

RESEARCH COMMUNICATION

Human Papillomavirus Testing for Suspected Cervical Cancer Patients from Southern Assam by Fast-PCR

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Abstract

World-wide epidemiological studies have shown that cancer of the uterine cervix is the second most common malignant disease in women. Virtually every cervical cancer (99.7%) is HPV-positive, indicating that the presence of HPV is an obligatory element in their development. The present study was conducted by Fast-PCR (within 15 min.) based diagnosis of HPV 16 and HPV 18 infection amongst patients of suspected cervical cancer, confirmed by cytological methods. Twelve women, out of a total of fifty studied cases who had positive cervical pap smears (24%) were found to be positive for HPV 16/HPV 18 infection when PCR based technique was applied. The results indicate, perhaps, a greater specificity of PCR based diagnosis, or presence of other HPV subtypes as etiological factors in the present study group confined to Southern Assam.

Keywords: Fast-PCR - cytological methods - cervical cancer - HPV - sensitivity - specificity - Southern Assam

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Introduction

World-wide epidemiological studies have shown that cancer of the uterine cervix is the second most common malignant disease in women (Ogunmodede et al. 2007). Virtually every cervical cancer (99.7%) is HPV-positive, indicating that the presence of HPV is an obligatory element in the development of cervical cancer (Walboomers et al., 1999). Specific anogenital types of human papillomavirus (HPV) cause the initiating infection that leads to cervical cancer. More than 100 HPV types are known, of which at least 70 infect the anogenital tract. Knowledge of HPV status is becoming increasingly important as a triage screen after detection of atypical cells of undetermined significance (Bollmann et al., 2003) and as a primary screen for cervical cancer detection (Cuzick et al., 2000).

HPV typing has an important prognostic or therapeutic value, as it can distinguish between HPV types of high and low oncogenic risks. Identification of high-risk HPV genotypes may permit selection of those patients who are at increased risk for disease and may therefore provide additional clinical value. An important requirement for this approach is that HPV testing and identification of high-risk HPV types should be highly sensitive and specific (Speich et al., 2004). The types associated with diseases of the anogenital tract can be classified on the basis of phylogenetic relationship (Zur Hausen, 1996) and of associated frequencies with benign or malignant cervical lesions as high-risk types (HPV-16, -18, -31, -33,

-35, -39, -45, -51, -52, -54, -56, -58, -59, and -66) and low risk types (HPV-6, -11, -34, -40, -42, -43, -44). The most common HR types are HPV-16, -18, -31, -33, and -45 (Bosch et al., 1995; Nobbenhuis, 1999). HPV genome can be divided into three different regions including early, late and long control regions (Moosavi et al., 2008). Early region proteins are classified into two groups: E1, E2, E3 and E4 proteins, and E5, E6 and E7 oncoproteins (Dyson, et al., 1989). E5 oncoproteins stimulate the growth of epithelial cells and in many cancers, lead to increased cellular mitosis and consequently cause papilloma lesions (Prayitino, 2006). E6 oncoprotein has been implicated in causing chromosomal abnormalities and progression of cells to neoplasia. E6 is also responsible for causing damage to P53 (Pei, 1996). E7 oncoprotein binds to Rb gene products and other similar proteins thereby inactivating them (Kim, 2001).

The detection and type-specific classification of HPV infection by in vitro viral culture is not possible and serological tests are still ineffective. Direct hybridization based assays, such as Southern blotting and in situ hybridization have been described, but lack sensitivity and specificity (Szuhai, 2001). It has been reported in many studies that target DNA amplification may offer more specificity and sensitivity in detecting HPV infection (Malloy, 2000). In this study we have compared the diagnostic efficacy of PCR based techniques over cytological examination in the direct detection of HPV infection involving HPV 16 and HPV 18 as well as evaluated the relevance as a screening technique.

