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New FRET-Base Approach for Detection of HPV High Risk Genotype by DNA Capturing

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ABSTRACT

Human Papillomavirus (HPV) is a highly prevalent virus responsible for several types of cancers, including cervical, throat, and anogenital cancers. Early detection and diagnosis are crucial for preventing the progression of HPV-related diseases. In this study, we introduce a new approach based on Förster Resonance Energy Transfer (FRET) method to identify viral DNA, which was designed for the conserved region of the L1 gene sequence in high-risk genotypes 16, 18, 31 and 33. In order to create suitable temperature conditions for the attachment and also to identify the fluorescent signal, real real-time PCR device was used. The results of the specificity test showed 100% specificity, and the limit of detection level of the method was reported to be 1000 copies/µl of the virus in the sample. The results of clinical sensitivity in the range of 86-96% between different genotypes and the rate of false negative results was in the range of 14-22%. Based on this, it can be said that maybe the developed method cannot be proposed as a suitable alternative, but due to the response time and lower cost, it can be proposed as a quick screening method.

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INTRODUCTION

Human papillomaviruses (HPVs) are a large and diverse group of epitheliotropic double-stranded DNA viruses (1). As one of the most common sexually transmitted infections globally, HPV poses significant public health challenges. HPV infection is widespread, with studies indicating that a significant majority of sexually active individuals will acquire HPV at some point in their lives. According to the World Health Organization (WHO), nearly 80% of sexually active people are expected to be infected with HPV at least once (2). The prevalence varies by geographical region, sexual behavior, and demographic factors. High-risk HPV types, particularly HPV 16 and 18, are responsible for the majority of cervical cancer cases, highlighting the need for targeted screening and vaccination efforts. The importance of early detection cannot be overstated, as it plays a crucial role in preventing the progression of HPV-related diseases, including cervical, anal, and oropharyngeal cancers (3). Understanding the epidemiology of HPV and the methods used for its detection is vital for effective prevention and management strategies (4). Based on WHO guidelines, Women should be screened for cervical cancer every 5–10 years starting at age 30. The global strategy encourages a minimum of two lifetime screens with a high-performance HPV test by age 35 and again by age 45 years (5). Precancers rarely cause symptoms, which is why regular cervical cancer screening is important, even if you have been vaccinated against HPV (6). Cervical

screening can detect multiple HPV types, including the highest risk types, HPV 16 and 18. HPV 16 and 18 have been linked to 70% to 80% of the cases worldwide (7).

The detection of HPV can be achieved through several methods, each with its own advantages and limitations. Pap smears have been the standard screening tool for cervical cancer (8). This method involves collecting cells from the cervix to identify any precancerous changes. However, it does not directly detect the virus. HPV DNA Testing is the more specific method for HPV detection; This method involves testing cervical samples for the presence of HPV DNA (9). This test is often recommended for women aged 30 and older as part of co-testing with Pap smears. HPV RNA Testing is the newer approach that detects the presence of HPV mRNA, which indicates active viral replication. This method can help differentiate between transient infections, which often resolve on their own, and persistent infections that may lead to cancer (10).

The basis of all methods based on DNA and RNA is the amplification of a specific part of the virus genome by PCR and then identifying the presence of the virus in the sample with electrophoresis or fluorescent probes or hybrid methods (11). In the meantime, the use of the real-time PCR method and the use of TaqMan probes is known as the gold standard. In this method, due to the diversity of the viral genome, its types can be separated. One of the disadvantages of this method is the time-consuming and high cost of testing (12). In this study, we introduce a new approach based on Förster Resonance Energy Transfer (FRET) method to identify viral DNA, which does not require a replication step and alternating temperature cycles. In this method, a special type of probe is used, which, unlike the TaqMan method, does not require hydrolysis, and the detection process can be monitored in real time using real-time PCR devices.

MATERIALS AND METHODS

Viral Variants and DNA Extraction

Pathogenic high-risk genotype of HPV viruses including 16, 18, 31 and 33 were collected from Razi Laboratory (Karaj, Iran). All the samples were taken in a period of three months from female patients who were positive for the desired genotypes by TaqMan real-time pcr test. The samples were vaginal swabs that were kept at refrigerator temperature. The viral DNA/RNA kit (FAVORGEN Biotech, Taiwan), according to the manufacturer's instructions performed DNA extraction. Extracted DNA was eluted with 50μl AE buffer and stored at -20°C until amplification.

