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Development of multiplex-PCR for identification of 8 genotypes of human papillomavirus (HPV)

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Abstract---Contamination with human papillomavirus (HPV) is one of the most significant risk factors for developing genital cancer. Since there is no possibility of detecting the virus and its subtypes using conventional cell culture, biochemical and serological methods, the molecular methods including multiplex Polymerase Chain Reaction (PCR) have specific importance in accurate, rapid and definite diagnosis of this virus. The present study was done to identify the 8 routine genotypes of the papilloma virus using the novel Multiplex-PCR (M-PCR). Twenty epithelial cells of vaginal samples were collected from the molecular diagnosis laboratory. Novel primers for diagnosis of 16, 18, 31, 33, 52, 53, 56, and 58 genotypes of the HPV were designed using the NCBI website and BLAST tool. We specifically designed and synthesized based on different gene regions (E6-7 and L2) and optimized the reaction using the samples of 8 investigated types. Genomic DNA was extracted from the samples and the M-PCR technique was used to detect HPV genotypes. Types 52, 53 and 31 were detected in 5 samples (25%). The M-PCR system developed in this project was able to identify 7 types with 100% specificity and without reacting with other types.

Keywords---human papillomavirus (HPV), genotypes, multiplex-PCR, detection.

Introduction

Microorganisms can cause severe health-threatening infections in human and animals (1-7). Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both women and men globally and is thought to be the most common sexually transmitted viral disease in the United States (8). It is estimated that the incidence of new infections in the United States ranges from 1 million to 5.5 million per year, and the prevalence is estimated to be as high as 20 million (9). HPV continues to be an important topic, as rates of infection appear to continue to be rapidly increasing.

Infection with the HPV is predisposing factor for the occurrence of human cervical carcinoma (10). There are more than 100 types of HPV, of which forty types are known to infect the genital tract and spread through sexual contact (11). Among these, persistent infection/integration of any of the 15 genotypes, namely, HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82 are implicated in causation of cervix carcinoma and are designated as high-risk HPVs (12). Unlike other DNA viruses, conventional cell cultures cannot detect HPV. Serological examination may also have false results in detection the HPV (13). However, molecular methods like polymerase chain reaction can directly detect the genomic DNA of the HPV in clinical samples (14). High sensitivity, specificity, accuracy and rapid procedure of detection were reported for the PCR method (15, 16). According to the high importance of HPV-related cervical carcinoma in Iran and absence of sensitive and specific method for detection of HPV virus, the present survey was done to develop the new M-PCR assay for detection of HPV routine genotypes in epithelial samples.

Materials and Methods

Ethics

The paper was approved by the ethical council (Ethics no. IR.IAU.TON.REC.1400.011). Authors tried to keep the personal information of patients secret.

Samples

Our study is of applied type paper. At this stage, in collaboration with the molecular diagnosis laboratory, a part of the epithelial cells of the vaginal samples of 20 patients was prepared in a period of 2 months. Also, with the cooperation of this laboratory, we prepared 8 positive controls for HPV types 16, 18, 31, 33, 52, 53, 56, and 58, which were used to optimize the reaction.

DNA extraction

According to the manufacturer's instructions, the genomic DNA was extracted from the isolates using the DNA extraction kit (Roche, Germany) (17-20).

Quality assessment of DNA

The purity (A260/A280) and concentration of the extracted DNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA) (21-25). Furthermore, the DNA's quality was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany) (26-30).

Primer design and M-PCR assay

Primer selection follows simple rules: primer length is 18-24 bp or more and GC content is 35-60%, so annealing temperature is 55-58°C or higher. Longer primers (28–30 bp) allowed the reaction to proceed at higher annealing temperatures and produced fewer products. To test for possible duplicate sequences, many of the primers used were checked against sequence databases at the National Center for Biotechnology Information (NCBI) using BLAST programs. Genome sequences were prepared using gen bank according to table 1.

Table 1. Access code of genomic sequences in gene bank

HPV types	Access code of genomic sequences
16	NC_001526
18	GQ180792
31	HQ537687
33	HQ537707
52	HQ537751
53	EF546482
56	EF177181
58	FJ385268

Considering the removal of parts of the HPV genome after integration into the human genome, especially the viral E1, E2 and L1 regions, it is better to design primers for other parts of the virus genome. In the multiplex system, in addition to the fact that the primers must be highly specific for each type, they must also have no homology with the human genome and no interference with each other. In this system, in addition to paying attention to the above points, each type must also be distinguishable from other types with a different reproduction size. Table 2 shows the nucleotide sites of the HPV genes.

Table 2. Nucleotide sites of the HPV genes

HPV genes	Nucleotide site
E6-7	102-829
E1	835-2802
E2	2744-3859
E4	3276-3620
L2	4379-5770
L1	5673-7268

In order to achieve the sequencing of the whole genome, the above 8 types were done with DNAMAN Version 1.2 software. The design of primer pairs based on different nucleotide locations was manually designed and then checked with the online software of IDTD (Oligoanalyzer) in terms of melting point and the state of the stem loop and other things. Table 3 shows the list of primers used for detection of HPV types.

