

Short Communication

Detection of human papillomaviruses type 16 and 18 by PCR and RFLP in paraffin-embedded cervical cancer tissue specimens

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ABSTRACT

Human papillomaviruses (HPVs) infect mucosal and cutaneous epithelia and cause malignant and benign neoplastic lesions. Human papillomaviruses cause 250000 deaths per year from cervical cancer most often in developing countries. This major public health problem make them important targets in the search for Human papillomaviruses detection methods that may play a role in cervical cancer screening. In the present study, we used a combination of polymerase chain reaction (PCR) amplification method and Restriction fragment length polymorphism (RFLP) analysis in order to identify HPV types in cervical cancer samples. Two of the five samples were HPV positive that one of them was HPV16 positive and the other was positive for HPV18. The method could facilitate the sensitive identification of a broad spectrum of genital Human papillomaviruses.

Keywords: Human papillomavirus, PCR, RFLP

INTRODUCTION

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women worldwide (Lowey *et al* 2001). Papillomaviruses are ubiquitous and have been detected in a wide variety of animals as well as in humans and are specific for their respective

hosts (Streeck 2002, Lin *et al* 1992, Peh *et al* 2004). More than 200 types of HPV have been recognized on the basis of DNA sequence data showing genomic differences. Eighty-five HPV genotypes are well characterized. An additional 120 isolates are partially characterized potential new genotypes (Zur Hausen 1999, Burd *et al* 2003, Munger *et al* 2004). HPVs can infect basal epithelial cells of the skin or inner lining of tissues and are categorized as cutaneous types or mucosal types. Mucosal types

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infect the lining of the mouth, throat, respiratory tract, or anogenital epithelium (Burd *et al* 2003, Fang Zhang *et al* 2000). Based on their association with cervical cancer and precursor lesions, HPVs can also be grouped to high-risk and low-risk HPV types. Low-risk HPV types include types 6, 11, 42, 43, and 44. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70. Included in the high-risk group are some HPV types that are less frequently found in cancers but are often found in squamous intraepithelial lesions (Some authors refer to these HPV types as intermediate - risk) (Comerford *et al* 1991, Wakabayashi *et al* 2002, Michle *et al* 2002, Lowy *et al* 1998, Greenstone *et al* 1998, Michle *et al* 2002, Ngelangel *et al* 1998, Chen *et al* 1991, Breitburd *et al* 1995).

HPV is associated with a variety of clinical conditions that range from innocuous lesions to cancer. Cervical cancer is the second most common cancer in women worldwide (Cope *et al* 1997, Bosch *et al* 1995). In developing countries, cervical cancer is often the most common cancer in women and may constitute up to 25% of all female cancers (Harro *et al* 2001). HPV detection and typing in genital specimens is a diagnostic challenge, because of the numerous viral types that must be detected and distinguished. Furthermore, precision, accuracy and technical feasibility are required for large-scale epidemiological studies. PCR-based methods facilitated the sensitive and broad-spectrum detection of genital HPVs. PCR-based methods used consensus primer pair MY09/MY11 that amplify the L1 viral gene of most genital HPV types. There are many different kinds of commercial kits for HPV genotyping but they used high quality DNA extractions and paraffin embedded tissue specimens are not suitable for these methods. There are several reasons for the failure of the techniques using DNA isolated from paraffin-embedded tissues (Merkelbach *et al* 1997): (1) the degradation of target DNA, which may

occur due to long time lapses between surgical tissue removal and fixation, the type of fixative used, and the duration of the fixation; and (2) the fragmentation of nucleic acids due to formalin fixation (Lehmann *et al* 2001). The gold standard method for genotyping is sequencing and we confirmed accurate genotyping with this method.

MATERIALS AND METHODS

In our study, we used a combination of L1-consensus primer PCR amplification method and restriction fragment length polymorphism (RFLP) analysis in order to identify HPV types in cervical cancer specimens.

Sample collection. Five different paraffin-embedded tissue blocks were collected from women. All cases presented histological diagnosis of high grade intraepithelial lesions. 20 μm sections of each block were prepared and were used for DNA extraction.

