Iranian Journal of Microbiology
Volume 17 Number 3 (June 2025) 488-502
DOI: http://doi.org/10.18502/ijm.v17i3.18832

ORIGINAL ARTICLE

Design and comparison of PCR-ELISA reaction with other available hybridization methods to identify types 11, 16, and 18 of the human papillomavirus

Seyed Mohammad Amin Mousavi-Rad[1], Shohreh Zare Karizi[2], Hamid Sedighian[1], Seyed Ali Mirhosseini[1], Hadi Esmaeili Gouvarchin ghaleh[3], Jafar Amani[1*]

[1]Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Department of Biology, Faculty Member, Varamin-Pishva Branch of Azad University, Tehran, Iran
[3]Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Received: November 2024**, Accepted:** February 2025

ABSTRACT

Background and Objectives: Human papillomavirus (HPV) is a significant etiological agent in cervical cancer. This study aimed to evaluate the performance of PCR-ELISA for detecting HPV genotypes 11, 16, and 18 compared to the conventional hybridization methods.

Materials and Methods: PCR-ELISA was designed and optimized to detect target HPV genotypes using biotin-labeled probes. Sensitivity, specificity and reproducibility were assessed through intra-assay and inter-assay variability tests. Additionally, a cost-benefit analysis was performed to compare PCR-ELISA with RT-PCR and gel electrophoresis.

Results: PCR-ELISA demonstrated high sensitivity (HPV18: 94.92%, HPV16: 98.36%, HPV11: 93.75%) and specificity (100% for all genotypes), with Kappa values ranging from 0.84 to 0.92, indicating strong agreement with the reference standard. Reproducibility analysis showed intra-assay CVs below 5% for most samples and inter-assay CVs within acceptable limits. The cost-benefit analysis revealed significant reductions in reagent and equipment costs compared to RT-PCR, making PCR-ELISA a cost-effective alternative.

Conclusion: PCR-ELISA offers a reliable, sensitive and cost-effective method for HPV detection, particularly in resource-limited settings. Its simplicity and compatibility with existing workflows makes it a promising tool for routine diagnostic applications.

Keywords: Human papillomavirus; Polymerase chain reaction-Enzyme-linked immunosorbent assay; Molecular diagnostics; Hybridization techniques; HPV DNA detection

INTRODUCTION

Human papillomaviruses (HPV) represent an extensive range of viruses discovered in twenty diverse mammalian species and are classified into different types (1). Papillomavirus is a double-stranded DNA (dsDNA) virus with a 20-sided capsid capable of infecting skin, oral and genital epithelial cells (2). Human papillomavirus is classified into two categories according to tissue orientation (3).

*Corresponding author: Jafar Amani, Ph.D, Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-2182482556 Fax: +98-2188068924 Email: jafar.amani@gmail.com

The papilloma group comprises of a pathogenic virus with low risk. It primarily targets the keratin levels of the skin and leads to harm. The virus strains involved are 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 70, 71, 72, 74, 81, 83, 84 and 91 (4, 5). Another group comprises of papillomaviruses with a heightened risk of pathogenicity, which are more likely to cause infection (6). In the throat, airways, mucous membranes of the mouth and especially the genitals, certain members of this group result in common warts (7, 8). However, most members within this group result in injuries that lead to malignancies, such as types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73 and 82 (4, 9).

Cellular culturing and serologic techniques are not useful in identifying this virus and its variations (10). The most precise means for diagnosing HPV is through molecular techniques, specifically PCR and Real-Time PCR. The latter method is predominantly employed by diagnostic laboratories (11). Molecular testing represents the most advanced and specialized approach when it comes to detecting various types of HPV genomes by means of specific primers (12). Molecularly sensitive methods offer valuable insights into diagnosis of human papilloma virus (HPV) infection. These methods are capable of detecting various types of HPV in both symptomatic and asymptomatic individuals (13, 14).

PCR-ELISA combines the sensitivity of molecular diagnostics with cost-effectiveness and ease of implementation. Compared to RT-PCR, which is widely used for HPV detection, PCR-ELISA offers comparable sensitivity but requires less sophisticated equipment, making it accessible for routine diagnostics. Droplet digital PCR provides high precision in quantification, yet it remains expensive and resource-intensive (15). On the other hand, next-generation sequencing (NGS) delivers comprehensive data, including co-infections and mutations, but it is unsuitable for high-throughput settings due to its cost and complexity (16). PCR-ELISA strikes a balance by being cost-effective, rapid and capable of processing a large number of samples, thereby meeting the needs of diagnostic laboratories in resource-limited environments.

After analyzing molecular methods that use hybridization, including PCR, RT-PCR and PCR-ELISA, it was concluded that PCR-ELISA is a viable alternative due to its ability to diagnose small amounts of specific disease gene sequences at an acceptable speed and limit. The use of digoxigenin is also recommended as a non-radioactive diagnostic method for detecting PCR products. The present study employed the detection of digoxin-labeled products through an anti-DIG antibody conjugated to peroxidase. A PCR-ELISA was subsequently designed using a pair of primers and a specialized probe, aiming to detect HPV types 11, 16, and 18.

MATERIALS AND METHODS

Sample preparation. To detect HPV in paraffin-embedded specimens, the 5-µm sections were cut and treated with xylene for deparaffinization. Samples were then incubated with a Lysis mixture (200 µl) overnight at 37°C. Subsequently, a phenol-chloroform-isoamyl alcohol solution (100 µl; 25:24:1) was added, mixed thoroughly, and centrifuged to extract the top phase. Afterwards, a phenol-chloroform-isoamyl alcohol solution (100 µl; 25:24:1) was added, mixed thoroughly, and centrifuged to extract the top phase. DNA was precipitated by adding 1ml of 95% ethanol and 50 µl of 3.5 M sodium acetate (pH 3.5). After centrifuging at 13000 rpm, the pellet underwent two washes with 70% ethanol, dried at room temperature, and was ultimately resuspended in 30 µl of deionised water. Furthermore, the DNA extraction kit from bioneer Inc. (South Korea) was employed to extract 17 samples.

Selection of gene fragment and design of suitable primer for PCR and RT-PCR reaction. This was followed by primer design to isolate and amplify genes located in a specific region of the HPV genome, targeting genotypes 11, 16, and 18, through bioinformatics software. The designed primers and probes were evaluated using BLAST against the NCBI nucleotide database to confirm specificity. The results showed no significant alignment with unrelated sequences or closely related HPV genotypes, confirming their high specificity for HPV types 11, 16, and 18. After designing the primer, BLAST analysis was employed to determine its specificity, followed by the examination of Tm, ΔG, loop formation, and dimer primer through DNASIS and Oligo 5 software. Table 1 displays the primers designed for the desired HPV genomes.

Proper design of probes for PCR-ELISA procedure. A probe was developed to detect the HPV

PCR product using a PCR-ELISA reaction. Table 2 illustrates the probes for each of the 11, 16, and 18 HPV types. The characteristics of the probe, such as Tm temperature, GC percentage, and the probability of loop formation were assessed using Oligo and DNASIS-Primer3-BLAST software. Cross-reactivity was tested against HPV types 31, 33, 45, and 52 using PCR and PCR-ELISA. No amplification or signal was observed for these genotypes, demonstrating the specificity of the primers and probes. To further ensure specificity, off-target amplification tests were conducted under standard reaction conditions. No unintended products were observed, confirming the robustness of the primer and probe design. Upon final approval, the probe was ordered from Sina Clone and labeled with biotin.

