Infectious Agents and Cancer

METHODOLOGY

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Development and validation of a multiplex qPCR method for identification of high-risk genotypes of human papillomavirus

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Abstract

Cervical cancer is a significant public health concern, disproportionately affecting women in less developed regions due to limited access to screening and vaccination programs. Despite advancements in cervical cancer prevention and treatment, there remains a need for efficient and cost-effective diagnostic tools. This study aimed to develop a multiplex real-time PCR assay to rapidly and accurately identify 15 human papillomavirus (HPV) genotypes.The primary objective was to design a screening method capable of simultaneously detecting HPV types 16 and 18, which account for over 70% of cervical cancers, as well as other clinically relevant high-risk and probable/possible high-risk. To validate the assay's performance, we compared its results with those obtained using the commercially available INNO-LiPA HPV Genotyping Extra II Assay kit (FujireBio, Tokyo, Japan). The developed assay successfully identified 15 HPV genotypes in a single reaction. Analysis of 150 positive and 40 negative clinical samples demonstrated excellent concordance between the two assays. The in-house real-time PCR test exhibited a clinical sensitivity of 98% and a clinical specificity of 100%, indicating its reliability and accuracy for HPV genotyping. The multiplex real-time PCR assay is a cost-effective and efficient tool for HPV screening, detecting multiple genotypes simultaneously. It enhances screening efficiency and accuracy, improving early detection and management of HPV-related diseases.

Keywords Human papillomavirus, Screening, Multiplex real-time PCR

Background

Human papillomavirus (HPV) is one of the most prevalent sexually transmitted infections globally [1]. Approximately 800 distinct putative HPV types have been documented in public databases, with new types being discovered regularly. As of now, 225 HPV types have been officially recognized, which can be categorized as low-risk or high-risk based on their associated lesions [2–4]. Among these strains, 12 are classified as high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and 3 as probable/possible high-risk (66, 67, and 68), previously categorized as high-risk. These 15 strains are among the most clinically significant HPV types [5–7].

Most HPV infections are resolved spontaneously due to the host's immune response. However, persistent infection with a high-risk HPV genotype can increase the risk of developing malignant lesions [8]. HPV infection is a significant risk factor for cervical, vaginal, anal, and penile cancers. Among these, cervical cancer is particularly concerning, representing a significant public health burden, especially in less developed regions with limited

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access to screening and vaccination programs. Approximately 70% of cervical cancers are attributed to infection with human papillomavirus types 16 and 18 [9].

The most critical factors in transforming lesions related to HPV infection into malignant cancerous lesions are the E6 and E7 proteins of the virus. These two proteins can intervene in the process of cell proliferation and differentiation. The E6 protein can interact with ubiquitin ligase (E6AP) and p53, leading to the ubiquitination and subsequent proteasomal degradation of p53 [10], therefore, the inhibition of p53 by E6 prevents apoptosis [11]. The E7 protein is primarily localized to the nucleus, where it can interact with and inactivate the retinoblastoma protein (Rb), Retinoblastoma is a crucial regulator of the cell cycle. By interacting with pRb, E7 can override cell cycle control and promote premature entry into the S phase. Additionally, E7 can interfere with the activity of p21 and p27, which are additional cell cycle regulatory proteins [12].

The E2 gene of the virus functions as a negative regulator, inhibiting the expression of the viral oncogenes E6 and E7. The E2 gene region frequently integrates viral into the host genome. Integration of the viral genome can disrupt the E2 gene, leading to increased expression of the E6/E7 oncoproteins and contributing to the development of malignant lesions [13].

As of 2018, global HPV immunization coverage was estimated to be 12.2% [14]. Consequently, cervical cancer remains a significant public health concern globally, particularly in less developed regions. A primary challenge in managing HPV infection is the screening and identification of lesions caused by high-risk HPV genotypes [8].

Nucleic acid amplification tests (NAATs) are currently the most reliable and efficient methods for HPV detection, demonstrating high sensitivity and specificity [15, 16]. Specific HPV diagnostic tests can simultaneously detect multiple HPV genotypes but may not be able to differentiate individual strains. Other HPV diagnostic tests can simultaneously detect multiple HPV genotypes while differentiating specific strains of particular clinical significance. Additionally, some tests can identify individual HPV genotypes [16–18].

This study aimed to develop a multiplex real-time PCR assay for the rapid and accurate identification of 15 high-risk and probable/possibly high-risk HPV genotypes: 12 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and 3 probable/possibly high-risk genotypes (66, 67, and 68).