DNA extraction from formalin-fixed and paraffin embedded tissues. Deparaffinisation was done using Xylene/ethanol. In brief, 1000 μl xylene (Merck Company, Germany) was added to 20 μm sections, agitated, heated for 15 min at 37°C then spun at 10500 g for 15 min. The supernatant was removed, fresh xylene added, and these steps repeated two times. Two identical washes with 100% ethanol (Merck Company, Germany) for 30 min, 37 °C, spinning at 10500 g for 15 min, was followed by air drying of the tissue pellet before digestion (Forsthoefel *et al* 1992). Digestion was performed by adding 150 μl of digestion buffer (Tris-Cl 100 mM pH=7.5, Tween 20 0.05% and Proteinase K 3 μl of 10 mg/ml (Fermentase)) to each tube and digested for 3 h at 55 °C, with gentle agitation every hour. Proteinase K was inactivated at 92.5 °C for 10 min (Forsthoefel *et al* 1992).

Purification was done by phenol/chloroform

(Merck Company, Germany) method. After gentle agitation in 150 μ l phenol/chloroform (1:1) mixture, samples were spun at 10500 g for 15 min. The upper phase was removed to a fresh tube and the step repeated two times. After centrifugation the upper phase was collected and added to 360 μ l 100% ethanol: 0.03 M sodium acetate (Merck Company, Germany) gently mixed and placed at -20°C for 2 h. The precipitated nucleic acid was pelleted by centrifugation at 10500 g for 15 min and, after twice washing with 70% ethanol, air dried. The samples were resuspended in 30 μ l sterile distilled water (Sambrook & Russell 2001).

Polymerase chain reaction DNA amplification.

Successful DNA extraction was assessed by PCR amplification of a 260bp fragment of β -globin gene using the primers GH20 and PCO4 (primers GH20 forward 5'- GAAGAGCCAAGGACAGGTAC -3' , PCO4 reverse 5'-CAACTTCATCCACGTTCCACC -3'), 2 μ l DNA sample, 1.5 mM MgCl₂ (Merck Company, Germany), 0.2 mM each dNTP (Cinagen, Iran), 5 pmol each primers, 1 U Taq polymerase (Cinagen, Iran). Amplification was carried out for 35 cycles (94°C for 30 s, 55°C for 45s, 72°C for 45 s) after an initial denaturation step of 94°C for 5 min, on a Techne Thermal Cycler (Techne, Genius, England). The cycles were followed by a 5 min extension at 72°C and the PCR product was visualized on a 1.5% agarose gel by ethidium bromide staining and UV photography (Uvidoc, BTS-20-M, EEC) (Resnick *et al* 1990).

The L1 region of HPV genome was detected by PCR using the consensus primers MY09/MY11 (MY09: 5'CGTCCMARRGGAWACTGATC 3', MY11: 5' GCMCAGGGWCATAAYAATGG 3', W= A+T, R= A+G, Y= C+T, M= A+C) , designed to amplify a segment of approximately 450 bp of the L1 gene of most genital HPV types. PCR mixture consisted of 50 pmol each primers, 2 μ l DNA sample, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 U Taq polymerase (Cinagen, Iran). Amplification was done for 35 cycles like β -globin gene

PCR(Resnick *et al* 1990) .

Restriction fragment length polymorphism (RFLP) analysis. A 70 μ l of the MY09/MY11 PCR product was used for restriction digestion. The enzymes for RFLP analysis of L1 region include: *BamH I*, *Dde I*, *Hae III*, *Hinf I*, *Pst I*, *Rsa I*, and *Sau III A I* with corresponding buffers (Roche, Germany). After incubation at 37°C for 3 h, digest products were electrophoretically resolved on 12% poly acryl amid gel (PAGE) and silver nitrate staining (Sambrook & Russell 2001).

RESULTS

The quality of DNA extracted by Beta-globin gene PCR amplification. To check the quality of DNA extracted from tumor samples, Beta-globin gene PCR was performed for 5 samples. The results in figure 1 indicated that the Beta-globin was identified in four of five samples. Figure 1 showed the 260 bp bands of the gene in sample 4, 5, 6, and 7, while 2 was negative.