RT-PCR and PCR reaction design. After ensuring the primer suitability through primer design software, we placed an order for manufacturing with Sina Clone Company (Iran). Subsequently, a PCR reaction was conducted to amplify 25 µl HPV genes of types 11, 16 and 18. Each reaction contained 0.4 micromoles of each primer, 0.5 units of Taq DNA polymerase enzyme (Sinaclone Co., Iran), 2.5 µl of X10PCR buffer, 3 mM MgCl2 concentration and varied concentrations of the genomic DNA. A RT-PCR Mastermix for SYBR Green was utilized with a 12 microliter concentration for the reaction. The temperature program for PCR and RT-PCR involves denaturation at 95°C for 10 minutes, denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. Additionally, PCR was carried out in 35 cycles and RT-PCR in 45 cycles. To visualise the PCR product, 5 µl of the final PCR product was electrophoresed on a 1% agarose gel prepared in TBE buffer. To determine the size of the PCR product, we utilised a 100 bp plus ladder indicator manufactured by Sinagen Company in Iran. The product was then viewed by staining the gel with ethidium bromide and preparing the accompanying image on the Gel document machine made by Omega in Germany.

Marking of genomic PCR product by DIG-dUTP. DIG-dUTP was utilised for labelling the PCR product of the HPV types 11, 16, and 18 genomes. This was achieved by replacing a portion of dTTP in the dNTPs mixture with DIG-dUTP. The Taq DNA polymer uses DIG-dUTP in certain regions instead of dTTP. The ratio of dTTP to DIG-dUTP used was 1:30. Alongside the original sample, a negative control sample was included in which the dNTP mixture without DIG-dUTP was used. The PCR reaction was prepared in a final volume of 25 µl, and the thermal cycles were conducted as previously described.

Detection by PCR-ELISA reaction. For the experiment, 5 µl of streptavidin was mixed with 95 µl of coating buffer in well A. In well B, 10 µl of PCR

Table 1. Primers designed for HPV type 11, 16 and 18 genes.

Virus strain		Sequence 5>3	Primer length	Nucleotide position (bp)	Expect Product size (bp)
HPV11	F HPV11	GGAATACATGCGCCATGTGG	20	6760-6740	360
	R HPV11	CGAGCAGACGTCCGTCCTC	19	7118-7099	
HPV16	F HPV16	ACCCAGTATAGCTGACAGTATAAAAAC	27	1623-1596	512
	R HPV16	CTCGTTTATAATGTCTACACATTGTTG	27	2108-2018	
HPV18	F HPV18	GCTTTGAGGATCCAACACGG	20	28-8	430
	R HPV18	TGCAGCACGAATGGCACTG	19	446-427	

Table 2. Probes of HPV types 11, 16 and 18 used in PCR-ELISA reaction

Virus strain	Sequence 5>3	Prob length (bp)	Nucleotide position (bp)
HPV+11	Biotin-CCTGTCAGAAACCCACACCTG	21	6937-6958
HPV+16	Biotin- GAGCCTCCAAAATTGCGTAG	20	1786-1806
HPV+18	Biotin- GATGCTGCATGCCATAAATG	20	184-204

product labeled with digoxin was mixed with 90 µl of coating buffer. In well D, 10 µl of normal (unlabeled) PCR product was mixed with 90 µl of coating buffer. In well E, 100 µl of coating buffer was added. Finally, 5 µl of normal PCR product was mixed with 95 µl of coating buffer in well F. The mixture was incubated for 1 hour at 37°C. The wells were then washed 3 times with PBST buffer (saline phosphate containing 0.05% tween). In the subsequent stage, 100 µl of blocking buffer containing 3% skimmed milk was introduced to each well, followed by an incubation period of 37 minutes at 37°C. The wells were then washed as previously directed. Simultaneously, 20 µl of DIG-labelled PCR product was added to 80 µl of SSC (saline-sodium citrate) hybrid buffer, and the combination was boiled for 5 minutes. The labelled picomole probe was subsequently added, with biotinylated 5 ends, and incubated at 55°C for 1 hour. The resultant mixture was transferred to well A and incubated at 37°C for an additional hour. After washing with PBST buffer for three times, 100 µl of peroxidase-labelled anti-digoxin-labelled antibody was added to each well with a dilution of 1000. The mixture was then incubated for 1 hour at 37°C. Following this, a substrate solution containing 5 mg OPD in 4 ml of phosphate citrate buffer with a pH of 5 was added to each well, along with 5 µl of 30% hydrogen peroxide. The well plate was left in a dark room for a few minutes before the reaction was stopped with 100 µl of 5.2M sulfuric acid. The device (ELISA Reader, Biotek USA) was used to read the light absorption at 492 nm. This process was executed individually for HPV types 11, 16, and 18.

Determination of sensitivity of RT-PCR and PCR reaction. To accomplish our objective, we implemented the dilution method. We initially purified the genome of the HPV following the aforementioned steps. To achieve this, we initially gauged the concentration of genomic DNA extracted from the HPV using the nano-APP. The measured concentrations were 127.9, 12.4, and 9.4 ng/µl, respectively. Subsequently, we extracted this DNA and produced a dilution series, employing DDW. Finally, these dilutions were utilized as a model in RT-PCR and PCR reactions to evaluate the sensitivity of the study.

Determination of minimum genomic concentration detectable by PCR-ELISA. Following the quantification of the PCR product of labelled HPV, serial dilutions were prepared for 11, 16 and 18 HPV types in nanograms. One well served as the negative control. 10 microlitres was added to each well, following which the other detection steps were conducted and measured using an ELISA reader.

Determining the specificity of PCR, RT-PCR, and PCR-ELISA methods. To assess the specificity of the primers designed, four distinct types were utilized in conjunction with the studied type. Subsequently, RT-PCR, PCR, and PCR-ELISA procedures were conducted to evaluate their efficacy.

RESULTS

PCR and RT-PCR process optimization. Under optimized reaction conditions and appropriate thermal cycles, PCR reactions were conducted with specific primers targeting HPV types 11, 16, and 18 genes. The observation of single bands at sizes of 360 bp, 512 bp, and 430 bp for types 11, 16, and 18 HPV, respectively was made. Fig. 1 presents the corresponding gel image. Similarly, RT-PCR reactions were carried out and the outcome is demonstrated in Fig. 2.

Upon examination and comparison of the obtained sequences with those in the gene sequence bank, it was confirmed that they are directly linked to the HPV gene. The BLAST software confirmed that the amplified fragment is entirely compatible with the HPV gene as evidenced by the sequencing results and alignment against sequences in the gene bank database.

Melting curve analysis of the Real-Time PCR reaction. After replication by the device, a melting

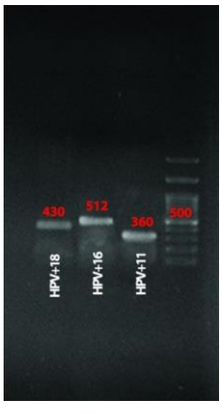

Fig. 1. Results of PCR reaction; lane 1: 100 bp DNA marker; lane 2: HPV + 11; lane 3: HPV + 16; lane 4: HPV + 18.

