Clinica Chimica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Development of reference materials to detect 15 different human papillomavirus genotypes

Jee Eun Rhee [a], Young Soon Kang [c], Hyun Hee Seo [c], Ju-yeon Choi [a], Mee-Kyung Kee [a], Tae-Jin Kim [d], Sung Ran Hong [b,*], Sung Soon Kim [a,**]

[a] Division of AIDS, Center for Immunology and Pathology, Korea National Institute of Health, Chungcheong-Bukdo, Republic of Korea
[b] Department of Pathology, Cheil General Hospital & Women's Healthcare Center, College of Medicine, Kwandong University, Seoul, Republic of Korea
[c] Office of Genome Research and Development, Cheil General Hospital & Women's Healthcare Center, College of Medicine, Kwandong University, Seoul, Republic of Korea
[d] Department of Obstetrics and Gynecology, Cheil General Hospital & Women's Healthcare Center, College of Medicine, Kwandong University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 28 November 2013
Received in revised form 29 January 2014
Accepted 17 February 2014
Available online xxxx

Keywords:
Human papillomavirus
Reference material
Genotype
Proficiency

ABSTRACT

Accurate human papillomavirus (HPV) typing is essential for evaluating and monitoring HPV vaccines in cervical cancer screening and in epidemiological surveys. In our country, different HPV DNA detection and genotyping methodologies have been established for diagnosing and monitoring HPV-related disease in clinical practice and for research. However, there is a lack of reference materials to standardize the methods for HPV detection and genotyping. In this study, we constructed candidate reference materials comprising 15 targets (13 types of high-risk HPV, two types of low-risk HPV). We evaluated whether the candidate reference materials could be used as the reference for HPV detection and genotyping using quantitative real-time polymerase chain reaction. Standard curves for the wide linear range (10^1–10^6 copies/μL) produced high correlation regression coefficient R^2 of 0.99. The reaction efficiencies were 96.3% to 101.2% for the standard curves, indicating highly efficient reactions. Specific genotypes were detected in single or multiple mixed samples. Our results suggest that these reference materials may provide useful standards for standardizing quality assurance for different HPV-typing assays and for proficiency testing in diagnostic laboratories.

1. Introduction

Cervical cancer is the second most common type of cancer among women aged 15–44 years worldwide, and human papillomavirus (HPV) infection is the cause of nearly 100% of cervical cancers [1–3]. The most important high-risk types are HPV-16 and -18, which account for about 70% of all invasive cervical cancers worldwide. HPV types 31, 33, 35, 45, 52, and 58 are associated with about 15% of cervical cancer cases [4].

The prophylactic vaccines against HPV-16 and -18 are Gardasil® (Merck and Co., NJ, USA; a quadrivalent vaccine for HPV-6, -11, -16, -18) and Cervarix® (GlaxoSmithKline Biologicals, Brentford, UK; a bivalent vaccine for HPV-16 and -18). These vaccines have been licensed by Korea Food and Drug Administration since 2007 and 2008, respectively. Both vaccines have been demonstrated to induce high titers of vaccine type-specific neutralizing antibodies and are effective in preventing persistent HPV infection and related cervical lesions [5–8]. These vaccines have increased the monitoring of HPV and have led to studies to document the effectiveness of the vaccines.

Accurate and internationally comparable HPV DNA detection and genotyping assays play an essential role in epidemiological studies of HPV surveillance, vaccination impact monitoring, and prevention of the development of cervical cancer from infection. Diverse methods for genotyping HPV DNA are used as the primary tool to measure HPV disease burden and vaccine impact. The accurate detection and genotyping of HPV DNA in clinical samples are important in ensuring that all HPV laboratories obtain results that are consistent, meaningful, and comparable.