Primer Designing for Viral Genomes

The mentioned high-risk genotype whole-genome sequences were obtained from The Papillomavirus Episteme (PaVE) was used to align the conserved domain of viral-specific genes (https://pave.niaid.nih.gov/). Using oligo7 (https://www.oligo.net/), a pairs of particular oligonucleotides for the L1 common conserved region was designed. Genescript, Inc. produced the oligonucleotide indicated in Table 1 (GeneScript, Jiangsu, China). At the 5' end of one of the oligonucleotides, the fluorescent FAM was placed as a fluorescent donor, and at the 5' end of the other oligonucleotide, the BHQ molecule was placed as a quencher. (Figure 1). If it is not attached to the template strand, the FAM dye will signal, and if both oligonucleotides are attached to the template, the fluorescent signal will be turned off due to the proximity of the quencher to the FAM dye.

Preparation of Standard Plasmid

In this research, 4 recombinant vectors were employed and were constructed as positive controls. To acquire the recombinant plasmids pcDNA3.1(+)/16, pcDNA3.1(+)/18, pcDNA3.1(+)/31, and pcDNA3.1(+)/33, particular target segments were first generated using the primers (Table 2), and then

Table 1. Primer sequence for amplification of the L1 gene region.

Genotype	Primer Sequence	Tm °C	PaVE ID
HPV 16 L1	5'- TTGCTGATGCAGGTGACTTT-3'	60	HPV16REF
	5'- CAAAAAGCATGCAACCGAAT-3'		
HPV 18 L1	5'- GCCCCTGCCTCTACACAGTA-3'	60	HPV18REF
	5'- ATAGCCCAACAAGCAACACC-3'		
HPV 31 L1	5'- GTGCCTGCGTGGAGTGAC-3'	60	HPV31REF
	5'- CCAGTGCTGCACATGTTTTT-3'		
HPV 33 L1	5'- CCACAGTGTACCTGCCTCCT-3'	60	HPV33REF
	5'- GGGTAGGGCAAGCAATACAA-3'		

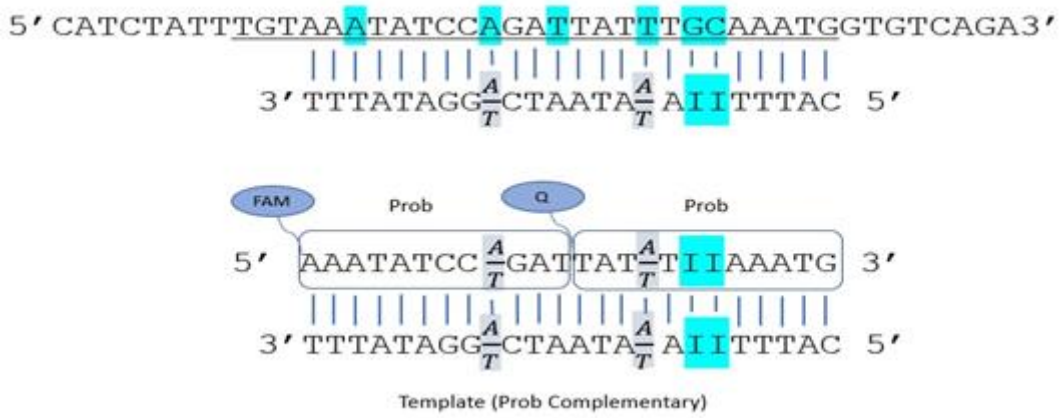

Fig1. The schematic diagram of the designed probe structure and the target sequence: as can be seen, the probe consists of two oligonucleotide fragments, one of which has a fluorescent molecule at the 5› end and the other a quencher molecule, so that when attached to the target sequence, the fluorescent signal is turned off, and the device can detect signal changes.

these sequences were introduced into the pcDNA3.1(+) plasmid (Shenzhen, China) (vector and genome map in figure 2). The vector copy number was determined by using the following equation: copy number (copies/µL) =NA (copies/mol) concentration (g/µL)/ MW (g/mol), where NA is Avogadro's number and MW is the reference times (https://www.technologynetworks.com/tn/tools/copynumbercalculator).

Set up the method

In this study, a real-time PCR device (ABI StepOnePlus, Thermo Fisher Scientific, USA) was used to establish the binding to template conditions and measure the fluorescent amount. Prepared PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) was used to create buffer conditions. The probe concentration was considered 10 mM for this study, and Temperature and time conditions were established according to Table 2.

Sensitivity and Specificity Analysis of the FRET Approach

a)Specificity analysis of the FRET: Probe blast analysis was used to assess the specificity of FRET. Also, HSV1, HSV2, and HIV were used to test the specificity of the FRET approach.

b)The FRET technique's efficiency: By using a 10-fold gradient dilution method, each one of the recombinant DNA vectors was diluted from 1 × 10^6 to 1 × 10^0 copies/µL. After that, FRET was carried out to determine the limit of detection (LOD). FRET reaction was performed for each sample with 3 replications.

c)Detection limit, analytic sensitivity, and normal range: Two inactive positive samples of 16, 18, 31, and 33 viruses at a concentration of 1 × 106 copies/µL were evaluated at the Virology Research Center to verify the technique. Each sample received 3 replications of the FRET procedure.