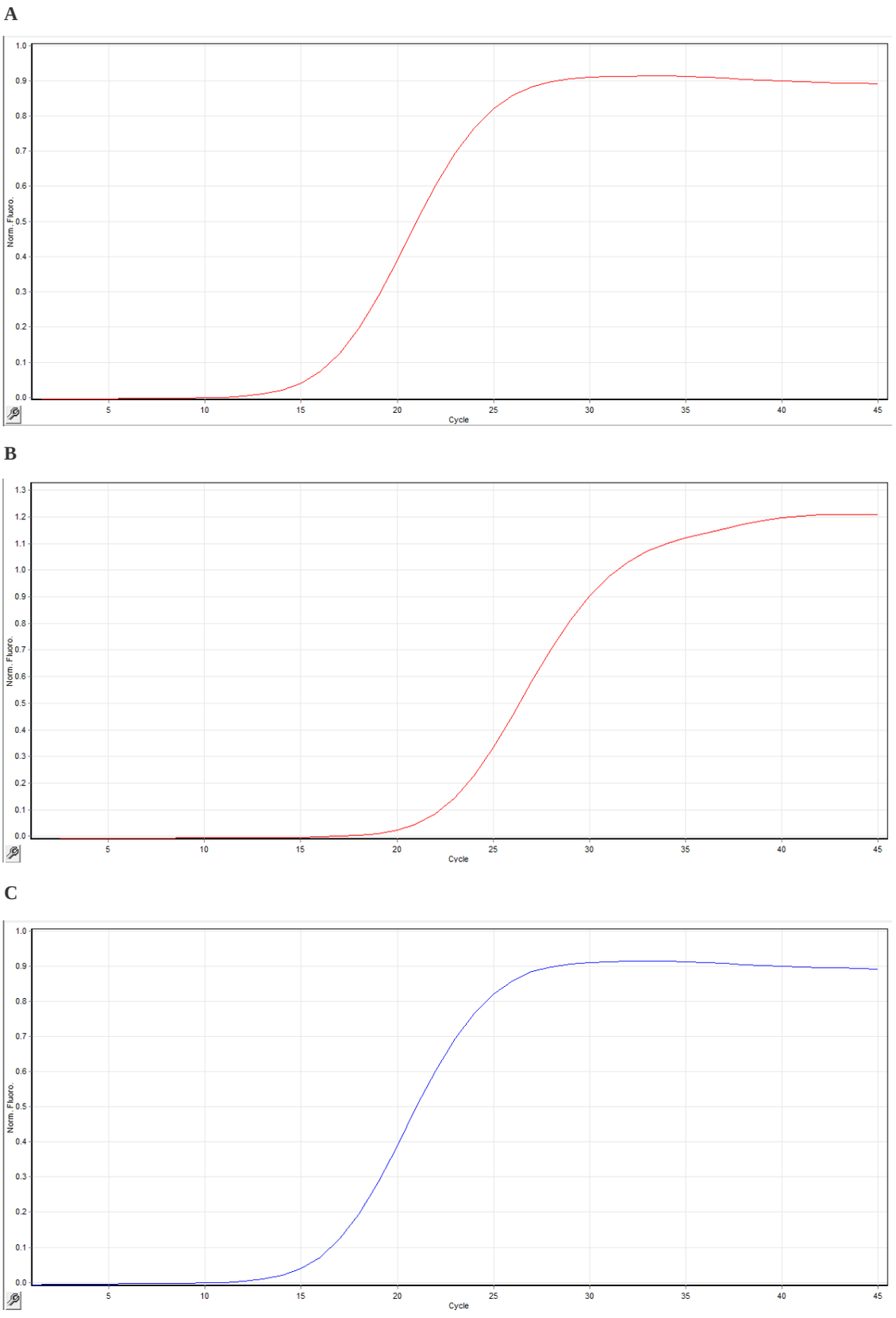

Fig. 2. Results of RT-PCR reaction (amplification plot) including; (A) HPV + 11; (B) HPV + 16; (C) HPV + 18.

curve analysis was carried out on the reproduction product to confirm the specificity and identity the replicated product. To perform this analysis, the temperature of the plate was gradually raised from 65°C to 95°C, while the device continuously recorded the fluorescence signal of the amplified product. Subsequently, the device generated a curve or graph of fluorescence intensity against temperature. By examining the unique and distinct peak of the studied product, specifically types 11, 16, and 18 of the HPV, the Real-Time PCR amplification's specificity was verified. Fig. 3 illustrates the analysis of the melting curve for the RT-PCR reaction for types 11, 16, and 18 of the HPV.

Based on the melting curve diagrams, it can be concluded that the fragment was present in the RT-PCR reaction and it was not contaminated. The absence of additional peaks in the curves confirms this. Furthermore, dimer primer was not detected during the confirmation process.

Determining the degree of specificity of designed primers. To establish the specificity of the primers utilized, every primer pair underwent testing with the genomes of four distinct HPV types. Electrophoresis showed that no band was present in these reactions, indicating that the primers used were highly specific. Fig. 4 indicates the specificity of HPV types 11, 16 and 18 with four different HPV types.

Determination of the sensitivity of PCR and RT-PCR reactions. Genomic DNA from HPV genome types 11, 16, and 18 was diluted and used to perform PCR and RT-PCR reactions. The PCR products were analyzed by electrophoresis on agarose gel, and the resulting image is presented in Fig. 5. Subsequently, the RT-PCR reaction was evaluated, and the results are displayed in Fig. 6. The optimized PCR reaction's detection limit was established for 11, 16, and 18 HPV types, as well as 1.9, 0.7, and 3 ng/µg of genomic DNA. Additionally, the detection limit was determined for all types in RT-PCR reaction, up to 80 pg of genomic DNA.

Labeling of PCR reaction product to perform PCR-ELISA reaction. DIG-dUTP was used to label the PCR product. Fig. 7 displays that the PCR product amplified with the digoxin gene is monoclonal and slightly larger than the PCR product amplified with normal dNTPs. This is due to the presence of the digoxin gene in the indicated PCR product, which increased the weight of the desired fragment. Therefore, performing PCR reactions in the presence of DIG-dUTP is recommended. Fig. 7 illustrates the findings of DIG-labeled products on a 10% agarose gel.

PCR-ELISA reaction. After labelling the PCR products, we conducted ELISA and measured light absorption at 492 nm using an ELISA Reader. Fig. 8 displays that the OD of the labelled HPV types 11, 16, and 18 PCR products were considerably higher than that of the control samples, indicating acceptability.

Evaluation of PCR-ELISA reaction sensitivity. In order to assess the sensitivity and establish the minimal detectable genomic concentration via PCR-ELISA diagnostic methodology, serial dilutions of the purified genome were subjected to fragment amplification by PCR, followed by detection via ELISA. As depicted in Fig. 9, the results obtained are both meaningful and acceptable.

Specificity of PCR-ELISA method. The diagnostic method's specificity was evaluated using four distinct types of HPV. The results indicated that the designed primers were specific. No bands were observed on the agarose gel for the other HPV tested in this study. Subsequently, the PCR-ELISA method's results were assessed and recorded in accordance with the data provided in Fig. 10. The light absorption of the samples for HPV types 11, 16, and 18 was greater than that of the reaction controls, whereas the light absorption for other HPV types was lower than that of the reaction control.

