Human Papillomavirus Genotyping by Dual Priming Oligonucleotide Technology And Its Clinical Efficacy in Cervical Cancer Management

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Abstract

Infection with oncogenic human papillomavirus (HPV) is a necessary source for the progression of cervical cancer. Several types of human papillomaviruses (HPVs) are associated with a range of benign and malignant lesions including genital condyloma and anogenital cancer as well as for the development of genital warts. Six patients came positive for Human papillomavirus. Patients with low grade squamous intraepithelial lesion (LGSIL) and high grade squamous intraepithelial lesion (HGSILS) were highest in number (05). LGSIL positive cases harbored HPV type 16 (03 cases) where as patients with HGSIL were HPV type 16, HPV type 18, HPV mixed types and HPV type 11 positive. A case with cervitis harbored HPV type 16. Discussion: Thus the conventional PCR using DPO technology is helpful for detecting and screening clinically significant HPV genotypes so that the existing vaccines for the particular genotype can be prescribed as well as for HPV types causing generalized and genital warts, proper treatment can be given to the patient. However more epidemiological research on the prevalence of various HPV types is required before the need for regionally-tailored vaccines is confirmed.

Introduction

Cervical cancer involves cells of the cervix which will become irregular and proliferate resulting in forming tumors (1). More than 60 types of Human Papillomavirus (HPV) are acknowledged to exist. Of these, fifteen are classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 73, and 82), and 12 as low-risk (6,11,40,42,43,44,54,61,70,72,81, and CP6108), but still some of the low risk HPV types may cause cancer (2). HPV types 16 and 18 are generally acknowledged to cause about 70% of the cancer cases. Most of the HPV infections clear up on their own; the infections could increase to major abnormalities and can lead to cervical cancer (3). The clinically significantly strains 16, 18 and 31 is the prime risk factor for cervical cancer. Several studies (Walboomers etal.1999) reported that the existence of HPV is a necessary situation for the development of cervical cancer. A virus cancer link with HPV has been found to trigger alternations in the cells of the cervix, leading to the development of cervical intraepithelial neoplasia and cancer. (4) The extensive introduction of the Papanicolaou test or Pap smear for cervical cancer screening has been attributed with noticeably reducing the incidence and mortality of cervical cancer in developed countries (5). The Pap smear suggests the presence of cervical intraepithelial neoplasia before cancer develops, allowing for further follow-up. Cervical cancer screening is presently done by finding abnormal cells in cervical smears (i.e., cervical cytology or Pap smears). Cervical cytology is insensitive for the detection of cancer and pre-cancer requiring many rounds of screening to achieve programmatic effectiveness. With the advent of Nucleic Acid Amplification Techniques (NAATs) and other molecular diagnostics tools, have markedly increased essential parameters like sensitivity and specificity for the detection of HPV. Detection of HPV genomes and transcripts can be achieved with hybridization procedures including Southern and Northern blots, dot blots, in-situ hybridization, signal-amplification molecular technology (Hybrid Capture assay, version hc2: Digene, Gaithersburg, MD, USA), and DNA sequencing (6). For proper treatment, follow-up and launching vaccination programmes, type specific HPV detection is very significant. There are several findings which showed a high frequency of multiple HPV infections in cervical carcinomas (7, 8). Researchers and R&D firms needs such type of informations for further HPV vaccine design and application. Regardless of the elevated incidences of cervical cancer reported from India, large-scale population based studies on the HPV prevalence and genotype distribution are very few (9). Present study includes HPV genotypes detection by Dual priming oligonucleotide technology in the patients showing cervical complications.

Materials and Method
For the present study, thirty cervical specimens from the department of Obstetrics & Gynecology of SMI Hospital, Patel Nagar, Dehradun were collected. The cervical specimens from the cervix were collected, using cytobrush and were transported in virus transport media (Digene Diag, Md) at 4°C to Molecular Research Laboratory. Genotyping and screening of HPV were done using multiplex PCR, applying DPO (Dual Priming Oligonucleotide) technology. This technique can simultaneously genotype (HPV-16 and 18) and screen out 16 high-risk HPV types (26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and HPV type 6 and 11. DPO technology is a new addition to the Nucleic acid amplification techniques (NAAT) which provides freedom in primer designing, PCR optimization and maximizes PCR specificity and sensitivity by fundamentally blocking non-specific sequences. The assay includes internal control for the validation of the protocol which is introduced into every amplification reaction and is co-amplified with target DNA from the clinical specimen. Major drawback of PCR technique is false positive due to amplicons generation. Amplicon contaminations can be prevented by using 8-methoxypsoralen (8-MOP) which will extinguish the template activity of contaminated DNA thus preventing false positive (10). For setting up the PCR reaction, add 4 µl of 5X HPV4A ACE PM (Primer Mixture primer pairs for HPV primer pair for internal control template of Internal control): 3 µl of 8-mop solution, 10 µl of 2X multiplex master mix (DNA polymerase, Buffer containing dNTPs {dATP, dCTP, dGTP, dTTP} MgCl₂, and stabilizers) with final addition of 3 µl of HPV DNA template into 0.2 ml of PCR tube. Cycling conditions for the amplification includes initial denaturation at 94°C for 15min, 30 cycles of denaturation at 94 °C for 0.5 minutes, annealing at 60°C for 1.5min and extension at 72°C for 1.5min. Give final extension at 72°C for 10 minutes followed by storage of amplicons at 4°C. After amplification, run the amplicons on 1.6% agarose gel by electrophoresis at 150 volts for 20 minutes. View the gel under U.V. transilluminator for interpreting the results. Amplicons size for Internal control at 1000bp, for HPV-16 at 588bp, for 16 high-risk HPV types screening (26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) at 465 bp, for HPV6/11 at 302bp, for HPV-18 at 230bp will be observed (Illustrations 4 &5.). Primers for DPO technology varies in structure from conventional primers and fundamentally differs. It comprises of two separate priming regions joined by a polydeoxyinosine linker. The linker forms a “bubble-like structure” which itself is not involved in priming, but rather delineates the boundary between two parts. DPO has two functional priming regions (one is longer than the other) separated by the poly (I) linker (as depicted in Illustration 2 and 3). These two unequally distributed priming regions generate dual priming reactions resulting in only target-specific products (Illustration 2 and 3). Deoxyinosine has a relatively low melting temperature compared to the natural bases, due to weaker hydrogen bonding so that the poly (I) linker will form a bubble-like structure at a certain annealing temperature and separates a single primer into two functional regions.