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Materials and Methods

Samples

Samples were collected from fifty patients attending Cachar Cancer Hospital, Silchar, Silchar Medical College and Hospital, Silchar and different infertility clinics of Southern Assam, who had positive pap smear during May-November, 2010. Considering the short duration of the study, no follow-up was done as part of this study in patients who were found positive for HPV infection by cytological/PCR methods except being referred for further appropriate clinical consultation. Samples were collected with informed consent of subjects.

Cytological Methods

Smears were collected with Ayer's spatula and immediately fixed in absolute alcohol for minimum 6 hours. The smears were then washed with distilled water and dipped in hematoxyline for 7 to 10 minutes. These were subsequently washed in running tap water and dipped in 1% acid alcohol for decolourization, and repeated twice. After third washing, the smears were dipped in 50% alcohol and transferred to O.G 6 solution and kept for 2 to 3 minutes. These were then dipped in absolute alcohol and transferred to EA 50 solution and kept for 20 to 30 minutes. After final dipping in absolute alcohol, the smears were cleared with xylene and mounted for viewing under optical microscope.

DNA extraction

Cervical samples were taken with sterilized swab and put in sterilized collection tubes. The samples were stored at 04°C until DNA isolation. To isolate DNA, sample was taken and mixed with 500µL of TES buffer. Then 30/50µL of 10% SDS and 2/5µL of proteinase K was added to it and kept in a water bath for 1Hr at 57°C. Equal amount of phenol chloroform isoamylalcohol (25:24:1) was added and centrifuged at 12000rpm for 8mins. The supernatant was taken and equal amount of chloroform isoamylalcohol (24:1) was added and further centrifuged. The supernatant was again taken and twice the amount of absolute alcohol was added and kept in refrigerator for some time. The refrigerated supernatant was next centrifuged, alcohol discarded and washed with 200 – 500 µl of 70% alcohol and kept for drying. After that 20 – 50µL nuclease free water was added and left overnight. Isolated DNA was checked under agarose gel electrophoresis in a 1% gel and observed in UV transilluminator for purity.

PCR Method: HPV DNA was demonstrated by performing PCR with consensus primers. To identify the specific HPV types (16 and 18) in the HPV positive specimens further PCR was performed using HPV 16 (5' GCC TGT GTA GGT GTT GAG G 3' -Forward and 5' TGG ATT TAC TGC AAC ATT GG 3' -Reverse) and HPV 18 (5' GTG GAC CAG CAA ATA CAG GA 3' -Forward and 5' TCC AAC ACG TGG TCG TTG CA 3' -Reverse) specific primers. The HPV 16 primer pairs were designed from the L1 region and HPV 18 primer pairs were from the E1 region of the HPV genome. Each amplification reaction mixture of GeneAmp® Fast PCR Master Mix (2X) [Applied Biosystem] 20µL contained buffer, Mg2+,

dNTPs, Gold enzyme, sets of Primers and isolated DNA from different cervical samples in 96 wells Veriti Thermal cycler (Applied Biosystem).

FAST PCR amplification using AmpliTaq GOLD (2X):The following PCR steps were employed for FAST PCR (AmpliGOLD-Applied Biosystem) of the total volume of 20 ul.

Step	PCR (30 cycle)				Final step	
	Reactivation of Enzyme	Denaturation	Annealing	Extension	RAM	Hold
Temp	95°C	95°C	58	68°C	72°C	4°C
Time	10 min	3 sec	3 sec	3 sec	15 sec	15sec

Total Time taken- Reactivation of Enzyme (10 min) + PCR for 30 cycles [3+3+3=9 second X30=270 sec (4½ min)] + 30 sec (½ min)= 15 min

Results and Discussion

In our study, samples were collected from fifty patients as described previously. To avoid false positive results, and more confirmation of HPV genotype, we use this new technique to detect HPV. The mean age of patients was forty five years (ranging from twenty to seventy) and the highest number of HPV positive cases belonged to patients aged forty to fifty years. The presence of amplifiable DNA, using primers for HPV 16 and 18 was confirmed in twenty four (24%) out of the fifty samples. Out of the twelve positive cases HPV type 18 was present in ten cases and HPV type 16 was present in two cases. The PCR was carried out using Fast-PCR method by Applied biosystem reagents (details in Materials and Methods) within in 15 min. with the primers specific to the subtype 16 and 18 and the product sizes were 246 bp (product from L1 region base 379 to 624 with forward – reverse primers) and 162 bp (product from E1 region base 1448 to base 1609 with forward – reverse primers) respectively, depicted in Figure 1.