Reproducibility analysis of PCR-ELISA intra-assay and inter-assay variability. To evaluate the reproducibility of PCR-ELISA, intra-assay and inter-assay variability were assessed using three positive HPV samples (HPV18, HPV16, and HPV11) over three days, with three replicates per day. The coefficient of variation (CV) was calculated for each condition.

For HPV18, the intra-assay CVs ranged from 2.63% to 4.37%, with an inter-assay CV of 8.92%. Similarly, for HPV16, the intra-assay CVs ranged from 2.63% to 10.48%, and the inter-assay CV was 9.71%. For HPV11, the intra-assay CVs were between 0.70% and 4.10%, and the inter-assay CV was 2.05%.

These results, summarized in Table 3, demonstrate the robust reproducibility of PCR-ELISA, with most

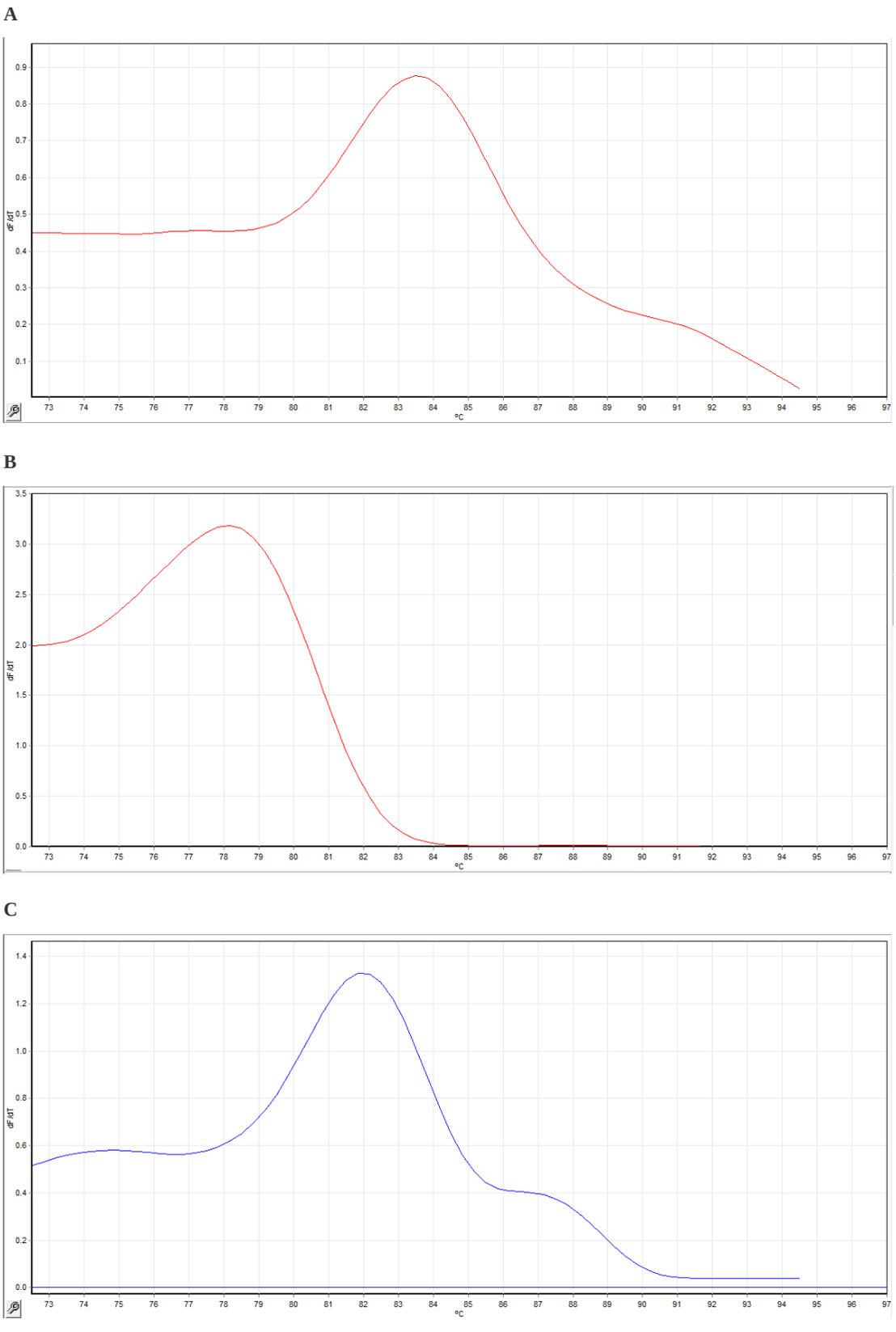

Fig. 3. Component connection temperature and the highest possible adsorption rate on the number of cycles in the Real-Time PCR reaction are; (A) HPV + 11; (B) HPV + 16; (C) HPV + 18, respectively.

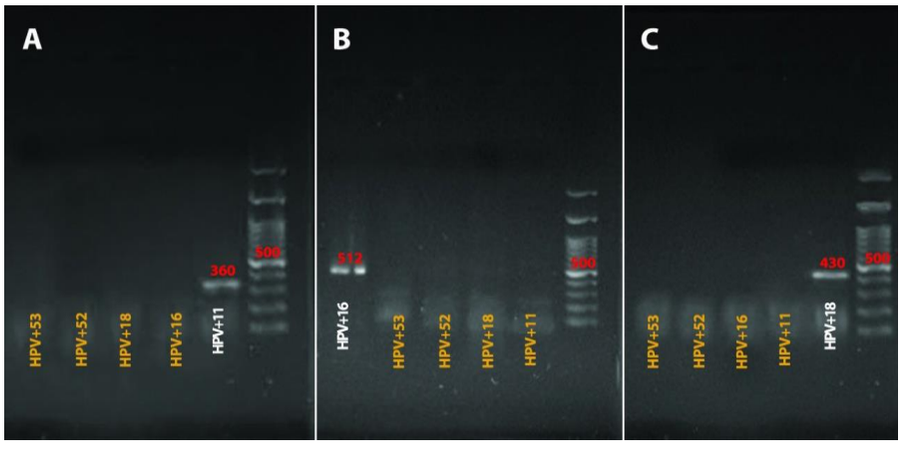

Fig. 4. Gel image of the gene bands of types 11, 16 and 18 of the HPV genotype along with four different HPV types to determine the specificity of the HPV; (A) HPV + 11; (B) HPV + 16; (C) HPV + 18.

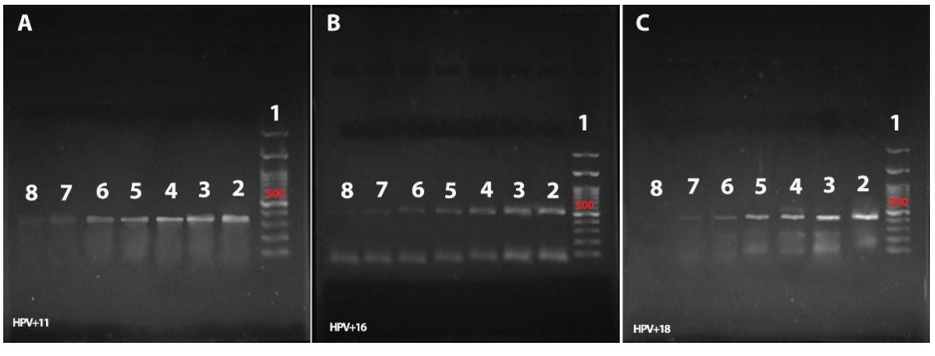

Fig. 5. Gel image showing sensitivity analysis. Lane 1: 100 bp DNA marker; Lanes 2–8: serial dilutions of HPV DNA concentrations for types 11, 16, and 18.

