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ORIGINAL ARTICLE

A Rapid, Sensitive and Type-Specific Detection of High-Risk HPV-16 and HPV-18

Sanjay Gupte[1,2] · Sreeja Parthasarathy[2] · Preeti Arora[2] · Sharvari Ozalkar[2] · Shweta Jangam[2] · Ketaki Rajwade[2] · Pradnya Nikam[2] · Sarjan Shah[2]

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Abstract

Human papillomavirus (HPV) infection, particularly infection with HPVs 16 and 18, is a major cause of cervical cancer. The current high-risk HPV screening or diagnosis tests use cytological or molecular techniques that are primarily based on qualitative HPV DNA detection. Comparative studies, however, revealed that different assays have varying sensitivities for detecting specific HPV types. Here, we developed and optimized a sensitive PCR (Polymerase Chain Reaction) assay for detection of high-risk HPV-16 and HPV-18. The PCR parameters were optimized, and analytical specificities were validated. Performance of developed PCR assay was evaluated in clinical samples ($n = 100$) which showed 100% specificity for both the assays and 96.97% and 94.12% sensitivity for HPV-16 and HPV-18, respectively. The developed assay demonstrated high sensitivity and specificity for detection of high-risk HPV-16 and HPV-18, making it applicable to routine HPV detection practices.

Keywords Human papillomavirus · PCR · Detection · Cervical cancer

Introduction

Human papillomaviruses (HPVs) are non-enveloped, double-stranded, circular DNA viruses having a genome size close to 8 kilobases [1]. HPV infection causes cutaneous and genital warts [2]. More than 200 HPV types are currently described which are further classified in to two major groups namely low risk (LR) (e.g., types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81) and high risk (HR) (e.g., types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) based on their ability to promote cell transformation, which leads to cancer development [3].

Cervical malignancy is the fourth most common malignancy in women worldwide and a significant cause of morbidity and mortality [4]. According to epidemiological studies, the leading etiological cause of cervical cancer is persistent infection with a high-risk HPV type. Among all HR HPV types, the most common is HPV 16 genotype (50–60%) responsible for causing cervical cancer followed by HPV 18 (11–15% of cervical cancer) [5]. Therefore, high-risk HPV testing may have implication for cervical cancer screening as well as management of women with cervical lesion. Early detection of the HR-HPVs using a simple and sensitive method is immensely important for therapeutic measure against cervical

Dr. Sanjay Gupte (MD) is a director Gupte Hospital, Postgraduate Institution and Centre of Research in Reproduction, Pune, India Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Sreeja Parthasarathy (MSc.) is a Senior Scientific Officer Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Preeti Arora (Ph.D.) is a Senior Genomic Scientist Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Sharvari Ozalkar (MSc.) is a Senior Scientific Officer Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Shweta Jangam (MSc.) is a Clinical Bioinformatician Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Ketaki Rajwade (MSc.) is a Senior Scientific Officer Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Pradnya Nikam (MSc.) is a Senior Scientific Officer Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India; Sarjan Shah (MD) is a Director Greenarray Genomic Research and Solutions of ADPL, Kothrud, Pune, India.

✉ Sanjay Gupte
drsanjaygupte@guptehospital.com

[1] Gupte Hospital, Postgraduate Institution and Centre of Research in Reproduction, Pune, India

[2] Research Division of Accurate Diagnostics Pvt. Ltd., Kothrud, Pune, India

cancer particularly in areas where specialized equipment is not available.

There are several tests available for HPV detection based on either cytological examination or HPV genotyping. The most commonly used HPV detection test employs signal amplification techniques (e.g., Hybrid Capture 2) to detect HPV DNA via an RNA DNA hybridization probe with chemiluminescence or fluorescence signals [6]. However, this method of detection is associated with certain limitations of high running costs and also requires dedicated equipment. Whereas, PCR-based detection of specific target DNA is one of the most sensitive and cost-effective method for HPV detection.

In this study, we have designed and evaluated a type-specific PCR assay targeting the E6/E7 regions for the sensitive detection of high-risk HPV-16 and HPV-18. The sensitivity and specificity of developed assay were also evaluated by testing clinical samples.

Materials and Methods

In-Silico Validation of PCR Primer

The primers were designed as described by Wu et al. [7]. *In-silico* validation of the primers was done using a Sequence Manipulation Suite: PCR Primer Stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) and IDT OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer). The specificity of the primer was checked using the MFEprimer-3.0 server (https://mfeprimer3.igenetech.com/spec).

