Journal of Microbiological Methods 97 (2014) 44–50

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Detection of major HPVs by a new multiplex real-time PCR assay using type-specific primers

Fatih Şahiner [a,*], Ayhan Kubar [a,1], Mehmet Yapar [a,2], Kenan Şener [a,3], Murat Dede [b,4], Ramazan Gümral [a,5]

[a] Department of Medical Microbiology, Gulhane Military Medical Academy, Ankara, Turkey
[b] Department of Obstetrics and Gynecology, Gulhane Military Medical Academy, Ankara, Turkey

ARTICLE INFO

Article history:
Received 20 November 2013
Received in revised form 13 December 2013
Accepted 13 December 2013
Available online 21 December 2013

Keywords:
Cervical cancer
Genotyping
Screening test
Multiplex PCR

ABSTRACT

In this study, we aimed to develop a cost-effective, practical, and sensitive method to be used for the diagnosis of HPV infections. The presence of HPV-DNA was investigated in cervical smear samples using three different methods: MY09/11 consensus PCR, TaqMan-based type-specific real-time PCR, and SYBR Green-based multiplex PCR. Of the 315 samples, 21.6% (68/315) were HPV-DNA positive by using at least one of the three methods. The relative sensitivities of MY09/11 PCR, type-specific PCR, and multiplex PCR were found to be 86.8% (59/68), 91.2% (62/68), and 91.2% (62/68), respectively. Genotyping analyses were successfully carried out in 62 of 68 HPV-DNA positive samples, and 77 isolates (8 low-risk and 69 high-risk HPV) were identified, while six samples were determined to be positive by consensus PCR only and could not be genotyped. The type distribution of the 69 high-risk HPV strains was as follows: 37.7% HPV 16, 13.0% HPV 52, 11.6% HPV 58, 7.2% HPV 18, 7.2% HPV 31, 7.2% HPV 68, 4.3% HPV 35, 4.3% HPV 39, 4.3% HPV 82, 1.4% HPV 33, and 1.4% HPV 45. Our data suggests that the diagnosis of HPV infections using only consensus PCR may lead to epidemiologically significant data loss, and that our multiplex PCR is more sensitive than consensus PCR and lower in cost than the type-specific PCR. We believe that the SYBR Green-based multiplex PCR may be useful and cost-effective for other microbiological fields. In addition, type-specific screening of HPV-DNA gives more reliable results, but it may also be used in combination with consensus PCR if the type spectrum of the test is not large enough.

1. Introduction

Human papillomaviruses (HPVs) belonging to the papillomaviridae family are non-enveloped viruses that are comprised of a double-stranded circular DNA within an icosahedral capsid (Jo and Kim, 2005; Lambert and Collins, 2008). To date, more than 200 HPV genotypes have been identified, and more than forty of these have been shown to cause anogenital infections in humans (Psyrri and DiMaio, 2008; zur Hausen, 1996). A sub-group of HPVs which are associated with pre-cancerous or cancerous lesions have been described as "high-risk" HPVs (Muñoz et al., 2003). Cervical cancer and other anogenital cancers may occur in a small percentage of people infected with high-risk HPVs, many years after the onset of infection (Psyrri and DiMaio, 2008). Cervical cancer is the second most frequently occurring cancer worldwide, and one of the ten most common cancers in Turkey (Ferlay et al., 2004). Screening programs that include Pap smears and HPV-DNA testing are very important for cervical cancer prevention, because this cancer is a serious but treatable disease (Psyrri and DiMaio, 2008).

HPV infection is mainly diagnosed using molecular methods based on the detection of viral DNA in tissue biopsies or exfoliated cells at the site of infection, because HPV cannot be grown in conventional cell cultures, and serological assays have only limited accuracy (Gravitt and Viscidi, 2004; Molijn et al., 2005; Mothershed and Whitney, 2006). Hybridization-based methods (e.g., filter in situ hybridization, southern blotting, and dot blot hybridization) and conventional PCR tests have been used for this purpose in the past (Gravitt and Viscidi, 2004). Today, numerous diagnostic techniques with increased sensitivity and specificity have been developed for the detection and/or genotyping of HPV in clinical samples. The most widely used of these methods can be grouped into three categories: signal amplification-based systems (Molijn et al., 2005; Stillman et al., 2009), real-time PCR-based methods that are based on target amplification by using consensus or type-specific primers (Depuydt et al., 2007; Gravitt and Viscidi, 2004; Sahiner et al., 2012), and different techniques for the detection and analysis of PCR products (Gravitt et al., 2000; Kleter et al., 1999; Qi et al., 2007; Shen-Gunther and Rebeles, 2013; Takács et al., 2008).