CVs within acceptable limits for clinical applications. The slightly higher inter-assay variability reflects day-to-day differences but confirms the method's reliability for large-scale diagnostics.

Diagnostic performance of PCR-ELISA sensitivity, specificity, and Kappa analysis. The diagnostic performance of PCR-ELISA was assessed for HPV types 18, 16, and 11. For HPV18, the assay achieved a sensitivity of 94.92%, specificity of 100%, and a Kappa value of 0.84, indicating strong agreement with the reference standard. Similarly, for HPV16, sensitivity was 98.36%, specificity 100%, and the Kappa value 0.92, reflecting excellent agreement. For HPV11, sensitivity was 93.75%, specificity 100%, and the Kappa value 0.84, highlighting the reliable performance.

These findings confirm the high accuracy and reliability of PCR-ELISA, with no false positives reported across all tested genotypes. Minimal discrepancies, primarily due to false negatives in low viral load samples, were observed. The results, summarized in Table 4, underscore PCR-ELISA's robust diagnostic performance.

Detection of clinical specimens using PCR-ELISA. For this study, we collected the genomes extracted from positive samples of HPV types 11, 16, and 18, along with control samples from healthy individuals, as referenced by TehranLab Lab. These results were then confirmed using the OPERON kit. We performed PCR programs with 30 cycles on these samples and measured them using an ELISA reader. Table 5 pertains to individuals who were referred to TehranLab Laboratory due to clinical signs of HPV for diagnosis. The types of HPV were identified using a kit from OPERON company and PCR-ELISA method.

The concordance between PCR-ELISA and the OPERON kit was 96.7%, with two negative samples missed by PCR-ELISA. Potential reasons for this discrepancy include low DNA concentrations in the

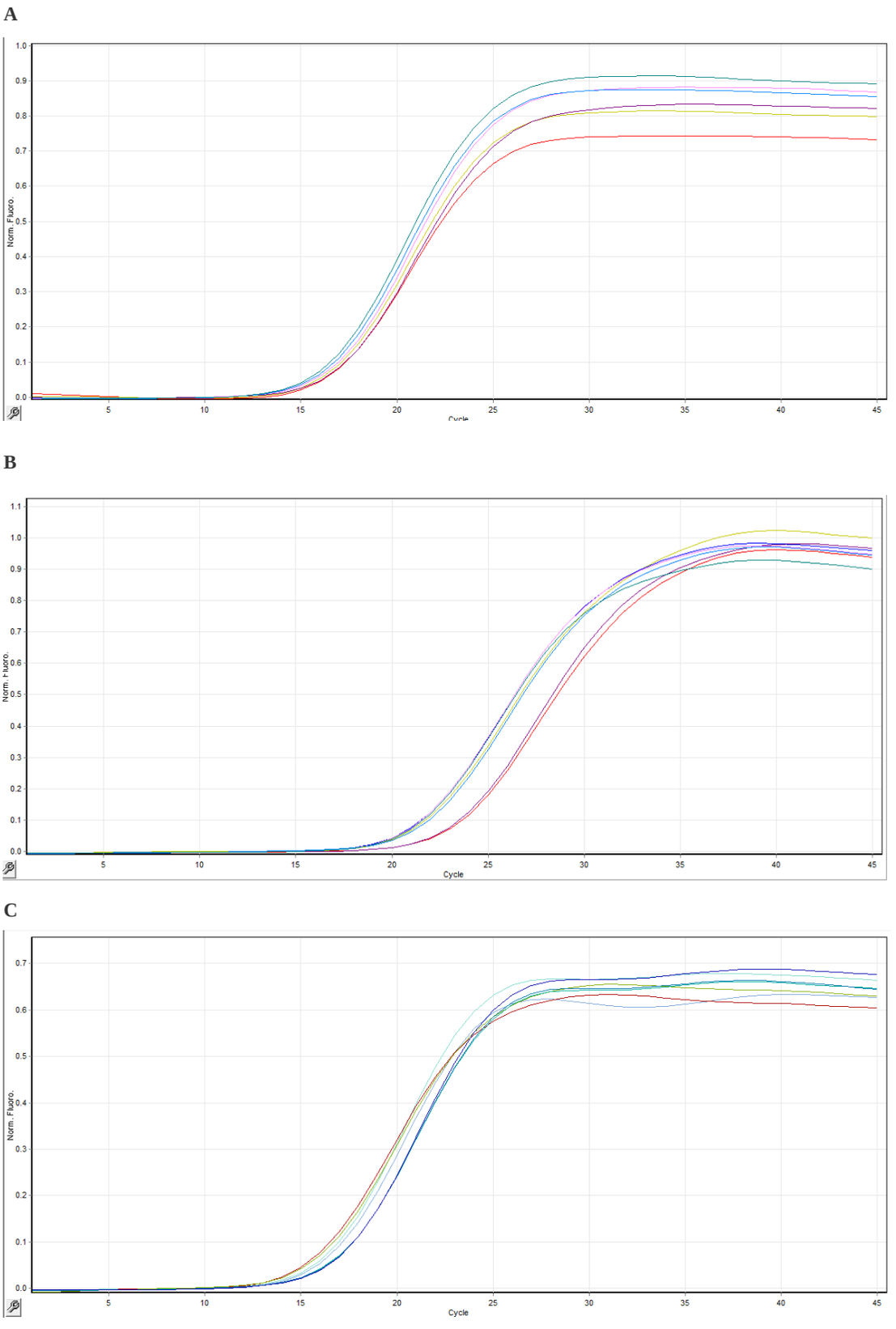

Fig. 6. The result of determining the reaction sensitivity in terms of the concentration of genomic DNA in the Real-Time PCR reaction, which includes; (A) HPV + 11; (B) HPV + 16; (C) HPV+18.

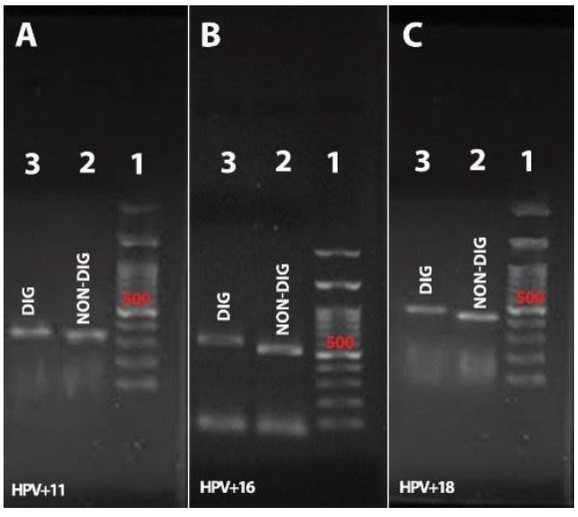

Fig. 7. PCR product labeling with DIG-dUTP including Fig. (A) HPV+11; Lane 1: DAN size indicator (100 bp DNA ladder), Lane 2: Unlabeled PCR product, Lane 3: PCR product labeled. Fig. B: HPV+16, Lane 1: DAN size indicator (100 bp DNA ladder), Lane 2: Unlabeled PCR product, Lane 3: Labeled PCR product. Fig. C: HPV+18, Lane 1: DAN size indicator (100 bp DNA ladder), Lane 2: Unlabeled PCR product, Lane 3: Labeled PCR product.