Methods

Samples

DNA extracts were obtained from swab specimens collected from individuals referred to medical diagnostic laboratories in Tehran, Iran. All samples were stored at -80 °C for 60 to 120 days following sampling and DNA extraction prior to analysis. HPV genotyping was conducted using the commercially available INNO-LiPA HPV Genotyping Extra II Assay kit (FujireBio, Tokyo, Japan). As the samples were anonymized for this study, only the genotype determined by the commercial kit is known.

Primer and probe design

The sequences for HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68, as well as the human beta-globin gene (used as an internal control), were obtained from the NCBI database. All primers and probes were designed using Allele ID software (PREMIER Biosoft, San Francisco, USA) to target the E6/E7 region of the HPV genome. The specific sequences are listed in Table 1. Specific primers and probes were designed for HPV genotypes 16, 18, and the human beta-globin gene. For the remaining 13 HPV genotypes, three pairs of universal primers and probes were designed, each capable of detecting at least four of these genotypes. To enable simultaneous amplification and identification of HPV genotypes 16, 18, all other genotypes, and the internal control within a single reaction, four distinct fluorophores were attached to the 5' end of each probe. In contrast, a quencher dye was attached to the 3' end of each probe. The probes targeting HPV genotypes 16 and 18 and the human beta-globin gene were labeled with distinct fluorophores. However, the three probes designed for the remaining high-risk and probable/possible high-risk HPV genotypes shared a common fluorophore. All primers and probes were synthesized by Metabion (Germany).

Single-plex

To optimize primer and probe concentrations, real-time PCR assays were performed in triplicate using three distinct primer and probe mixtures for each HPV genotype. Mixture A consisted of 1.5 pM forward primer, 1.5 pM reverse primer, and 0.5 pM probe. Mixture B contained 3.0 pM forward primer, 3.0 pM reverse primer, and 1.0 pM probe. Mixture C comprised 6.0 pM forward primer, 6.0 pM reverse primer, and 2.0 pM probe. Each mixture was tested against its corresponding target HPV genotype.

The real-time PCR reaction was performed using 4X CAPITAL™ qPCR Probe Master Mix (Biotechrabbit, Germany) and the Rotor-Gene Q MDx 5plex HRM device (QIAGEN, USA). The reaction setup and temperature profile were optimized according to the manufacturer's instructions(3 min at 95 °C for 1 cycle as the initial activation temperature, followed by 40 cycles of 10 s at 95 °C as the denaturation temperature and 30 s at 60 °C as the annealing and extension temperature.).

Table 1 Primers and probes

Target	Primers and probe(5′ to 3′)	fluorophore	quencher	Genbank ID
HPV16	F: AATGTTTCAGGACCCACAGG R: GTTGCTTGCAGTACACACATTC P: ACCACAGTTATGCACAGAGCTGCA	FAM	BHQ-1	K02718
HPV18	F: ACCCTACAAGCTACCTGATCT R: ACCTCTGTAAGTTCCAATACTGTC P: ACGGAACTGAACACTTCACTGCAAGA	HEX	BHQ-1	X05015
HPV 31,33,35,52,59,67	F: GACTATGTATTAGATTTGGAACC R: GACCAAGACGAACAGGTCGA P: TGACCTATACTGCTATGAGCAATT	ROX	BHQ-2	J04353 M12732 X74477 X74481 X77858 D21208
HPV 39,45,58,68	F: TTATGTATAGTGTATAGAGA R: AGACGACCTGTGTTACCAAAGTC P: GATATTACTCGGACTCGGTATATG	ROX	BHQ-2	M62849 X74479 D90400 DQ080079
HPV 51,56,66	F: ACACCGCAAACTGAAATTGAC R: CCATGCACTTAATCCATTGTG P: GAGCAATTGGACAGCTCAGAGGA	ROX	BHQ-2	M62877 X74483 U31794
β globin	F: GCAAGGTGAACGTGGATGAA R: GTCTCCACATGCCCAGTTTCT P: GGTGGTGAGGCCCTGGGCAGGTTGGT	CY5	BHQ-2	AH001475.2

To optimize the annealing temperature for amplification of all HPV genotypes, the reaction was performed at 57, 58, 59, 60, and 61 degrees Celsius, and the amplification efficiency was evaluated for each temperature.

Multiplex

Following optimization of test conditions, the developed multiplex assay was evaluated. Optimized primer and probe concentrations for all target HPV genotypes and the internal control (human beta-globin gene) were combined into a single reaction mixture. Subsequently, the assay was re-tested against all targeted strains to confirm accurate and efficient multiplex amplification and detection. To compare the performance in both single-plex and multiplex modes, single-plex reactions were conducted for each strain using its corresponding primers and probes. This was performed in parallel with a multiplex reaction where all primers and probes were present simultaneously.