Comparative test with the reference method

To validate the FRET technique in this study, an IVD mark kit for high-risk HPV (Sacace, Italy, CAS No. V67-100FRT) was prepared. FRET technique and IVD mark kit were performed on 100 clinical samples. The results obtained from the FRET technique in this study were compared with the results of the IVD mark kit was repeated 3 times.

RESULTS

Construction and Identification of Recombinant Plasmids

Virulence genes of 16, 18, 31, and 33 genotypes were cloned into the eukaryotic expression vector pcDNA3.1(+), as shown in Figure 2, separately. DNA sequencing revealed that the gene sequences from the 4 recombinant plasmids were identical to the 16, 18, 31, and 33 genotypes. BamHI and EcoRV were used to digest the plasmids that had been constructed. The digestion products were separated electrophoretically, indicating that the recombinant plasmid was successfully constructed.

Table2. Time and temperature conditions.

Temperature	Time	Number of cycles
95°C	1min	10
65°C	1min	

*Data collection at 65°C and on the FAM Channel

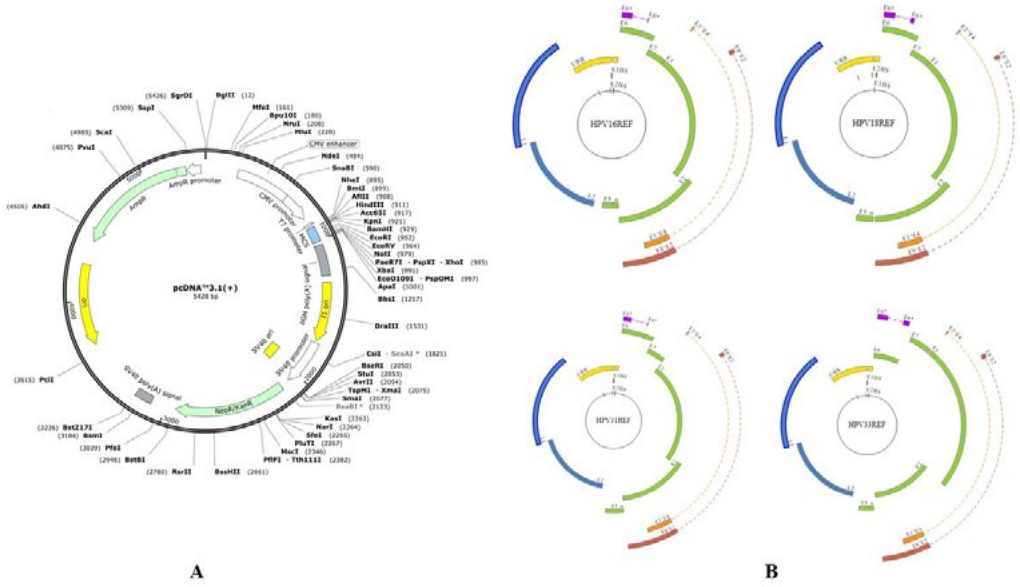

Fig2. A) Schematic structure of the plasmid used to create the positive control vector. B) Genome structure of four high-risk HPV types (16,18,31,33) in this study probe was designed for the conserved region of the L1 gene.

The FRET Approach Has a High Level of Specificity

These 4 viruses, as well as HSV 1/2 and HIV cDNA, were used to test the specificity of the FRET approach. The curve for each viral pathogen was apparent for FRET evaluation. The extracted genome of the viruses was evaluated separately with the HPV specific probe and under the same conditions, and the results showed that no signal of binding to their genome was recorded.

The FRET Technique's Efficiency and LOD measurement

The FRET approach efficiency for each viral sample was assayed by a positive control (Recombinant plasmids). According to the results of the minimum detectable amount of virus in this kit is 1000 (1 × 10^3) copies. Therefore, the performance of this kit is equal to 1000 copies of the number. In the next step, this approach was tested for verification with virus samples.

Compare with the reference kit and use Clinical Samples

FRET developed technology and IVD-marked real-time PCR kits were used to investigate a total of 120 clinical specimens. 30 samples for each high-risk genotype and 100 healthy samples as a negative control. Table 3 shows the positive rate of each virus. As can be seen in Table 3, the reference kit was able to fully identify all positive and negative samples (100% rate), compared to the FRET technique, 86.6% for genotype 16, 96.6% for genotype 18, 86.6% for genotype 31, and 83.3% for genotype 33 of positive samples have been identified and all negative samples were reported completely without signal.