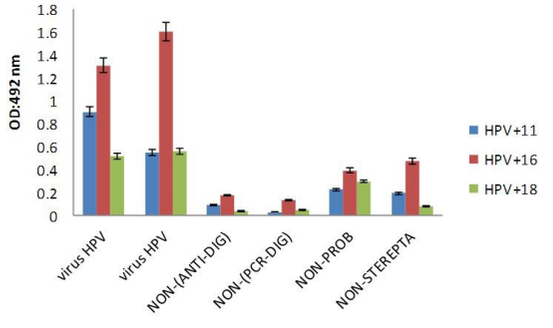

Fig. 8. Detection of avidin-containing microtiter plates using HPV strain 11, 16, and 18 gene product in comparison to PCR-ELISA reaction

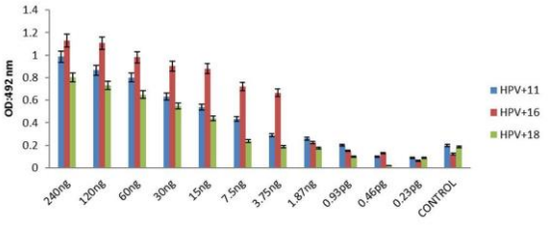

Fig. 9. Determination of PCR-ELISA sensitivity using PCR product for each of the 11, 16, and 18 genes of the HPV.

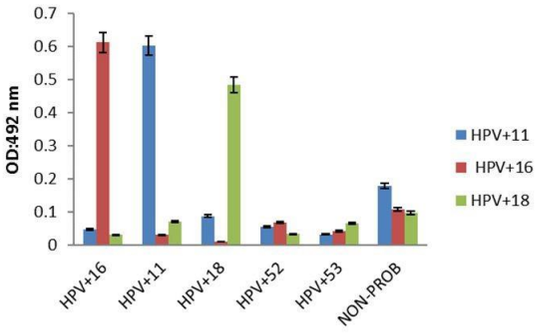

Fig. 10. The result of OD reading of PCR product of HPV genome types 11, 16, and 18 with different types to evaluate the specificity of PCR-ELISA reaction.

samples, possible degradation of DNA in paraffin-embedded tissues, and minor mismatches in probe design that could reduce binding efficiency. Operator variability during sample preparation and handling may also have influenced the results.

To address such issues in future applications, we recommend optimizing protocols for low-concentration samples, refining probe design to improve target binding, and ensuring standardized training for laboratory personnel. These measures could further enhance the sensitivity and reliability of PCR-ELISA in diagnostic workflows.

DISCUSSION

Human papillomavirus (HPV) is a DNA virus with over 100 known types, with over 40 of them affecting the genital area. The virus is transmitted through sexual contact and is the leading cause of cervical cancer in women. The most effective way to reduce the risk of HPV infection is through vaccination and safe sexual practices. It is important to raise awareness of the dangers of HPV and the importance of prevention measures (17). One of the primary means of HPV transmission is sexual contact. Each HPV strain is identified by a unique numerical or alphabetic type designation (18). Of the 100 types of HPV, about 60 cause warts in areas such as the hands and feet (19). Another 40 or more HPV types can enter the body through sexual contact and attach to the mucous membranes surrounding the genitals (20). The typical means of identifying PCR products involves gel electrophoresis, followed by staining with ethidium bromide and hybridization (21). All

Table 3. Summary of intra-assay and inter-assay variability for PCR-ELISA across HPV types.

HPV Type	Day	Intra-assay Mean	Intra-assay CV (%)	Inter-assay Mean	Inter-assay CV (%)
HPV+18	Day 1	0.873	2.63	0.796	8.92
	Day 2	0.732	4.37		
	Day 3	0.783	3.70		
HPV+16	Day 1	0.943	4.03	0.958	9.71
	Day 2	1.059	10.48		
	Day 3	0.873	2.63		
HPV+11	Day 1	0.892	3.70	0.876	2.05
	Day 2	0.878	4.10		
	Day 3	0.857	0.70		

Table 4. Sensitivity, specificity, and Kappa coefficients of PCR-ELISA for detecting HPV types

HPV Type	Sensitivity (%)	Specificity (%)	Kappa
HPV18	94.92	100	0.84
HPV16	98.36	100	0.92
HPV11	93.75	100	0.84

of these approaches present their own limitations, from restricted timeframes for detecting samples to dangerous substances such as ethidium bromide and agarose gel. Presently, various diagnostic methods are utilised to ascertain and substantiate HPV human papillomavirus infection. Many of these involve molecular techniques, such as PCR - RT - PCR – RFLP (22, 23). The approach employed here utilizes general primers MY11/MY09 and GP5+/6+ for L protein and the E1 region of the HPV papillomavirus genome, respectively. This allows for increased objectivity and precision in analysis (24). The use of GP5+/6+ instead of primer MY11/MY09 resulted in amplified DNA and consequential discrepancies in diagnosis. These inconsistencies were due to variations in target protein expression across different HPV types and DNA positioning in cancer cell genes, as well as an inability to detect the target DNA (25). Due to the extensive range of the HPV genome, it has become quite challenging to accurately identify the virus and select the appropriate primer considering the variety of virus types.

PCR-ELISA has been employed in diagnosis of herpesvirus infections (26), leishmaniasis (27), diarrhea-causing *Escherichia coli* isolates (28), *Mycobacterium tuberculosis* (29), and others, but not much has been done to diagnose HPV infections using PCR-ELISA. In 1998, this method was used to diagnose HPV by Venturoli and Zorbini (30, 31). MV09/MYII primers and digoxigenin-labelled nucleotides were used in the PCR reaction to hybridise the biotinylated probe to the product. This technique involves the use of a streptavidin plate as the overlay component.

The resulting hybrid was added to a microtiter plate and obtained through the interaction of biotin and streptavidin. Scientists are currently investigating the detection of the complex bound by anti-Dig antibody to a conjugated enzyme, as well as the addition of a suitable substrate (30). One of the most significant benefits of utilising PCR-ELISA in contrast to alternative methods is that the ELISA approach requires a series of incubation stages which can be easily accomplished due to the procedure's simplicity (30, 32). The specificity of the methodology can be improved through the application of a specialised probe. Large scale analysis, as well as the use of UV and darkroom, are no longer necessary. The method presents several advantages such as a reduced environmental impact in comparison with other techniques, like Saturn staining. Additionally, it is imperative to avoid radioactive and carcinogenic substances such as ethidium bromide, due to their adverse implications to human safety and health (32). The PCR-ELISA method eliminates the use of hazardous chemicals such as ethidium bromide, commonly employed in gel electrophoresis. In contrast, PCR-ELISA reduces hazardous waste production by over 90%, as it relies on non-toxic reagents like biotin-labeled probes and enzyme substrates. Gel electrophoresis requires the use of agarose gel and running buffers, which collectively produce approximately 100g of solid and liquid waste per batch. In comparison, PCR-ELISA uses pre-coated ELISA

Table 5. The number of patients displaying clinical symptoms who were diagnosed with HPV types using the OPERON kit and PCR-ELISA method at the Tehran Lab Laboratory.