Analytical specificity

To assess analytical specificity, the developed assay was evaluated against a panel of control samples, including Adenovirus 2, Herpes simplex 1 and 2, HIV, HBV, HCV, CMV, Bacteroides fragilis, Candida albicans, Escherichia coli, Klebsiella pneumoniae, and Lactobacillus acidophilus, which are potential interferents in the assay. All positive control samples, extracted and purified genomes of microorganisms, were generously donated by the Pasteur Institute of Iran (Tehran, Iran) for the purpose of assessing specificity and cross-reactivity in this study. The final concentration of each microorganism's genome in each real-time PCR reaction was as follows: Adenovirus 2 (70,000 copies/reaction), Herpes simplex 1 and 2 (500,000 copies/reaction), HIV, HBV (100,000 copies/reaction), HCV (500,000 copies/reaction), CMV (100,000 copies/reaction), Bacteroides fragilis (60,000 copies/reaction), Candida albicans (500,000 copies/reaction), Escherichia coli (70,000 copies/reaction), Klebsiella pneumoniae (80,000 copies/reaction), and Lactobacillus acidophilus (50,000 copies/reaction).

Analytical sensitivity (limit of detection)

To determine the limit of detection (LOD) of the developed assay, the desired amplicon was amplified using strain-specific primers on a thermocycler (Peqlab, South Korea). Subsequently, gel electrophoresis (Bio-Rad, USA) was employed to purify the target amplicon. The concentration of each amplified HPV genotype was then quantified using a NanoDrop One^{c} Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). The number of amplified fragments corresponding to each genotype was calculated based on the spectrophotometric measurements.

$$Copy\,number\,(molecules) = \frac{X(ng) \times (6.0221 \times 10^{23})}{(N \times 660\,(g/mol)) + (10^{9}(\frac{ng}{g}))}$$

Where:

$$X\ (ng) = Amount\,of\,amplicon$$

$$N = Length\ of\ dsDNA\ amplicon$$

$$660\ \left(\frac{g}{mol}\right) = \text{Average mass of 1bp dsDNA}$$

$$6.0221 \times\ 10^{23} = Avogadro's\ constant$$

$$10^{9}\left(\frac{ng}{g}\right) = Conversion\ factor$$

Serial dilutions (1:10) of each amplified HPV genotype were prepared. Real-time PCR was then performed on these dilutions and the lowest dilution at which the assay could accurately identify 95% of replicates was determined as the limit of detection (LOD).

Clinical sensitivity and specificity.

To evaluate the performance of the developed assay, 150 HPV-positive and 40 HPV-negative samples were analyzed using the INNO-LiPA HPV Genotyping Extra II Assay kit (FujireBio, Tokyo, Japan) and the developed method. Clinical sensitivity and specificity were calculated based on the concordance between the two methods.

Results

Optimization

The results of real-time PCR assays, performed using a range of primer and probe concentrations for each HPV genotype to optimize assay performance, are summarized in Table 2, indicate that a concentration of 3 pM for both forward and reverse primers and 1 pM for the probe consistently yielded the lowest average cycle threshold (Ct) values, comparable to other tested concentrations.

Among the tested annealing temperatures (57, 58, 59, 60, and 61 degrees Celsius), 60 °C consistently yielded the lowest average cycle threshold (Ct) values, indicating optimal amplification conditions.

Multiplex

After optimizing primer and probe concentrations and annealing temperature in singleplex mode, the validated conditions were applied to multiplex PCR. Results obtained in multiplex mode were generally consistent with those observed in singleplex mode. However, comparison of multiplex and singleplex reactions revealed that competition between primers in the multiplex format resulted in slightly lower sensitivity and marginally higher Ct values. Detailed results are not included in this study. The results presented in Tables 3 and 4 represent the multiplex mode. The optimized conditions for multiplex PCR were determined to be 3 pM for each primer and 1 pM for each probe, with an annealing temperature of 60 °C.

Analytical specificity

The designed primers and probes were subjected to in silico analysis using the NCBI BLASTn tool against the human genome and potential interfering viruses and bacteria to assess specificity. Additionally, in vitro specificity was evaluated using control samples, including HPV types 6 and 11 (two common low-risk genotypes), Adenovirus 2, Herpes simplex, HIV, HBV, HCV, Bacteroides fragilis, Candida albicans, Escherichia coli, Klebsiella pneumoniae, and Lactobacillus acidophilus. The developed assay did not exhibit cross-reactivity with any of these control samples.