In our country, different HPV DNA detection and genotyping methodologies have been established for diagnosing and monitoring HPV-related disease in clinical practice and in research. However, we lack well-characterized reference materials to evaluate the performance of these laboratories and to determine the sensitivity and specificity of HPV DNA detection and quantification. In response to the need, we constructed recombinant HPV plasmid DNAs that encode the full-length L1 protein of 15 different HPV genotypes and evaluated whether these could be used as the reference materials for HPV genotyping. We assessed their suitability for use in assays to detect and genotype HPV DNA in HPV clinical laboratories and in the research field.

* Correspondence to: S.R. Hong, Department of Pathology, Cheil General Hospital & Women's Healthcare Center, College of Medicine, Kwandong University, Republic of Korea. Tel.: +82 2 2000 7661; fax: +82 2 2000 7779.
** Correspondence to: S.S. Kim, Division of AIDS, Center for Immunology and Pathology, Korea National Institute of Health, Republic of Korea. Tel.: +82 43 719 8410; fax: +82 43 719 8459.
E-mail addresses: srh7661@naver.com (S.R. Hong), sungskim@korea.kr (S.S. Kim).

http://dx.doi.org/10.1016/j.cca.2014.02.013
0009-8981/

2. Materials and methods

2.1. HPV infectious samples

To obtain the L1 gene of the 15 HPV genotypes (HPV-6, -11, -16, -18, -31, -33, -39, -45, -51, -52, -56, -58, -59, -66, and -68b), we used cervical DNA collected from the Korea HPV Cohort Study [9]. The Korea HPV Cohort Study is a multicenter, open, cross-sectional and prospective cohort study of participants in four metropolitan cities in Korea. The cohort recruits women who have been shown to be HPV positive with a cytological abnormality on a Pap smear; the HPV test results were obtained from the participating hospitals. Cervical DNA was extracted from exfoliated cervical cells after clinical examination. The target HPV-infected cervical DNAs of at least one of the 15 target genotypes collected in the cohort study were selected. We confirmed their genotypes using the Linear Array HPV genotyping test (Roche Diagnostics, Basel, Switzerland).

2.2. Construction of the specific genotype-targeted plasmid DNA

2.2.1. Polymerase chain reaction (PCR) amplification

The HPV L1 gene was amplified with specific genotype primers (Table 1). To amplify the 15 individual L1 genes, 1 U of Taq DNA polymerase, 250 μM dNTP, 1.5 mM $MgCl_2$, 40 pmol of corresponding primers (Table 1), and 10 ng of cervical DNA were added to a 20 μL reaction volume. The optimized PCR program for the L1 gene was as follows: 94 °C for 5 min; then 30 cycles at 94 °C for 30 s for denaturation, 55 °C at 30 s for annealing; 72 °C for 1 min, and 30 s for extension; followed by 72 °C for 10 min. The amplified products were separated on a 1.5% agarose electrophoresis gel and purified using the QIAquickGel Extraction Kit (Qiagen, Hilden, Germany).

2.2.2. Cloning and sequencing of the L1 gene into pGEM-T vector

The full-length L1 gene of the 15 genotypes was purified from the agarose gels. Each purified DNA was ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109. The nucleotide sequences of the plasmid DNA fragment were determined by primer walking (Macrogen, Seoul, Korea). Comparisons of the nucleotide and deduced amino acid sequences were conducted using BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information (NCBI)). The purified plasmids were then linearized with *Eco*RI for 3 h at 37 °C, and their expected size was confirmed.