Strain examined	**HPV18**				**HPV16**				**HPV11**			
Total number of samples examined	**60**				**40**				**30**			
Detection method	**PCR-ELISA**		**operon**		**PCR-ELISA**		**operon**		**PCR-ELISA**		**operon**	
Diagnosis (positive & negative)	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**
Results of the experiment	2	58	-	60	-	40	-	40	-	30	-	30

plates, which significantly leads to less waste. Furthermore, the absence of UV visualization equipment reduces energy consumption and operational risks. From a safety perspective, PCR-ELISA eliminates the need for personnel to handle carcinogenic substances like ethidium bromide and minimizes exposure to UV radiation during gel visualization. This makes PCR-ELISA a safer alternative for routine diagnostic workflows. Cost-benefit analysis Table 6 showed that PCR-ELISA significantly reduces reagent and equipment costs while maintaining high sensitivity and specificity. Its simplified workflow also lowers labor costs compared to RT-PCR, making it a cost-effective alternative for diagnostic applications.

PCR-ELISA requires specialized equipment such as ELISA plate readers and thermal cyclers, which may pose challenges in resource-limited settings. However, these devices are increasingly accessible due to their versatility and cost-effectiveness. The method also requires trained personnel for proper execution and interpretation, a challenge that can be addressed through targeted training programs. Despite these limitations, PCR-ELISA integrates seamlessly into existing diagnostic workflows in laboratories equipped with ELISA and PCR systems. Its compatibility with standardized reagents and protocols minimizes the need for additional infrastructure, making it a practical option for many laboratories.

This approach lifts the testing constrains for the quantity of pandemic samples. This dissimilarity appears to be rooted in the detection method employed, since in PCR only the size of the target sequence is used to ascertain its accuracy and the existence of the virus in the sample. This study discovered that PCR-ELISA is more sensitive than PCR and RT-PCR using the SYBR Green method. This dissimilarity appears to be rooted in the detection method employed, since in PCR only the size of the target sequence is used to ascertain its accuracy and the existence of the virus in the sample. In RT-PCR using the SYBR Green method, the criterion for detection is the synthesis of two strands and the presence of SYBR Green dye between them. This criterion, however, is not entirely definitive. In contrast, PCR-ELISA employs a probe as the detector of the fragment, which detects the specific linkage of bases to determine the exact reason for the presence of the fragment and identify the infection with the virus under question. The study demonstrated the PCR-ELISA technique's ability to detect minute quantities of DNA in a sample in terms of detectability. This heightened sensitivity may be partially attributed to the detection method and apparatus employed. The enzyme molecule that binds to the biotin in the probe can irreversibly modify the substrate molecules, generating a dye in the surrounding environment. This is of particular significance when the initial pattern value is minute. Despite its high sensitivity and specificity for PCR and RT-PCR, this method necessitates real-time devices, gel electrophoresis, and UV light with a dark room. Furthermore, it does not

Table 6. A comparative cost-benefit analysis of RT-PCR and gel electrophoresis.

Parameter	**PCR-ELISA**	**RT-PCR**	**Gel Electrophoresis**
Reagent Cost (per test)	$2.5	$5.0	$1.5
Equipment Cost	Moderate	High	Low
Labor Requirement	Moderate	High	High
Throughput (samples/day)	96	48	24
Hazardous Waste	Minimal	Moderate	High

employ materials such as ethidium bromide. As previously stated, this technique allows for the analysis of a large number of samples simultaneously. This technology can be automated, providing benefits for clinical use. Conducting the initial amplification and diagnosis in a tube reduces contamination and other errors. PCR-based methods generally minimize direct handling of clinical specimens, lowering risk for personnel. Furthermore, samples can be stored at -20°C until needed. As there is no requirement for a contagious virus, only intact DNA, there is no need for extreme precautions during the collection and storage of samples. Any physician can easily collect, store, and send samples to a laboratory if necessary.

The results of this study demonstrate that PCR-ELISA provides excellent diagnostic performance, as evidenced by high sensitivity and specificity across all tested HPV types. The calculated Kappa values (ranging from 0.84 to 0.92) further indicate strong agreement with the reference standard. These results suggest that PCR-ELISA is a reliable method for detecting HPV genotypes, particularly in clinical settings where accuracy is critical.

While the assay showed minimal discrepancies (e.g., a few false negatives for low viral load samples), the overall performance underscores its potential as a cost-effective and accurate alternative to existing methods such as RT-PCR. Future studies may focus on optimizing the assay for low-concentration samples to further enhance its sensitivity.

Multiplex assays allow for the simultaneous detection of multiple HPV genotypes in a single reaction, which increases throughput and reduces reagent usage. However, these assays require more complex instrumentation and optimization to avoid cross-reactivity between the targets. In contrast, PCR-ELISA focuses on a smaller set of targets with high specificity and lower costs, making it suitable for laboratories with limited resources.

Digital PCR provides absolute quantification of HPV DNA and is highly sensitive, even at very low viral loads. While this precision is advantageous for clinical research, the high cost and technical complexity of digital PCR limit its widespread use. PCR-ELISA offers a practical alternative by balancing sensitivity with cost-effectiveness, particularly in high-throughput diagnostic settings.

As HPV diagnostics continue to evolve, PCR-ELISA serves as a bridge between traditional methods like gel electrophoresis and more advanced technologies such as digital PCR and NGS. Its adaptability and affordability ensure its relevance in both research and clinical laboratories, especially in resource-limited settings.

Despite its advantages, PCR-ELISA has limitations, including reliance on high-quality DNA samples, lower throughput compared to automated methods and potential false negatives in low viral load cases. Addressing these issues in future studies could enhance its clinical applicability.

CONCLUSION

Therefore, it can be concluded that the PCR-ELISA technique offers various advantages over alternative diagnostic methods. Firstly, the combination of PCR product with a complementary cross-reaction step has enhanced the accuracy of the work. Secondly, the use of a plate enables the examination of a larger number of samples. Thirdly, this technique has higher sensitivity and specificity than agarose gel electrophoresis and RT-PCR by the SYBR Green method. Further benefits of this methodology include the absence of a gel electrophoresis apparatus or darkroom requirement, the potential for reduced environmental contamination compared to Saturn staining protocols and the employment of cost-effective materials in the real-time assay. While PCR-ELISA demonstrates significant potential for HPV diagnostics, addressing its current limitations will further enhance its reliability and applicability.

REFERENCES

1. McBride AA. Human papillomaviruses: Diversity, infection and host interactions. *Nat Rev Microbio*l 2022; 20: 95-108.
2. Smith HB (2013). Handbook on human papillomavirus: prevalence, detection and management (Virology Research Progress: Public Health in the 21st Century). Nova Science Pub Inc; 1st edition. https://www.amazon.com/Handbook-Human-Papillomavirus-Prevalence-Management/dp/1626186723
3. Santos-López G, Márquez-Domínguez L, Reyes-Leyva J, Vallejo-Ruiz V. General aspects of structure, classification and replication of human papillomavirus. *Rev Med Inst Mex Seguro Soc* 2015; 53 Suppl 2: S166-S171.