Positive Controls

Plasmid constructs containing type-specific HPV-16 and HPV-18 DNA (Eurofins Genomics) were used as positive controls.

Clinical Samples and DNA Extraction

After obtaining written informed consent, clinical samples for 100 patients were obtained from Gupte Hospital.

Cervicovaginal samples were collected in the Digene HC2 kit. Qiamp DNA mini kit was used for extraction according to the manufacturer's instructions. Extracted DNA was stored at − 80 °C till further processing.

Optimization of PCR Conditions

PCR assay was optimized for different parameters such as annealing temperature (for HPV-16: 54 -56 °C and HPV-18: 52 to 54.5 °C), template concentration (0.5 µl, 1 µl, 2 µl) and primer concentration (0.5 µl, 1 µl, 1.5 µl, 2 µl).

PCR Amplification

PCR reaction was performed in 25 µl mixture containing 12.5 µl of Emerald Amp GT PCR master mix (Takara Bio) which has dye marker for visualization on gel, 0.5 µl forward and reverses HPV 16, 18 and β-globin gene primers. β-globin gene was also analyzed as internal control in order to check extraction efficiency.

PCR amplification conditions were as follows: an initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 1 sec, annealing for 1 min at 56 °C for HPV-16 and β-globin gene and 53.5 °C for HPV-18 and extension at 72 °C for 1 min followed by final extension of 10 min at 72 °C. 10 µl of PCR product was analyzed in 2% agarose gel electrophoresis.

Performance Evaluation of the Assay with Clinical Samples

Performance of designed PCR assay was evaluated using 100 clinical cervico-vaginal samples. Out of 100 samples, 50 were HPV negative and 50 were HPV positive by Hybrid Capture method. Out of these 50 HPV positive samples, 33 were identified as HPV-16 positive and 17 were HPV-18 positive by RT-PCR-based genotyping test performed using a commercially available TRUPCR® HPV 16 & 18 Detection Kit. Specificity of designed assay was checked by Sanger sequencing of PCR product.

Sequencing of PCR Products

PCR products positive for HPV 16/18 were sequenced by using BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems) on an ABI 3500 Genetic Analyzer (PE Applied Biosystems). The sequences obtained were subjected to NCBI BLAST to check the analytical specificity of primers.

Results

Primer Analysis

The primers were validated *in-silico* for parameters such as GC content, primer homo/hetero-dimerization, hairpin loop formation, etc. Non-specific amplicons were not observed upon *in-silico* specificity analysis of the

type-specific primers. The sequences for all primer pairs and their predicted product size are given in Table 1.

Optimization of PCR Conditions

Different primer concentrations, template concentrations, and annealing temperatures were optimized for the type-specific HPV primer sets. Figures 1 and 2 demonstrate PCR amplification under various conditions for HPV-16 and HPV-18, respectively. The Optimum annealing temperature was observed at 56 °C for HPV-16 and 53.5 °C for HPV-18 as indicated by the intense band on 2% agarose gel.

Table 1 HPV type-specific and β-globin gene primer pair sequences

Primer sequence 5'-3'	Product size in BP
HPV16-F: AGGGCGTAACCGAAATCGGT	632
HPV16-R: CTGAGCTGTCATTTAATTGCTCA	
HPV18-F: GGGAGTAACCGAAAACGGT	662
HPV18-R: TCCTCTGAGTCGCTTAATTGCTC	
β-globin-F: CAACTTCATCCACGTTCACC	268
β-globin-R: GAAGAGCCAAGGACAGGTAC	

Using different concentrations of template DNA, almost similar intense bands were observed for HPV-16, but 1 µl was selected as the optimum template concentration for the assay since a higher template concentration may result in non-specific amplification. For HPV-18, intense band was observed at 2 µl template concentration.

High-intensity bands were observed at 0.5 µl primer concentration for both HPV-16 and HPV-18.

Performance Evaluation of the Assay with Clinical Samples

100 clinical cervicovaginal samples were tested using an optimized PCR assay. The result for HPV-16 and HPV-18 PCR assay for all known 50 HPV-negative samples was observed to be in concordance. Out of 33 HPV-16 positive samples detected by the standard Hybrid-capture method, 32 were tested positive by the developed HPV-16 assay. 16 samples for HPV-18 were tested positive out of total 17 known HPV-18 positive samples by the developed HPV-18 assay. Only 1 false negative result was observed for the developed HPV-16 and HPV-18 assay.