Our result showed that the occurrence of affected percentage of HPV type 18 is high in comparison to the HPV type 16 in our geographical region. In nineteen cases (76%), it was not possible to identify the virus genotype which we attributed to possibly false positive results of cytological tests or may be due to the presence of other

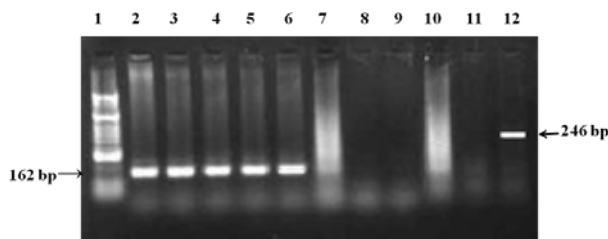


Figure 1. Agarose Gel Electrophoresis (2%) of PCR Products of HPV Type 16 (246 bp) and 18 (162 bp). Lane-1-DNA marker, Lane- 2,3,4,5 and 6-amplified DNA (162 bp) of HPV18 and Lane-7, 8, 9, 10 and 11- no amplified (absence of HPV), 12- amplified DNA (246 bp) HPV type 16

genotypes.

Molecular (PCR) tests may accurately identify different types of HPV (of low and high cancer risks) in cells from cytological screening of cervical lesions and, due to their high sensitivities, have been the focus of attention of many studies (Bauer et al., 1992). The purpose of our study was to detect the HPV 16 and 18 strains by PCR technique with the help of two pairs of primers. The advantage of this PCR based assay, unlike the other currently available assays, is that it is able to report the actual genotype of the HPV detected, rather than issue a broad based ambiguous diagnosis of HPV infection. In the present study, cervical smears were simultaneously subjected to cytological examination as well as PCR technique of detection. The traditional way of classifying tumors is by histopathology. The ability to analyze change in the levels of the transcripts and/or protein products for literally thousands of genes promises interesting possibilities as a research tool for understanding the underlying molecular mechanisms, but also for automated tissue diagnosis (Drain et al., 2002; Oh et al., 2009). HPVs frequently infect humans. They are classified into categories of low risk types responsible for the most common sexually transmitted viral infections and high risk types which are crucial etiological factors in cervical cancer development (McFadden and Schumann, 2001). The direct detection of HPV in cervical specimens may offer an alternative (?) or complement to population-based cytological screening. Recent studies have demonstrated that HPV test results are more sensitive (although they are less specific) than Pap smears in detecting high-grade dysplasia in older women (Schiffman et al., 2000; Wright et al., 2000). In most scenarios women with positive HPV tests still have Pap tests or a diagnostic procedure to provide cytological or histological confirmation of their disease.

We conclude that PCR based diagnosis may be a more specific test for detection of HPV subtypes. Contrary to many studies, it seems that PCR based diagnosis of HPV may not be ideal as a screening test in comparison to conventional pap smear studies, although it may be complementary. However, the use of PCR technique, covering all strains, may be a more sensitive diagnostic procedure that can help to differentiate between suspicious lesions and confirmed HPV infection at risk of subsequent malignancy. We suggest that further studies, covering larger study groups in other geographical locations, are required to draw a firm conclusion as to the diagnostic significance/advantage of PCR techniques over conventional methods.