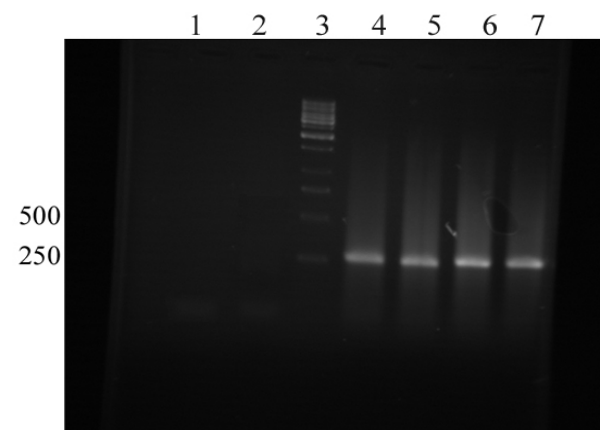


Figure 1. Beta-globin gene PCR. *Lane 1:* negative control of PCR, *Lane 2:* Beta-globin gene negative. *Lane 3:* DNA size marker. *Lane 4, 5, 6 and 7:* a 260 bp fragment of β -globin gene.

Determination the presence of HPV DNA in DNA extractions. To assess the presence of HPV in the Beta-globin positive samples, four positive

samples were analyzed for HPV L1 PCR. The results in figure 2 showed that two of four was HPV L1 positive and they showed about 450 bp fragments in PCR using MY09/MY11 consensus primers. Figure 2 indicate that samples 4 and 5, showed a positive band while samples 1 and 2 were negative.

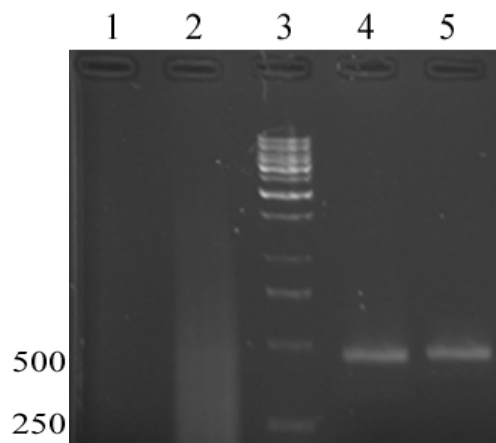


Figure 2. HPV L1 gene PCR. Lane 1 and 2: negatives, Lane 3: DNA size marker, Lane 4 and 5: 450 bp fragment of HPV L1 gene.

Restriction fragment length polymorphism (RFLP) for MY09/MY11 PCR product analysis.

The pattern of restriction fragment lengths from the L1 450 bp PCR product for the major HPV types are different. This pattern for HPV16 and HPV18 is followed: *BamH I*, *Dde I*, *Hae III*, and *Hinf I* enzymes are non-cutter for HPV16 L1 fragment. *Pst I*, *Rsa I*, and *Sau III A I* enzymes cut HPV16 L1 fragment and three fragments (216nt, 210nt, and 26nt with *Pst I*; 310nt, 72nt, and 70nt with *Rsa I*; 369nt, 63nt, and 20nt with *Sau III A I*) were produced.

For HPV18 L1 fragment, *Hae III* and *Hinf I* enzymes are non-cutter but *BamH I*, *Dde I* and *Pst I* enzymes cut it and two fragments (372nt and 83nt with *BamH I*; 432nt and 23nt with *Dde I*; 242nt and 213nt with *Pst I*) are resulted. *Sau III A I* enzyme cut HPV18 L1 fragment and produce three fragments (372nt, 63nt, and 20nt). HPV18 L1

fragment is cut with *Rsa I* like HPV16 L1 fragment but in HPV16 three fragments (as previously described) and in HPV18 five fragments (135nt, 125nt, 85nt, 72nt, and 38nt) are resulted (Schieffman et al 1991).

RFLP analysis was performed on MY09/MY11 PCR product and HPV typing by RFLP was successful in two of positive cases that one of them was HPV16 positive (figure 3) and the other was HPV18 positive (Figure 4).

