_2F/DP1. The interaction of E6 with membrane associated granulocytes kinase family (MAGUK) protein

and PSD-95/DLG/ZO-1 (PDZ) causes transformation of the DLG protein and disruption of its suppressor function.[6,7] Overall, E6 serves to impede normal protein activity in such a way to allow cell to grow and multiply at an increased rate characteristics of cancer.

Human papillomavirus (HPV) types 16 and 18 are found in 50-80% of squamous intraepithelial lesions and up to 90% of invasive cancers.[8] HPV-16 is the major carcinogenic type in almost all the countries surveyed;[9] it persists longer than any other type and also found in association with other anogenital and oropharyngeal cancers.[10] Most cervical infections with HPV are latent or sub-clinical. They can be detected with Pap smear as Low grade Squamous Intraepithelial Lesions (LSILs) or high grade Intraepithelial Lesions (HSILs). The conventional Pap smear is however cheap, easy to perform, with a specificity as high as 99%[11] but the sensitivity is as low as 58%, giving a high false negative result. Moreover, Papillomaviruses cannot be grown in conventional cell culture and serological assays have limited accuracy as antibodies may not be suitable for distinguishing present and past infection. Thus, the accurate diagnosis of HPV infection is based on molecular methods. We investigated the frequency of HPV-16/18 E6 genes using Polymerase Chain Reaction (PCR) in women with normal and abnormal Pap smear test results in Kano, where the incidence of cervical cancer is amongst the highest in the country.[12] This study was aimed to molecularly determine the presence of HPV 16/18 and expression of E6 gene in squamous intraepithelial lesions. The study of this type would be helpful in developing effective preventive and therapeutic vaccines of local relevance and would ultimately contribute to control of cervical cancer.

METHODS

Sample collection

This study was cross sectional in nature. Cervical cells were exfoliated from endo- and ectocervix (using Ayer's spatula) from 50 eligible women who were selected in accordance with the International Agency for Research on Cancer HPV survey protocol.[13] Samples were stored in phosphate buffered saline at 4°C until processing. In addition Pap smear test was carried out to check for cytological evidence of HPV infection. Nucleotide sequences of choice were obtained from a gene bank and the primers (Table 1) were designed using Bioedit software and verified with ABI TM calculator. Samples were processed and analyzed in the Biotechnology Research Center of Ahmadu Bello University, Zaria.

Table 1: Showing the organisms, primers, target gene, base pair in the PCR.

Organism	Primer sequence	Target gene	Product size
HPV16	F:5'- TGCCCATTAACAGGTCTTCC-3'	E6	164bp
	R:5'- CAGCTCAGAGGAGGAGGATG-3'		
HPV18	F:5'- GTGCCAGAAACCGTTGAATC-3'	E6	165bp
	R:5'- GGCAGATCGTCAGTCAGTCA-3'		

HPV16-Human Papillomavirus type 16, HPV18-Human Papillomavirus type 18, bp-Base pair, F-Forward primer, R-Reverse primer

DNA extraction

DNA extraction was carried out using the ZR genomic DNA™ tissue mini prep kit (Zymoresearch Co-operation, South Africa). Samples were first spun (10000 g, 5 mins) in effendurf tubes and the supernatant discarded. Cell samples were lysed in a buffer containing proteinase-K (20 mg/ml). Following washing to remove contaminants, DNA was eluted in 50µL elution buffer. The final concentration of DNA was on average 5 µg/100µL. It was stored at -20°C according to the manufacturer's instruction.

Detection of HPV-16/18 E6 genes using PCR

The amplification was carried out using the primer set (Inqaba Biotechnical Industries Pty Ltd, South Africa). The reaction was performed according to the PCR master mix manufacturer's protocol (Fermentas Life Science, South Africa). The final reaction volume was 50 µL; containing 5 µL DNA template, master mix 20 µL (containing 0.05 U/µL tag DNA polymerase, reaction buffer: 4 mM $MgCl_2$, 0.4 mM each of dNTP, 1 mM each of forward and reverse primer and 23 µL nuclease free water. The thermal program was started with pre-heat at 95°C for 5 mins, 35 cycles of suitable annealing temperature and extension. Final extension was at 72°C for 5 mins (Table 1).