The consensus PCR assays (MY09/11, GP5+/6+, PGMY09/11, and SPF-10) have been widely used throughout the world due to their cost-effectiveness and ease of application. MY09/11 PCR is one of the

* Corresponding author. Tel.: +90 312 304 3481; fax: +90 304 34 02.
E-mail addresses: fsvirol@gmail.com (F. Şahiner), akubar@hotmail.com (A. Kubar), myapar@gata.edu.tr (M. Yapar), ksener@gata.edu.tr (K. Şener), mdede@gata.edu.tr (M. Dede), rgumral@gata.edu.tr (R. Gümral).
[1] Tel.: +90 312 304 3414.
[2] Tel.: +90 312 304 3436.
[3] Tel.: +90 312 304 3420.
[4] Tel.: +90 312 304 5811.
[5] Tel.: +90 312 304 3433.

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http://dx.doi.org/10.1016/j.mimet.2013.12.012

most commonly used consensus PCR methods, and it targets a consensus region (approximately 450 bp lengths) of the HPV L1 gene (Depuydt et al., 2007). This method is considered to detect over 40 different genital HPV types (Gravitt and Viscidi, 2004). The type-specific real-time PCR assays are another diagnostic technique commonly used (Depuydt et al., 2007; Sahiner et al., 2012). These methods are highly sensitive and able to detect, quantify, and genotype HPV-DNA; however, carrying out type-specific PCR on all samples for each HPV genotype may be difficult and costly, because a large number of different HPV types can cause genital infections. The complexity of HPV detection and typing leads to the development of multiplex PCR to detect various HPV types in one PCR reaction (Poljak and Kocjan, 2010; Takács et al., 2008). SYBR Green-based PCR was used for the detection and quantification of nucleic acid targets, and have been adapted for use in multiplex real-time PCR protocols (O'Mahony and Hill, 2002; Pérez et al., 2012; Varga and James, 2005). This method works on the principle of measuring fluorescence signals that are generated following the binding of SYBR Green to PCR products. The specificity of fluorescence signal can be determined melting curve analysis, which is dependent upon the length and the G-C content of the amplicons (O'Mahony and Hill, 2002). Although numerous SYBR Green-based real-time PCR protocols have been described to date, SYBR Green-based duplex (De Crignis et al., 2010; Gibellini et al., 2006; Jothikumar et al., 2003) and multiplex PCR methods (Guion et al., 2008; Pérez et al., 2012; Varga and James, 2005) were described only in a few studies. In this study, we aimed to develop and optimise a cost-effective, practical, and sensitive real time PCR assay based on multiplex SYBR Green detection for the diagnosis and follow-up of HPV infections, and screening associated lesions.

2. Materials and methods

2.1. Study subjects

This study was conducted after approval from the local ethical committee (Gulhane Military Medical Academy, Ankara, Turkey; Decision number: 1491-61-11/1539-1535). Cervical smear samples were obtained from 315 women during routine gynecological examinations, after informed consent was obtained from the study subjects. Repeat samples from the same patients were excluded from the study.

2.2. DNA extraction

HPV-DNA was extracted from smear samples using the standard phenol-chloroform-isoamyl alcohol method (Sambrook et al., 1989). Briefly, cervical smear samples were suspended in 500 μl of TE buffer (10 mM Trishydrochloride, 1 mM EDTA, pH 8), and homogenized by vigorous mixing on a vortex. A 100 μl of mixed specimen was placed in 10 μl of protease solution (65 mg/ml) (Sigma–Aldrich Corp, St. Louis, MO, USA) and 250 μl of K buffer for 60 min at 45 °C. Following centrifugation at 10,000 *g* for 10 min at 12 °C, DNA was extracted from the supernatant using a mixture of 250 μl alkali phenol and 250 μl chloroform-isoamyl alcohol (24: 1), and then precipitated using 500 μl isopropyl alcohol. DNA was washed in 75% ethyl alcohol at 10,000 *g* for 5 min at 4 °C, air-dried at 37 °C, and dissolved in 100 μl distilled water.