For HPV-16 and HPV-18, both assays were found to be 100% specific and 96.97% and 94.12% sensitive, respectively.

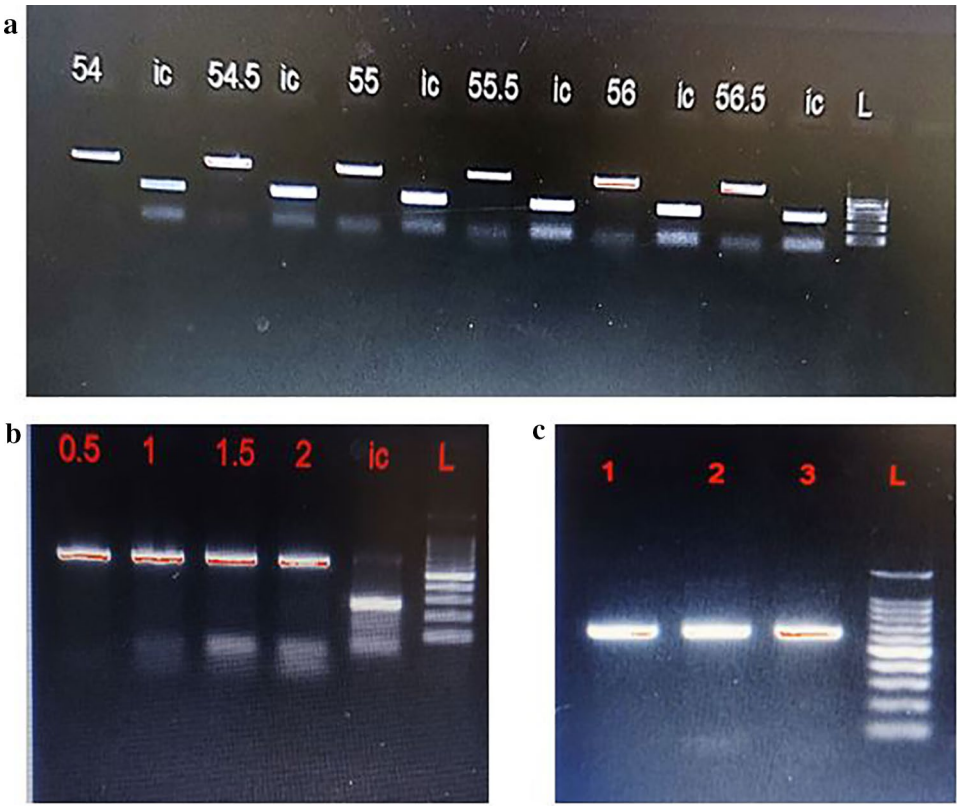

Fig. 1 PCR optimization for HPV-16. **a** Annealing Temperature Optimization: Optimum annealing temperature was observed at 56 °C **b** Primer Concentration Optimization: Optimum primer concentration was observed to be 0.5 µl. **c** Template Concentration Optimization: Optimum template concentration was observed to be 1 µl