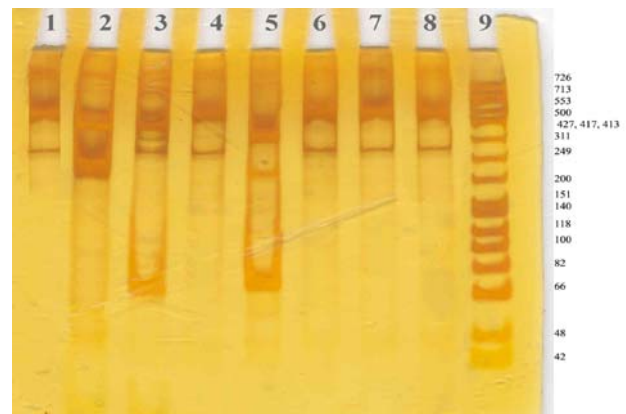


Figure 3. RFLP analysis on MY09/MY11 PCR product. It showed HPV16 genotype. Lane 1: un-cut fragment (negative control), Lane 2: *Pst I*, Lane 3: *Sau III*, Lane 4: *HAE III*, Lane 5: *Rsa I*, Lane 6: *Hinf I*, Lane 7: *BamH I*, Lane 8: *Dde I* and Lane 9: DNA size marker.

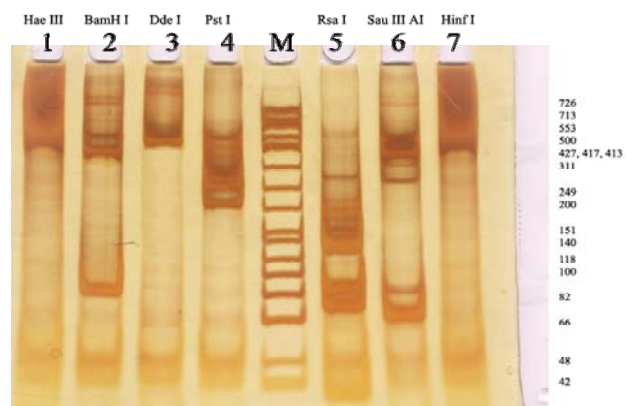


Figure 4. RFLP analysis on MY09/MY11 PCR product. It showed HPV18 genotype. Lane M: DNA size marker

DISCUSSION

Cervical cancer is one of the most common cancers in women, killing about 0.25 million women per year (Frazer *et al* 2004, Bernard *et al* 2004). A sub set of anogenital HPVs, the high-risk HPVs, which include HPV16 and HPV18, are associated with more than 90% of cervical carcinoma, a leading cause of death by cancer among women worldwide (Hausen 1991). Because HPV is considered to be an etiological agent of cervical cancer, screening of women for this agent could be potentially applicable means for the early detection of the disease.

High quality DNA extraction from paraffin-embedded tissue specimens is problematic because of degradation of target DNA and fragmentation of nucleic acids due to formalin fixation. In the present study, we identify a better method for DNA isolation from the samples. Digestion protocol and its buffer is also an important factor, and the yield of extraction is depended on it.

There are several methods for HPV detection in patient samples. In situ hybridization method is very specific and reliable method for HPV typing and the technique dose not required confirming by sequencing but the sensitivity of this method is 50-200 copy DNA per cell that it is not enough and some positive samples may be become false negative (Coutlee 1995, Cope 1997, Resnick 1990). In additional this method is very time consumption and difficult. PCR technique is very sensitive method for detecting HPV sequences in clinical samples. It can be detect one copy of HPV DNA in 10^5 cells, but in some cases, it may be become false positive (Zazove *et al* 1998). Application of PCR alone that used in some studies (Maleknejad *et al* 2006a, Farjadian *et al* 2003, Mokhtari-Azad *et al* 2006) may have non specific bands and should be confirmed by sequencing (Nadji *et al* 2006). PCR-RFLP using one or two enzymes (Maleknejad & Shahsavan 2006b) may have some error and should

be sequenced for confirming, but PCR-RFLP using several enzymes is reliable and it can be used for HPV typing without needing sequencing. PCR-RFLP, that we used, is sensitive and a rapid detection method for HPV typing. The technique allowed quantifying the prevalence of the more oncogenic HPV types in population to detect infection with less frequent HPV types (such as 45, 52, 53, and 54). This method can be used for the detection of HPV from clinical samples such as a fresh or paraffin-embedded samples. Some samples are β -globin gene PCR positive but HPV L1 negative. Its means is not that the sample is really HPV negative, it may be HPV positive and the difficulty of amplify the L1 fragment which is the larger (450 bp) might be partly attributed to the degradation of DNA in paraffin-embedded.

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