2.3. Real time PCR analyses

All smear samples were tested using three different real time PCR assays: MY09/11 consensus PCR, TaqMan-based type-specific real-time PCR, and SYBR Green-based multiplex PCR. All PCR processing and analyses were performed using an ABI Prism 7500 Sequence Detection system (Applied Biosystems, USA).

2.3.1. MY09/11 Consensus PCR

All samples were tested by a previously described SYBR Green-based real-time PCR method using MY09/11 primers (Bauer et al., 1992) (MY09: 5′-cgtccmarrggawactgatc-3′) (MY11: 5′-gcmcagggwcataay aatgg-3′). After the completion of 40 PCR cycles, the melting-curve data were obtained by continuous fluorescence acquisition from 55 to 95 °C, with a thermal transition rate of 0.1°C/s. The melting temperature of the MY09/11 products was determined to be 82 ± 1.5 °C using optimization studies.

2.3.2. TaqMan-based type-specific real-time PCR

All samples were tested in separate tubes for each genotype, regardless of consensus PCR results. PCR analyses were performed by using the type-specific primers and TaqMan probes that were designed from the L1, E6, and E7 genes of 16 different HPVs (HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 52, 53, 66, 58, 59, 68, and 82) (Table 1). The reaction mixture was prepared as follows: 1.25 U Hot Start *Taq* DNA polymerase (Bioron, Germany), 10 pmol of each primer, 2.5 pmol TaqMan probe, 0.2 mM dNTP mix, and 2.5 mM $MgCl_2$. PCR amplifications were carried out after the addition of 5 μl of the sample containing template DNA in a final volume of 25 μL of the PCR reaction mixture. The amplification conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s each at 95 °C and 1 min at 60 °C (annealing-extension step) (Kubar et al., 2004). The primers and probes were designed by using the OligoYap 4.0 software program (Yapar et al., 2005), and confirmed by using a new software program (OligoBee 1.0) that was developed in our laboratory for molecular biology research. All primer and probe sequences were analyzed with the GenBank BLAST database for specificity, and were synthesized by MWG-Biotech (Ebersberg, Germany). The TaqMan probes were labeled with fluorescent reporter dyes (FAM: 6-carboxy fluorescein and JOE: 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein) at the 5′ end, and with black hole quencher (BHQ) as the non-fluorescent quencher at the 3′ end. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, and the PCR mixture without template DNA was used as a negative control. HPV isolates used as a positive control were originated from the strains of our laboratory. Firstly, genomic DNAs extracted from clinical samples were amplified using type-specific primers, and then these amplicons were sequenced in both directions using PCR primers on an ABI PRISM 3130XL Genetic Analyzer. After compatible results with TaqMan-based type-specific real-time PCR and sequencing, type-specific amplicons of each of the 16 HPV genotypes and the GAPDH amplicon were cloned into plasmid vectors using a TOPO TA Cloning System (Invitrogen, USA). We didn't sequence all positive samples detected by TaqMan-based type-specific real-time PCR, since this method includes sequence-based detection system. Serial plasmid dilutions (10^8–10^1 copies/ml) were prepared for the determination of detection sensitivities of PCR assays.

2.3.3. SYBR Green-based multiplex PCR

Multiplex PCR reactions were performed for 16 different HPV types within the four reaction tubes by using the same primers that were used in the TaqMan-based type-specific real-time PCR (Table 1). PCR reactions were carried out in a total of 25 μl volumes containing 10 pmol of each primer, a final concentration of 1X SYBR Green, 0.25 mM dNTPs, 2.0 mM $MgCl_2$, and template DNA. PCR amplification cycles were as follows: a single cycle for 10 min at 95 °C (hot-start *Taq* DNA polymerase activation), followed by 40 amplification cycles of 15 s each at 95 °C and 1 min at 60 °C (annealing-extension step) (Kubar et al., 2004). After completion of the 40 PCR cycles, the melting-curve data were obtained by continuous fluorescence acquisition from 55 to 95 °C, with a thermal transition rate of 0.1°C/s. Positive melting temperature values of four different reaction tubes were determined by optimization studies while trying out different combinations of HPV types. The samples, which have Tm values within the determined ranges, were accepted