Gel electrophoresis

Single strength (1x) Tris Acetate EDTA (TAE) was first prepared using 8 ml of the 50x in 400 ml of distilled water. The gel was prepared using 100 ml of the single strength TAE and 5 µLs (100 mg/ml) ethidium bromide added. Amplified DNA fragments were separated in 1% agarose gel via electrophoresis and read by comparison with DNA molecular weight ladder. Amplicons were also visualized and captured using documentation unit.

Statistics

Data were entered, processed and analyzed using SPSS version 16.0 statistical packages.

RESULTS

The results of gel electrophoresis showing HPV 16 and 18 E6 genes are depicted in Figure 1a and 1b.

HPV prevalence

In present study, 38 (76%) of samples were detected as HPV positive. Out of HPV positive cases, 32 (64%) were infected with HPV-18, 29 (58%) with HPV-16 and 23 (46%) had co-infection with HPV-16 and 18 (Table 2).

HPV positivity in the cervical lesions

The total HPV positivity in the abnormal Pap test results was 83.3% (Table 3). SCC was associated with 100% positivity (50% HPV-16; 50% HPV-18), HSILs 50% (co-infection with HPV-16/18), 90% for the LSILs (80% HPV-16; 70% HPV-18; 60% co-infection) and ASC-US was associated with 75% positivity (75% HPV-16; 50% each for HPV-18 and co-infection) (Table 3 and Figure 2). In addition, 71.9% of samples suggestive of negative Pap test results were found positive for the infection (Table 2).

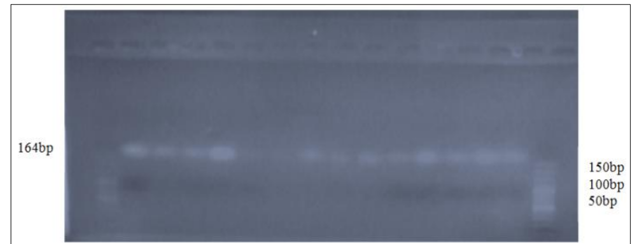

Figure 1a: Agarose gel (1%) showing 164 bp long PCR product obtained by amplification of E6 gene region in HPV-16 using type-specific primer pair.

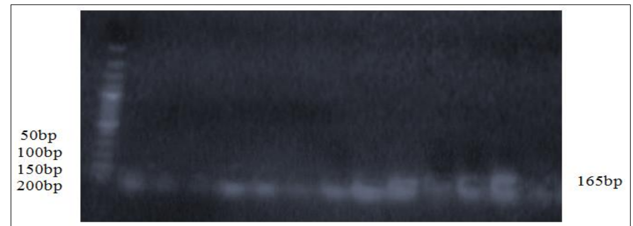

Figure 1b: Agarose gel (1%) showing 165 bp long PCR product obtained by amplification of E6 gene region in HPV-18 using type-specific primer pair.

Table 2: Overall prevalence of HPV infection.

HPV type	Cytology result								
	Normal			Abnormal			Total		
	Single	Co-inf.	Total (%)	Single	Co-inf.	Total (%)	Single	Co-inf.	Total (%)
HPV$^-$			09 (28.1)			03 (16.7)			12 (24)
HPV$^+$	09	14	23 (28.1)	06	09	15 (83.3)	15	23	38 (76)
HPV-16	02	14	16 (50)	04	09	13 (72.2)	06	23	29 (58)
HPV-18	07	14	21 (65.6)	02	09	11 (61.1)	09	23	32 (64)

Total prevalence=76% (95% CI=61.8-86.9); Prevalence of HPV-18=64% (95% CI=49.1-77.1); Prevalence of HPV-16=58% (95% CI=43.2-71.8); Prevalence of co-infection with HPV 16 and 18=46% (95% CI=31.8-60.7); Positivity in normal cytology=71.9% (95% CI=53.3-86.3); Positivity in abnormal cytology=83.3% (95% CI=58.6-96.4)

Table 3: Cumulative percentage HPV positivity in abnormal Pap test results.