DISCUSSION

Human papillomavirus (HPV) is a major cause of cervical cancer, genital warts, and other anogenital and oropharyngeal cancers. Due to the importance of early detection of HPV infection in managing and reducing the risk of cancer caused by this virus, screening programs have been put on the agenda of the health systems of all countries (13). Early and accurate detection of HPV infection is crucial for prevention, timely treatment, and improving patient outcomes. Traditional methods for HPV detection include PCR (Polymerase Chain Reaction), in situ hybridization, and serological assays, but emerging molecular techniques such as Fluorescence Resonance Energy Transfer (FRET) offer a promising approach for sensitive and specific detection (14).

Molecular and epidemiologic studies have solidified the association between high-risk strains of genital HPV and squamous cell carcinoma of the cervix. The incidence of cervical cancer and its associated mortality have declined in recent years, largely due to the widespread implementation of screening programs (15). Screening for cervical cancer remains an important public health and economic concern throughout the world. Large-scale studies to evaluate management options for women with abnormal Pap smear results have been conducted, and these studies highlighted the potential utilization of HPV DNA testing in the management of women with Undetermined Significance (ASCUS) Pap smear results (16).

Golden standard method that used detection and

Table 3. Compare with reference kit and use Clinical Samples results

Method	16 Pos	18 Pos	31 Pos	33 Pos	Neg
IVD Mark Kit	30/30 (100%)	30/30 (100%)	30/30 (100%)	30/30 (100%)	100/100 (100%)
FRET Approach	26/30 (86.6%)	29/30 (96.6%)	26/30 (86.6%)	25/30 (83.3%)	100/100 (100%)

genotyping HPV is TaqMan probe real-time PCR, Taqman probes also follow the FRET system, so that the donner molecule is at a certain distance from the quencher, which causes no signal to be emitted, but after attaching to the template sequence and during the DNA polymerization process by the polymerase enzyme, the probe is hydrolyzed and the molecule The donner is released from the quencher trap and the fluorescent signal is detected by the device (17). The requirement of this technique is the amplification of the target sequence by PCR. This makes even small amounts of virus detectable in the sample because it increases during the PCR process. But the use of TaqMan probes, in addition to the high cost, requires an amplification process, which makes the screening process time-consuming.

Several FRET-based methods have been proposed to identify different pathogens. In this study, we developed a special type of FRET probes, which do not require an amplification process to use them, and it costs less for testing, because a large number of genotypes can be identified using one probe (18-20). This probe actually consists of two labeled oligonucleotide sequences, whose binding site is on the sequence of the template, and if it is attached to the template strand, as the donor molecule is placed next to the quencher, the signal is turned off, exactly the opposite of the action of TaqMan probes (21). In a similar study conducted by Rezanejad et al., this method was used to identify the COVID-19 virus. In this specific study, the researchers designed a FRET-based probe by conjugating fluorescent molecules to DNA sequences that are complementary to the target COVID-19 viral RNA or DNA. When the probe binds to the target sequence, a change in fluorescence occurs due to the energy transfer between the fluorophores, which can be measured. This method provides a rapid and highly sensitive detection system, useful for diagnosing infections by detecting viral genetic material without the need for complex equipment or lengthy procedures (22).

In this study, the desired probe was designed for the conserved region of the L1 gene sequence in high-risk genotypes 16, 18, 31 and 33. In order to create suitable temperature conditions for the attachment and also to identify the fluorescent signal, real real-time PCR device was used. Vectors containing target sequences were used to perform sensitivity, specificity and limit of detection tests. The results showed that this method could detect the amount of viral particles up to 1000 copies/µl. Also, the results of the clinical analysis test and comparison with the reference kit showed that the sensitivity of this method is in the range of 86-96% and the rate of false negative results was in the range of 14-22%.

The false negative results were significantly higher than the reference method. Based on this, it can be said that maybe the developed method cannot be proposed as a suitable alternative, but due to the response time and lower cost, it can be proposed as a quick screening method. TaqMan probes, which rely on PCR amplification, can detect extremely low viral loads; the FRET probe's lack of amplification reduces its ability to detect minimal amounts of HPV DNA, potentially leading to false negatives in cases with low viral titers. This trade-off between speed and sensitivity highlights the need for further refinement of FRET technology to ensure that diagnostic accuracy is not compromised in favor of efficiency. Consequently, while the FRET probe holds potential as a rapid diagnostic tool for high-risk HPV strains, its lower sensitivity may limit its application in certain clinical scenarios, particularly in early or low-level infection.

CONCLUSION

In conclusion, the FRET-based HPV detection method offers significant advancements in real-time, PCR-independent diagnostics, but its reduced sensitivity compared to TaqMan probes requires careful consideration, especially in settings where detecting low viral loads is critical. Continued research is necessary to optimize these probes for broader clinical use. The development of this method using real-time fluorescent signal detection devices that have a higher detection power can be effective in increasing the sensitivity of this method.

Declarations

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors's Contribution

Ghazal Emadian: Conceptualization editing and review. The author read and confirmed the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Conflict of Interest

The authors declared no conflict of interest.

Consent for publication

Not Applicable

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