2.3. Real-time PCR

For TaqMan real-time PCR, primers and probes corresponding to each specific genotype L1 were designed using Primer V3 software (Table 2) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). The probe contained a reporter dye 5′,6-carboxyfluorescein (for HPV-6, -16, -31, -45, -52, -58, and -66) or 5-hexachlorofluorescein (for HPV-11, -18, -33, -39, 51, -56, -59, and -68b) at the 5′ end and a quencher dye (NFQ-MGB) at the 3′ end. TaqMan real-time PCR was performed in a 20 μL final volume containing 1 μL of each serially diluted recombinant DNA (1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 1×10^1 copies/μL), 10 μL of 2× LightCycler 480 Probe Master Mix (Roche), 0.5 μL of 10 pmol HPV genotype-specific forward primer, 0.5 μL of 10 pmol HPV genotype specific reverse primer, 0.1 μL of uracil DNA glucosidase (NEB Ltd., Hertfordshire, UK), 0.1 μL of HPV DNA type-specific TaqMan MGB probe, and 7.8 μL of H_2O. The PCRs were all run on a LightCycler 480 II (Roche) using the following program: 95 °C for 10 s and 45 cycles at 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. The fluorescence signals were measured once during each cycle at the extension step, and the data were then analyzed. Each reaction was repeated three times in triplicate.

2.4. Linear array assay

Extracted recombinant DNA was tested using the Linear Array HPV Genotyping Assay (Roche). The linear array genotyping system uses PCR amplification of DNA, followed by a reverse line blot hybridization assay to detect the amplified DNA products. The reaction volume

Table 1
Oligonucleotides used to amplify genotype-specific L1 genes.

Oligonucleotide	Oligonucleotide sequence (5′ → 3′)	Size (bp)	GenBank accession no.
HPV-6F	ATGTGGCGGCCTAGCGACAGCAC	1503	AF092932
HPV-6R	TTACCTTTTGGTTTTGGCGCGCTTAC		
HPV-11F	ATGTGGCGGCCTAGCGACAGCAC	1506	U55993
HPV-11R	TTACTTTTTAGTTTTGGTGCGCTTAC		
HPV-16F	ATGCAGGTGACTTTTATTTACATCC	1596	K02718
HPV-16R	TTACAGCTTACGTTTTTTGCGTTTAG		
HPV-18F	ATGTGCCTGTATACACGGGTCCTG	1707	X05015
HPV-18R	TTACTTCCTGGCACGTACACGCAC		
HPV-31F	ATGTCTCTGTGGCGGCCTAGCGAGG	1515	JO4353
HPV-31R	TTACTTTTTAGTTTTTTACGTTTT		
HPV-33F	ATGTCCGTGTGGCGGCCTAGTGAGG	1500	M12732
HPV-33R	TTATTTTTTAACCTTTTTGCGTTTT		
HPV-39F	ATGGCTCTGTGGCGGTCTAGTG	1518	JN104070
HPV-39R	TTATTTAGACACACGTTTACGTTTGTG		
HPV-45F	ATGGCACACAATATTATTTATGGCC	1620	X74479
HPV-45R	TTATTTCTTACTACGTATACGTACA		
HPV-51F	ATGGCATTGTGGCGCACTAATGAC	1515	M62877
HPV-51R	TTACTTTTTAACACGTTTACGTTTGGC		
HPV-52F	ATGGTACAGATTTTATTTTACATCC	1590	X74481
HPV-52R	TTACCTTTTAACCTTTTTCTTCTTT		
HPV-56F	ATGATGTTACCCATGATGTATATATAC	1605	X74483
HPV-56R	CTACCGCCTTTTACGTTTTGCTG		
HPV-58F	ATGGTGCTGATTTTATGTTGCACC	1575	JX313772
HPV-58R	TTATTTTTTAACCTTTTTGCGTTTGGTGG		
HPV-59F	ATGGCTCTATGGCGTTCTAGTGACA	1527	X77858
HPV-59R	CTATTTTCTGGAAGACTTGCGACGC		
HPV-66F	ATGGCGATGTGGCGGCCTAGTGACA	1512	U31794
HPV-66R	CTATCGTTTTTTACGTTTAGCTGGT		
HPV-68bF	ATGGCATTGTGGCGCTCTAGCGAC	1518	FR751039
HPV-68bR	TTACTTTGACACACGTTTACGTTTGTGC		

Please cite this article as: Rhee JE, et al, Development of reference materials to detect 15 different human papillomavirus genotypes, Clin Chim Acta (2014), http://dx.doi.org/10.1016/j.cca.2014.02.013