Table 3. Primer sequences for detection of HPV types.

Genes	Primer sequence (3'-5')	Product size (bp)	Volume of reaction (50)	Temperature and time
Hp18-F	GAA CGA CTC CAA CGA CGC	122	1 cycle: 15 min: 95 °C 35 cycles: 60 s: 95 °C 30 s: 62 °C 2 min: 72 °C 1 cycle: 5 min: 72 °C	10X PCR buffer: 5 µL Mgcl ₂ : 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL
Hp18-R	TAG AAG GTC AAC CGG AAT			
Hp31-F	AAC AAC ACC ACC ACA TCG	180		
Hp31-R	TGC ACT GAT AAC CCC ACA			
Hp53-F	ACA TCC ATG GAT CGT CGG	292		
Hp53-R	TTA GTG CTT CCA GGC TAG			
Hp52-F	CCT ACA ACC ACC ACA GAA	418		
Hp52-R	ATA TYA CAA TGA CAT GAC			
Hp33-F	GCA CCA ACA TCT GTA CCT	562		
Hp33-R	TTC TAC TGT ACC TAA TGC			
Hp16-F	GAC ACA ACA AGA GTA ATG	610		
Hp16-R	ATA GTT TGA ATA GAT GAC			
Hp58-F	GGG TTG TAT <u>TAT</u> ATA CAT	693		
Hp58-R	CAAAAATAGGTAATATCA			

A programmable DNA thermocycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions (31-34). In addition, amplified samples were analyzed by electrophoresis (120 V/208 mA) in a 2.5% agarose gel stained with 0.1% ethidium bromide (0.4 µg/ml) (35-37). Besides, UVI doc gel documentation systems (Grade GB004, Jencons PLC, London, UK) were used to analyze images (38-40).

Data analysis

Data analysis was performed by SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's exact two-tailed tests were performed to assess any significant relationship between the HPV types distribution (41). Besides, P-value < 0.05 was considered statistically significant (42, 43).

Results

Sequencing results of E6-7 and L2 genes of HPV

To design suitable primers for the Multiplex-PCR system, these primers must have no homology with the human genome and also interference with each other. For this purpose, 3 gene regions E6-7 and L2 were investigated (Figure 1).



Figure 1. Exhibition the ranking of only the E6-7 and L2 gene regions of the six studied types. Conserved regions are shown as colored bases, and in the absence of homology, the bases are shown as colorless

Optimization results

In the optimization phase, multiplex reaction mixing was performed with 8 clinical samples, each of which was infected with one of the studied types, during which the best PCR thermal cycle conditions and the values of each of the reaction components were obtained. As you can see in the figure 2, each type of replication tape shows exactly according to the sizes determined during the primer design.

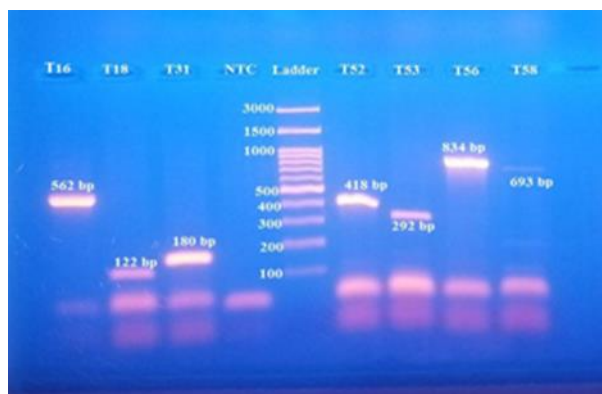


Figure 2. Typing of HPV and performing a multiplex reaction with a sample infected with only one of the studied types

Seven types of human papillomavirus and performing a multiplex reaction with a sample that was infected with only one of the studied types, and due to the rarity of type 33, we could not prepare a positive control sample for it. Each type shows

exactly the size of the specified reproduction product. The molecular weight in the other well is 100 to 3000 match pairs of South Korea's SMOBIO company. M-PCR results on a number of samples to test the molecular screening of the HPV. At this stage, samples were extracted in the previous season, their total DNA was extracted and developed through the multiplex PCR reaction, and their results can be seen. The results of these 20 samples are shown in figures 3 and 4.

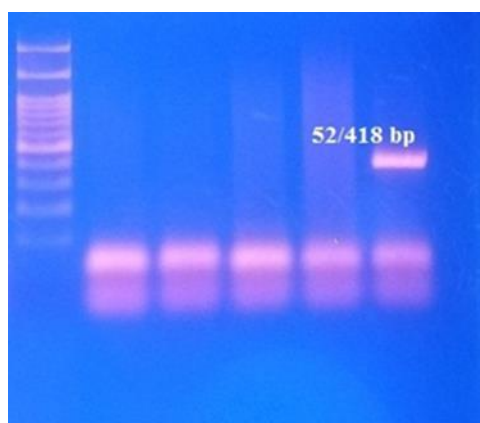


Figure 3. M-PCR results of a number of clinical samples. From right to left: patient 1 has type 52, and patients 2 to 5 lack one of the 8 investigated types. The last well of marker size 100 to 3000 SMOBIOI.