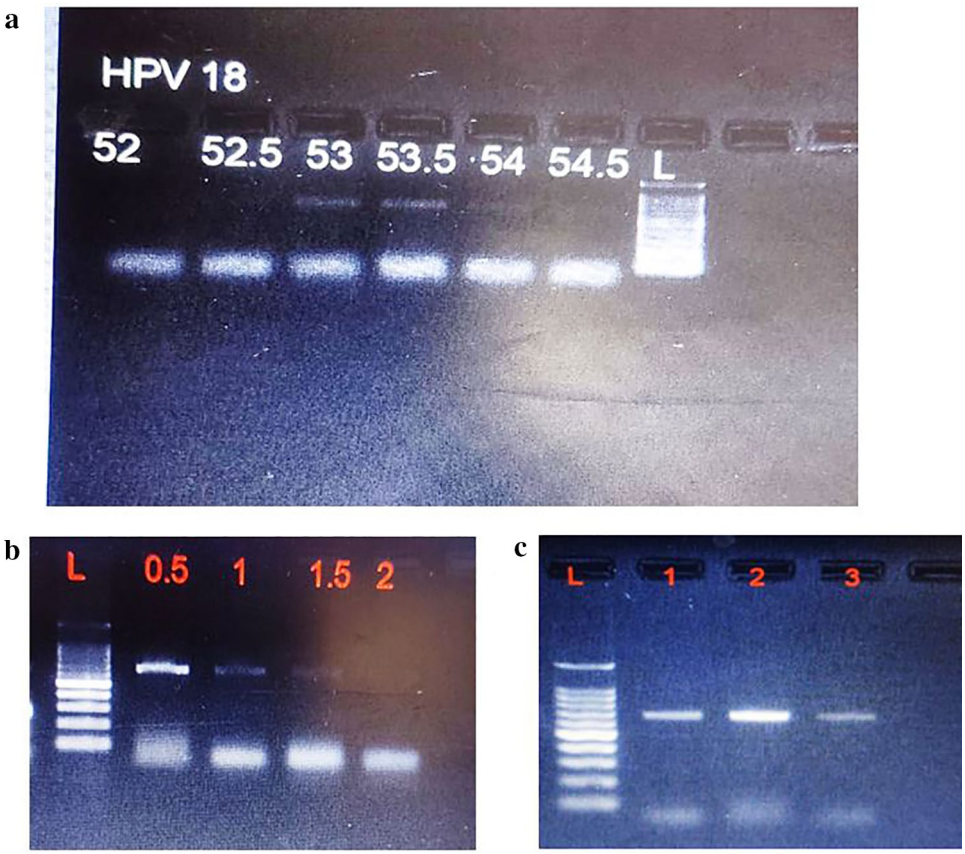

Fig. 2 PCR optimization for HPV-18. **a** Annealing Temperature Optimization: Optimum annealing temperature was observed at 53.5 °C. **b** Primer Concentration Optimization: Optimum primer concentration was observed to be 0.5 µl. **c** Template Concentration Optimization: Optimum template concentration was observed to be 2 µl

Amplicons obtained from all the samples were subjected to Sanger sequencing. The sequences were later verified using NCBI BLAST and were found to be type specific for HPV-16 and HPV-18.

Discussion

Cervical cancer is one of the most known causes of death among women in developing countries [8]. Human Papilloma Virus (HPV) is known to induce cervical cancer if left untreated [9]. High mortality rate due to poor diagnosis of HPV infection is major concern in developing as well as under developed nations. It is the need of the hour to have efficient diagnosis for early detection and treatment. Detection of viral DNA helps in diagnosis of the infection. Among the various HPV infection types, the high-risk types such as 16, 18, 33 and 58 are known to be most prevalent [10].

Initial on set and progression of the cancer are dependent on two main oncogenes E6 and E7 and are expressed constitutively [11]. As these oncogenes contribute as carcinogenic factors, targeting this conserved region would be effective in testing for HPV and subsequently in cervical screening. Targeting highly conserved regions in the viral genome enables to develop PCR primer sets such as MY09-MY11, PGMY09-MY11, GP5 + -GP6 + , SPF10, and LCR-E7 [12]. Typically, HPV16 and HPV18 are the subtypes with the highest infection rate worldwide and classified as "high-risk" type [13]. Globally HPV 16 and 18 affects between 60 to 90% people and which is also reason for HPV cancer [14]. Rather than considering all conserved regions, our study focuses on targeting the most prevalent region known for HPV 16 and 18. Targeting conserved regions allows in specific detection of various HPV genotypes.

We have developed and validated PCR-based assay which is type-specific and sensitive in detection of HPV 16 and 18 infections based on the amplification of conserved viral region of E6 and E7. This method combines the method of type-specific detection and sensitivity which was validated by testing on clinical samples. This assay helps in detection of high-risk types HPV-16 and HPV-18 by targeting E6/E7 region as primer binding site. The assay also shows sensitivity and specificity when performed with clinical samples. Because the developed assay demonstrated high sensitivity and specificity for detection of high-risk HPV-16 and HPV-18, it is applicable to routine HPV detection practices.

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Declarations

Conflict of interest We have no conflicts of interest to disclose.

Ethical Approval We ensure that this work is original and has not been published elsewhere, nor it is currently under consideration for publication elsewhere. We also acknowledge that all authors have substantially contributed to the underlying research and drafting of this manuscript and agree with the content of the manuscript.

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