4. Kashefi F, Alizadeh M, Kalaki NS, Ejlalidiz M, Yazdani S, Shayestehpour M, et al. Molecular Epidemiology of human papilloma virus (HPV) in women's Cervical samples from Tehran, Iran. *Asian Pac J Cancer Biol* 2024; 9: 169-174.
5. Galati L, Chiantore MV, Marinaro M, Di Bonito P. Human Oncogenic viruses: Characteristics and prevention Strategies-Lessons learned from human Papillomaviruses. *Viruses* 2024; 16: 416.
6. Sabatini ME, Chiocca S. Human papillomavirus as a driver of head and neck cancers. *Br J Cancer* 2020; 122: 306-314.
7. Singh S, Ahmad S, Srivastava AN, Misra JS. A review on role of human Papilloma virus (HPV) in Health-Related diseases. *Adv Med Dent Health Sci* 2020; 3: 34-40.
8. Niya MHK, Tameshkel FS, Motamed N, Miri SM, Mortazavi H, Ajdarkosh H, et al. Detection of human Papilloma virus (HPV) infection in Colorectal cancer and viral Genome Integration Status. *Shiraz E Med J* 2022; 23(10): e118289.
9. Qureshi QUA, Qureshi NUAZ, Qureshi PAAA, Abbasi N. Human papilloma virus status among women presenting with vaginal discharge at a tertiary care hospital. *J Soc Obstet Gynaecol Pak* 2021; 11: 87-90.
10. Hahn A, Podbielski A, Meyer T, Zautner AE, Loderstädt U, Schwarz NG, et al. On detection thresholds–a review on diagnostic approaches in the infectious disease laboratory and the interpretation of their results. *Acta Trop* 2020; 205: 105377.
11. Rotondo JC, Oton-Gonzalez L, Mazziotta C, Lanzillotti C, Iaquinta MR, Tognon M, et al. Simultaneous detection and viral DNA load quantification of different human papillomavirus types in clinical specimens by the high analytical droplet digital PCR method. *Front Microbiol* 2020; 11: 591452.
12. Mühr LSA, Guerendiain D, Cuschieri K, Sundström K. Human Papillomavirus detection by Whole-Genome Next-Generation sequencing: Importance of validation and quality assurance procedures. *Viruses* 2021; 13: 1323.
13. Molijn A, Kleter B, Quint W, van Doorn L-J. Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol* 2005; 32 Suppl 1: S43-S51.
14. Kingma DW, Allen RA, Caughron SK, Melby M, Moore WE, Gillies EM, et al. Comparison of molecular methods for detection of HPV in oral and oropharyngeal squamous cell carcinoma. *Diagn Mol Pathol* 2010; 19: 218-223.
15. Borkakoty B, Jakharia A, Singh P, Khan SA (2024). Trends of Diagnostic Methods for Human Viral Diseases. Viral Infectious Diseases Annual Volum 2024. https://www.intechopen.com/chapters/89145
16. Naegele S, Ruiz-Torres DA, Zhao Y, Goss D, Faden DL. Comparing the diagnostic performance of quantitative PCR, digital droplet PCR, and next-generation sequencing liquid biopsies for human Papillomavirus–associated cancers. *J Mol Diagn* 2024; 26: 179-190.
17. Iftner T, Eberle S, Iftner A, Holz B, Banik N, Quint W, et al. Prevalence of low-risk and high-risk types of human papillomavirus and other risk factors for HPV infection in Germany within different age groups in women up to 30 years of age: an epidemiological observational study. *J Med Virol* 2010; 82: 1928-1939.
18. Boccardo E. New approaches for infective HPV detection, quantification and inactivation: Preventing accidental virus transmission in medical settings. *EBioMedicine* 2021; 64: 103222.
19. McBride AA. Human papillomaviruses: Diversity, infection and host interactions. *Nat Rev Microbiol* 2022; 20: 95-108.
20. Sinal SH, Woods CR. Human papillomavirus infections of the genital and respiratory tracts in young children. *Semin Pediatr Infect Dis* 2005; 16: 306-316.
21. Bagul KA, Khandelwal N, Naphade ME, Panchbudhe SA, Mishra V, Tripathy S, et al. Detection of human Papillomavirus among cervical cancer patients by qualitative polymerase chain reaction on formalin-fixed, paraffin-embedded tissue: A retrospective observational analysis. *Nat J Lab Med* 2023; 12: PO10-PO13.
22. Bharadwaj M, Hussain S, Tripathi R, Singh N, Mehrotra R (2020). Chapter 6: The clinico-molecular approaches for detection of human papillomavirus. Animal Biotechnology (Scond Edition): Elsevier. p. 103-130. https://www.sciencedirect.com/science/article/abs/pii/B9780128117101000069
23. Comar M, De Seta F, Zanotta N, Del Bue S, Ferrante P (2020). New Diagnostic Approaches to viral Sexually transmitted infections. Sexually Transmitted Infections: Springer. p. 107-148.
24. Mousavi T, Rafiei A, Reza Haghshenas M, Sadeghian-Kiadehi SF, Valadan R. Molecular prevalence and phylogenetic analysis of human papillomavirus in normal cervical samples from northern Iran. *Gene Rep* 2020; 21: 100958.
25. Sharmin S, Zohura FT, Islam MS, Shimonty A, Khan MA, Parveen R, et al. Mutational profiles of marker genes of cervical carcinoma in Bangladeshi patients. *BMC Cancer* 2021; 21: 289.
26. Daly P, Doyle S. The development of a competitive PCR–ELISA for the detection of equine herpesvirus-1. *J Virol Methods* 2003; 107: 237-244.
27. Pereira LQ, Ferreira-Silva MM, Ratkevicius CMA, Gómez-Hérnandez C, De Vito FB, Tanaka SCSV, et al. Identification of *Leishmania infantum* in blood donors from endemic regions for visceral leishmaniasis. *Parasitology* 2021; 148: 110-114.
28. Jangpatarapongsa K, Saimuang K, Polpanich D, Thi-

ramanas R, Techakasikornpanich M, Yudech P, et al. Increased sensitivity of enterotoxigenic *Escherichia coli* detection in stool samples using oligonucleotide immobilized-magnetic nanoparticles. *Biotechnol Rep (Amst)* 2021; 32: e00677.

29. Khan A, Singh R, Sharma S, Singh V, Sheoran A, Soni A, et al. Diagnosis of osteoarticular tuberculosis by immuno-PCR assay based on mycobacterial antigen 85 complex detection. *Lett Appl Microbiol* 2022; 74: 17-26.
30. Zerbini M, Venturoli S, Cricca M, Gallinella G, De Simone P, Costa S, et al. Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA. *J Clin Pathol* 2001; 54: 377-380.
31. Bartosik M, Moranova L, Izadi N, Strmiskova J, Sebuyoya R, Holcakova J, et al. Advanced technologies towards improved HPV diagnostics. *J Med Virol* 2024; 96(2): e29409.
32. Musiani M, Venturoli S, Gallinella G, Zerbini M. Qualitative PCR–ELISA protocol for the detection and typing of viral genomes. *Nat Protoc* 2007; 2: 2502-2510.