Cytological classification	Number	Percentage positivity
SCC	2	2 (100)
HSIL	2	1 (50)
LSIL	10	9 (90)
ASC-US	4	3 (75)
Total	18	15 (83.3)

SCC: Squamous cell carcinoma; HSIL: High grade squamous intraepithelial lesions; LSIL: Low grade squamous intraepithelial lesions; ASC-US: Atypical squamous cells of undetermined significance

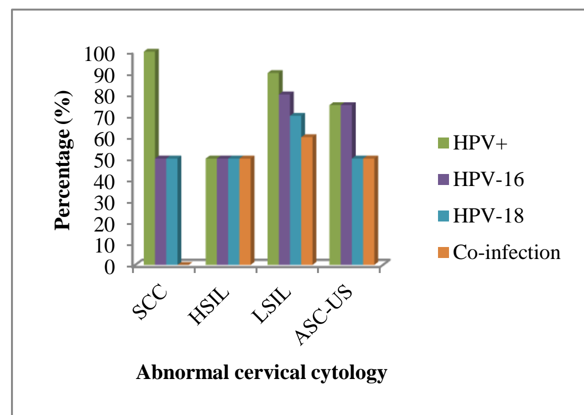

Figure 1: HPV type specific in positive Pap tests.

DISCUSSION

Epidemiological, clinical and molecular studies have implicated HPV as the aetiologic agent of cervical cancer. However, the infective mechanism were more closely with high risk HPV types (HPV 16 and 18), with over expression of several onco-proteins, especially during the early stage, most notably E6 and E7.[14] Although Pap smear has yielded substantial reduction in the incidence and mortality of cervical cancer, additional HPV-based assays have been introduced because of their high sensitivity and negative predictive value for HPV typing, which rely on either demonstration of viral nucleic acids or cellular antigens indicative of oncogenic risk in the cytology or biopsy specimens.[15]

In this study, E6 gene was targeted using polymerase chain reaction, because of its multiple functions in HPV-16 and 18 associated lesions which are likely to be absent in low risk types.[2]

A very high prevalence was found in this study, most especially in cytology positive samples, though there is no significant difference when compared with the normal Pap test results. A prevalence of 76% found in current study agree with what was obtained in another African country reported by Odida et al.,[16] closer to what was obtained in Italy (67%) and Central and South America (70%) Smith et al.[17]

The report that expression of E6 proteins is higher in advanced lesions[15] was testified by present findings; cervical cancer was associated with 100% positivity, followed by LSILs and ASC-US with 90% and 75% respectively. These also agree with the findings of Bosch et al.,[18] Clifford et al.,[19] Dienne et al.,[20] and Szostek et al.[21] It has also been observed that 88.3% of abnormal cytology test results were positive for the viral DNA. This conforms to reports of Gargiulo et al.,[22] Gul et al.[23] and slightly lower than that of Chen et al.[24] who reported 92.9% in patients with CIN2+ lesions. But in contrast with what was found by Piras et al.,[25] Thomas et al.[13] and Zhang et al.[26] However, the differences could be explained by larger samples and methodologies employed by the three researchers.

In conclusion, the findings in this study show the effectiveness of PCR-based assay for the detection of HPV DNA in majority of cervical intraepithelial lesions and even among the cytology negative test results. Thus, HPV testing improves the accuracy and sensitivity of cervical cancer screening. In order to enhance the sensitivity and specificity of cytology there is need to incorporate HPV DNA detection, most especially in low resource countries where cervical cancer screening is not routinely available. This would improve outcome of test results and guide towards effective and efficient treatment options.

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