Table 2 Primer and probe concentration optimization

target	Mean CT in 1.5,1.5,1 pM Primer F, Primer *R*, probe concentration	Mean CT in 3,3,1 pM Primer F, Primer *R*, probe concentration	Mean CT in 6,6,2 pM Primer F, Primer *R*, probe concentration
HPV 16	24.37	21.97	22.46
HPV 18	27.14	25.39	26.08
HPV 31	22.70	21.78	22.89
HPV 33	24.43	24.67	25.47
HPV 35	26.97	26.12	28.31
HPV 39	28.69	25.32	25.19
HPV 45	22.62	20.74	23.47
HPV 51	20.86	21.24	22.75
HPV 52	30.11	28.67	29.39
HPV 56	24.01	22.49	24.41
HPV 58	22.98	19.81	20.82
HPV 59	27.70	26.35	25.73
HPV 66	27.85	27.09	27.44
HPV 67	24.67	23.16	23.48
HPV 68	20.52	17.21	19.34
β Globin	20.41	16.55	16.28

Table 3 Analytical sensitivity(or LOD)

Sample Concentration(copy/μL)		50	25	10	1
Genotype-Specific Positive Rates(%)	HPV 16	100	100	100	90
	HPV 18	100	100	100	95
	HPV 31	100	100	95	65
	HPV 33	100	95	95	70
	HPV 35	100	100	95	45
	HPV 39	100	100	100	90
	HPV 45	100	95	95	50
	HPV 51	100	100	100	70
	HPV 52	100	100	95	45
	HPV 56	100	100	100	45
	HPV 58	100	100	100	90
	HPV 59	100	95	95	40
	HPV 66	100	100	100	65
	HPV 67	100	100	95	80
	HPV 68	100	100	95	75

Table 4 Comparison of the developed Multiplex Real-time PCR assay with a commercial kit

Detected by INNO-LiPA HPV Genotyping Extra II Assay	number	Multiplex Real time PCR	
HPV 16	27	27	100%
HPV 18	15	15	100%
HPV HR	108	106	98%
HPV negative	40	40	100%

Analytical sensitivity

To determine the limit of detection (LOD), serial dilutions (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 50, 25, 10, and 1 copies/μL) were prepared for each HPV genotype and Real-time PCR was performed on these dilutions. The limit of detection (LOD) of the method was determined as the lowest concentration at which more than 95% of the replicate tests yielded positive results. As indicated in Table 3, the lowest concentration that consistently produced positive results for all target strains was 10 copies/μL so the detection limit (LOD) for the developed assay was determined to be 10 copies/μL for all HPV genotypes evaluated.

Clinical sensitivity and specificity

To evaluate the performance of the developed assay, 190 DNA samples were analyzed. This included 150 HPV-positive samples, with at least five samples representing each target genotype. The developed real-time PCR method successfully detected 188 out of 190 samples, demonstrating high concordance ($kappa = 0.9884$) with the INNO-LiPA HPV Genotyping Extra II Assay kit (FujireBio, Tokyo, Japan), Table 4. Our method's clinical specificity and sensitivity were calculated to be 100% and 98%, respectively. All samples associated with HPV strains 16 and 18 were correctly identified by the assay. The two cases with discordant results were associated with other HPV strains, specifically HPV 59 and HPV 68.

$$Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive} \rightarrow \frac{40}{40} = 100\%$$

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative} \rightarrow \frac{148}{150} = 98\%$$

Discussion

The developed multiplex real-time PCR assay offers significant advantages over traditional HPV genotyping methods. By simultaneously detecting 15 high-risk and probable/possible high-risk HPV genotypes in a single reaction, this assay improves efficiency and reduces the need for multiple tests. The assay's high sensitivity and specificity ensure accurate identification of HPV infections, even in low-resource settings. Recent advancements in viral diagnostics have led to the development of nucleic acid-based methods. Real-time PCR is a cutting-edge technique that offers several advantages, including rapid results, high accuracy, and the elimination of post-PCR processing steps [19]. Several HPV genotyping methods that utilize multiple tubes, primers, and probes have been developed, which can increase costs and reduce efficiency [20–22]. In contrast to existing HPV genotyping methods, this study presents a multiplex assay capable of simultaneously detecting 15 high-risk and probable/possible high-risk HPV genotypes in a single reaction. This approach offers the dual advantages of high throughput and low cost, which are essential criteria for effective screening tests.