Results

Thirty cervical specimens were processed by utilizing multiplex PCR out of which five came positive for Human papillomavirus. Patients showing low grade squamous intraepithelial lesion (LGSIL) and high grade squamous intraepithelial lesion (HGSILS) were the highest in numbers (05). Patients with LGSIL positive case harbored HPV type 16 where as patients with HGSIL were HPV type 16, HPV type 18, HPV mixed types and HPV type 11 , where as the case with cerviitis harbored HPV type 16 (Illustration 6).

Discussion And Conclusion

The significant risk factor in the progression of cervical cancer is infection with a high risk strain of human papillomavirus (11). The presence of HPV strains 16, 18 and 31 are the chief risk factor for cervical cancer, walboomers et al., in 1999 reported that for the development of cervical cancer the presence of HPV is a necessary condition. The outcome or the findings of molecular diagnostics assay for HPV is very important for further management as well as for proper follow up of this disease. Women with HPV result negative can safely return to Pap smear routine screening and their risk of cancer is negligible. Only women with HPV positive result require instant reference for colposcopy. Women with an equivocal Pap smear or low grade cervical dysplasia are advised to have HPV test. Those women having HPV result negative can be safely return to Pap smear routine screening their risk of cancer is negligible (12). The women with High Grade Squamous Intra Epithelial Lesion (H.G.S.I.L.) have more chances of HPV infection. In our study most of the HPV positive cases showed the presence of genotype HPV16. HPV type 6 and 11 are less harmful & these genotypes causes genital warts & mostly do not cause the cancer (13).
With the emergence of HPV vaccines and large-scale clinical trials proposed and under way, awareness in the distribution of HPV genotypes prevalent in the Indian population has gained clinical significance. HPV vaccines Gardasil (Merck) and Cervarix (GSK) predominantly target HPV type 16 and 18. Cervarix is an HPV16/18 L1 virus-like particle vaccine that offers complete protection against HPV type-16 and HPV type-18 associated precancerous lesions as well as substantial protection against types 45 and 31 (Harper et al 2006). Gardasil is a quadrivalent vaccine composed of major capsid protein (L1) of high-risk HPV types 16 and 18, and low-risk types 6 and 11, which has been found to prevent 100% CIN2/3 and adenocarcinoma in situ associated with HPV16 and 18 infections (Schmiedeskamp and Kockler 2006). These vaccines may prove to be effective against a majority of cervical cancers, but may not benefit women infected with rarer HPV types (14, 15). Primary approaches to prevent HPV infection include both risk reduction and development of HPV vaccines. Another challenge for programs that ultimately offer an HPV vaccine is how to “position” and promote the vaccine. Should it be described as an “anti-cancer” vaccine (which would appeal primarily to women) or an anti-STD vaccine (which raises difficult social issues in most cultures) or even an anti-wart vaccine (which may broaden the vaccine’s appeal, particularly in young, sexually-active populations) (16, 17). These questions are important ones; planning how to promote the vaccine would have a major impact on its ultimate success or failure. At a minimum, any vaccine must be safe and effective. Some researchers have promoted the development of regional vaccine formulations that are tailored to prevent locally prevalent types of HPV. More epidemiological research on the prevalence of various HPV types is required before the need for regionally-tailored vaccines is confirmed, however. Thus the use of several genotyping methods like conventional PCR utilizing Dual Primer Oligonucleotide Technology is very helpful as it can detect different genotypes of HPV so that the existing vaccines for the particular genotype can be prescribed as well as for HPV types causing generalized and genital warts, proper treatment can be given to the patient.

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Illustrations

Illustration 1

Genomic composition of HPV Type 16 and functions of important genes
Illustration 2

Conventional primers and DPO primers (Photo courtesy Seegene)

Illustration 2. Conventional primers and DPO primers (Photo courtesy Seegene).
Illustration 3

PCR mechanism of Dual priming oligonucleotide technology (Photo courtesy Seegene)
Illustration 4

Gel Picture

Illustration 5

Results Interpretations
Illustration 6

HPV Type specific detection by multiplex PCR in patients with several clinical complications.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Pap Smear Status</th>
<th>No. of cases Positive</th>
<th>Multiplex PCR Results by dual priming oligonucleotide technology</th>
<th>HPV Type/s detected</th>
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<td>2</td>
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<td>01</td>
<td>Positive</td>
<td>Type 16</td>
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<tr>
<td>3</td>
<td>H.G.S.I.L (07)</td>
<td>04</td>
<td>Positive</td>
<td>Type 16, Type 18 and HPV mixed types HPV Type 11</td>
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<tr>
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<td>01</td>
<td>Positive</td>
<td>HPV type 16</td>
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<td>5</td>
<td>Infertility(03)</td>
<td>-</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td><strong>Total (30)</strong></td>
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<td></td>
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<td></td>
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<td><strong>Mixed type infection = 01 HPV Type 11 = 01</strong></td>
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