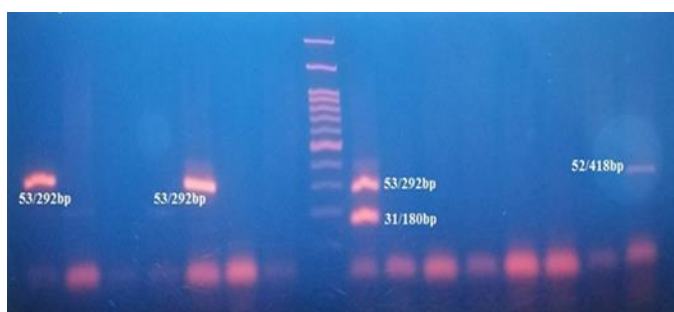


Figure 4. M-PCR results of a number of clinical samples. From left to right: patient 1 with type 53, sample 5 with type 53. Sample 8 has types 53 and 31, sample 15 has type 52. Other samples lack one of the 8 investigated types. As indicated in the above results, in the 20 samples we examined, there were only types 52 with size bp418, type 53 with size bp292 and type 31 with size bp180, and the rest of the samples had types other than the 7 types we examined. which could not be recognized by our system. On the other hand, in the system optimized by us, the 7 types selected for examination are completely recognizable by the system designed by us.

Discussion

HPV is the cause of warts in all human populations and sometimes the infection caused by this virus leads to cancer (44). These viruses infect the skin and mucous epithelial tissue of different anatomical areas. To date, more than a hundred different types of human papillomavirus have been identified, $\frac{1}{3}$ of which

infect the epithelial cells of the genital tract (45). HPV types that infect the genital tract are divided into two categories: low-risk types such as HPV-6 and HPV-11 cause benign warts and do not progress to cancer and usually recover, and high-risk types include HPV types 16, 18, 33, 31, and 45 are related to the development of genital cancer and are found in 99% of cervical cancers, among which HPV-16 is more prevalent (46). Infection with high-risk types of HPV is not limited to the genitals, and for example, about 20% of oropharyngeal cancers contain high-risk types of papilloma viruses (47). The HPV genome is functionally divided into three regions: the first region is the non-coding region called the long control region (LCR). This region includes the main promoter and regulatory sequences of DNA replication. Also, there is the highest prevalence of viral genome in this region. The second region is the premature region, which includes E6, E5, E3, E2, E1 and E7 and is involved in viral replication and carcinogenesis. The third region is the lag region that encodes the structural proteins L1 and L2 for the viral capsid (48, 49).

In the present survey, E6-7 and L2 regions were used to identify the 16, 18, 31, 33, 52, 53, 56, and 58 genotypes of the HPV using a novel M-PCR method. Our findings showed the high potential of the designed M-PCR method for detection of HPV genotypes. Several similar surveys have been conducted in this field globally. Zendinia et al. (2016) (50) used M-PCR for rapid and sensitive detection of HPV 16, 18, 31, 33 and 45 Genotypes in cervical cancer. The study was conducted on 100 cervical lesions of women. Among the 100 evaluated samples, 82 cases were found to be HPV positive. Among them, the highest rate of infection was HPV 16. Also, 30 HPV positive samples had two or more HPV types. The M-PCR method used in their study can provide a rapid, sensitive and economical method for detecting viral infections and can be used for small volumes of vaginal samples. Masoumalinjad et al. (2018) (51) performed the molecular identification of HPV in tissue samples from cervical cancer patients using the M-PCR method. Among the 60 samples, 19 HPV positive cases and 41 negative cases were confirmed. This study showed that M-PCR using specific primers for HPV-16 and HPV-18 is a suitable and accurate method for detecting this virus. Kiani et al. (2015) (52) identified and typed HPV by the M-PCR in cervical cancer. Total DNA of 66 samples were extracted and nested M-PCR was used with first round PCR using primers designed based on GP-E6/E7 conserved regions to amplify genomic DNA of all known mucosal HPV genotypes, and second round PCR was performed by a mixture of multiplex PCR primers. Human papillomavirus infection was observed in 78.8% of the samples, the highest prevalence of HPV was 16%, while simultaneous infections with two types were observed in 10.6%. The NMPCR method is more convenient and easier to analyze the results, which is important for rapid diagnosis and patient management.

Conclusion

The best way to prevent the spread and emergence of HPV-related cancers is to create different screening programs using molecular methods with high sensitivity and specificity. Also, diagnosis is the basis of effective treatment and the country's need for development. It is necessary to use and launch new diagnostic methods with sensitivity and clinical specificity, fast and cost-effective to identify common high-risk genotypes of HPV virus. Certainly, the identification and

examination of genetic polymorphisms as predictive markers play a significant role in planning, determining the policy of treatment strategies and improving the level and development of health and treatment in the country, so we believe that the use of multiplex PCR technique and design Primers based on different gene regions can be useful for HPV virus detection, although in our research using this system we were only able to detect 7 common types of this virus, but in future research, this system can be developed to use It is possible to identify more types of this virus and provide a better treatment system for patients using it.

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