While some HPV detection assays focus solely on quantitative detection of HPV types 16 and 18 [23], it is important to note that other high-risk HPV genotypes also pose a risk of cancer and disease progression. The developed multiplex assay addresses this limitation by simultaneously identifying 15 high-risk and probable/possible high-risk HPV genotypes, including types 16 and 18, making it a suitable screening tool with high sensitivity.

Several commercial kits for HPV identification and genotyping based on real-time PCR have been developed by reputable companies, including the Hybrid Capture 2 HPV DNA Test kit from Qiagen, APTIMA HPV Assay from Hologic Inc., and BD Onclarity HPV Assay from Becton Dickinson. However, these kits are often expensive and require specialized equipment that may only be readily available in some laboratories, particularly in developing countries.

The integration site of the viral genome can influence the oncogenic potential of HPV. Viral oncogenesis may be reduced if the breakpoint disrupts the E6/E7 genes.

Episomal HPV genomes typically maintain a balanced ratio of E2 and E6/E7 genes, which can suppress E6/E7 expression. However, integrating the viral genome into the host genome may lead to a reduction in E2 copies or disruption of the E2 gene, potentially promoting increased E6/E7 expression and oncogenesis. Increased expression of E6 and E7 can contribute to cellular transformation and carcinogenesis. Additionally, viral genome integration may disrupt the E6/E7 genes, potentially leading to false negative results in diagnostic tests that rely on detecting these gene regions [24]. In this study, primers and probes were designed to target the E6/E7 region of the HPV genome to avoid false negatives.

Specific detection of HPV types 16 and 18, which are strongly associated with dysplasia, may be more clinically valuable than other genotypes. Additionally, genotyping can provide insights into the management of HPV infections by differentiating between persistent infections and reinfections with new HPV types [25].

While a direct correlation between viral load and disease progression within the cervical intraepithelial neoplasia (CIN) spectrum has not been established, significant differences have been observed when comparing CIN-positive individuals to those without CIN [26]. However, it is important to note that low viral load does not necessarily correlate with decreased clinical significance. For instance, HPV type 18 may exhibit a lower viral load (indicated by a higher CT value) compared to other types, but it remains a high-risk type with significant clinical implications [27].

Given the clinical significance of HPV types 16 and 18, we focused on developing a qualitative screening method to detect these specific genotypes.

In conclusion, this study demonstrates the feasibility and clinical utility of a multiplex real-time PCR assay for HPV genotyping. The assay offers several advantages, including high sensitivity, specificity, efficiency, and cost-effectiveness. By providing rapid and accurate HPV detection, this assay can contribute to improved HPV screening and early diagnosis of HPV-related diseases.

While the multiplex nature of the assay could potentially impact sensitivity, the method demonstrated a high limit of detection (LOD) of 10 copies/μL, comparable to that of the cobas HPV test and BD Onclarity HPV assay, which are considered commercially available and highly sensitive assays [28].

According to the study's primary objective of developing a cost-effective screening method, only HPV types 16 and 18 were specifically detected. The remaining 13 strains were not individually differentiated, which may be considered a limitation of this study. Furthermore, due to the low prevalence of certain HPV strains, the anonymous nature of sample collection, and cultural sensitivities, the sample size in this study was limited. Additionally, the lack of patient information and long-term follow-up data restricted our ability to investigate the association between specific HPV genotypes and disease progression. Therefore, future studies should aim to include larger sample sizes and incorporate detailed patient information to further explore the relationship between HPV genotypes and lesion development.

Conclusions

The developed multiplex real-time PCR assay is a highly efficient and cost-effective tool for HPV screening, offering exceptional sensitivity and specificity. Compatible with any real-time PCR platform capable of detecting four distinct fluorescent channels, this assay provides a versatile and accessible diagnostic solution. By simultaneously detecting multiple HPV genotypes, including the clinically significant types 16 and 18, as well as other high-risk genotypes, this comprehensive approach can significantly enhance the efficiency and accuracy of HPV screening programs, ultimately improving the early detection and management of HPV-related diseases.

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Author contributions

A.M. and E.L. and F.K. and F.G. and M.B. are contributed in design of the study. A.M. wrote the main manuscript text and drafted the manuscript. All authors reviewed the manuscript. E.L. and F.K. and F.G. make revise.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

It was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Affiliated Hospital of Iran University of Medical Science (IR.IUMS.REC.1398.696). To use their HPV DNA samples and data, all patients and healthy volunteers gave their informed consent.

Competing interests

The authors declare no competing interests.

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