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References

Bauer HM, Greer CE, Manos MM (1992). Determination of genital HPV infection using consensus PCR. In *Diagnostic Molecular Pathology: a Practical Approach*, pp. 131–152.

Edited by C. S. Herrington & J. O. McGee. Oxford: Oxford University Press.

- Bollmann R, Méhes G, Torka R, et al (2003). Determination of features indicating progression in atypical squamous cells with undetermined significance. *Cancer Cytopathol*, **99**, 113-7.
- Bosch FX, Manos MM, Muñoz N, et al (1995). Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst*, **87**, 796-802.
- Cuzick J, Sasieni P, Davies P, et al (2000). A systematic review of the role of human papilloma virus (HPV) testing within a cervical screening programme: summary and conclusions. *Br J Cancer*. 83(5):561-565.
- Drain PK, Holmes KK, Hughes JP, Koutsky LA (2002). Determinants of cervical cancer rates in developing countries. *Int J Cancer*, **100**, 199-205.
- Dyson N, Howley PM, Münger K, Harlow E (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, **243**, 934-7.
- Kim YW, Hur SY, Kim TE, et al (2001). Protein kinase C modulates telomerase activity in human cervical cancer cells. *Exp Mol Med*, **33**, 156-63.
- Malloy C, Sherris J, Herdman C (2000). HPV DNA Testing: Technical and Programmatic Issues for Cervical Cancer Prevention in Low-Resource Settings. *Path* : 1 – 29.
- McFadden SE, Schumann L (2001). The role of human papillomavirus in screening for cervical cancer. *J Am Acad Nurse Pract*, **13**, 116-25.
- Moosavi SS, Saeed S, Mojghan S (2008). A comparison between cytological method and PCR in the diagnosis of HPV infection among patients with cervical cancer. *Biotechnology*, **7**, 798-802.
- Nobbenhuis MA, Walboomers JM, Helmerhorst TJ, et al (1999). Relation of human papillomavirus status to cervical lesions and consequences of cervical cancer screening: a prospective study. *Lancet*, **354**, 20-5.
- Ogunmodede F, Yale SH, Krawisz B, Tyler GC, Evans AC (2007). Human papillomavirus infections in primary care. *Clin Med Res*, **5**, 210-217.
- Oh JK, Franceschi S, Kim BK, et al (2009). Prevalence of human papillomavirus and Chlamydia trachomatis infection among women attending cervical cancer screening in the Republic of Korea. *Eur J Cancer Prev*, **18**, 56-61.
- Pei XF (1996). The human papillomavirus E6/E7 genes induce discordant changes in the expression of cell growth regulatory proteins. *Carcinogenesis*, **17**, 1395-401.
- Prayitino A (2006). Cervical cancer with human papillomavirus and Epstein barr virus positive. *J Carcinog*, **5**, 13-8.
- Schiffman M, Herrero R, Hildesheim A, et al (2000). HPV DNA testing in cervical cancer screening: results from women in a high-risk province of Costa Rica. *JAMA*, **283**, 87-93.
- Speich N, Schmitt C, Bollmann R, Bollmann M (2004). Human papillomavirus (HPV) study of 2916 cytological samples by PCR and DNA sequencing: genotype spectrum of patients from the West German area. *J Med Microbiol*, **53**, 125-8.
- Szuhai K, Sandhaus E, Kolkman-Uljee SM, et al (2001). A novel strategy for human papillomavirus detection and genotyping with SybrGreen and molecular beacon polymerase chain reaction. *Am J Pathol*, **159**, 1651-60.
- Walboomers JM, Jacobs MV, Manos MM, et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*, **189**, 12-9.
- Wright TC Jr, Denny L, Kuhn L, Pollack A, Lorincz A (2000). HPV DNA testing of self-collected vaginal samples compared with cytologic screening to detect cervical cancer. *JAMA*, **283**, 81-6.
- Zur Hausen H (1996). Papillomavirus infections - a major cause of human cancers. *Biochim Biophys Acta*, **1288**, 55-78.